THE POTENTIAL OF CHROMATOGRAPHIC TECHNIQUES
FOR THE MANIPULATION OF VIABLE MICRO-ORGANISMS

by

Pradip Dahyabhai Patel, M.Sc., M.I.Biol.

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Dept. of Microbiology
University of Surrey

Dept. of Applied Microbiology
Leatherhead Food RA
Randalls Road
Leatherhead
Surrey

5928088

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SUMMARY

Interaction of viable micro-organisms with negatively charged cation exchange resin, Bio-Rex 70 and with hydrophobic Phenyl-sepharose gel was investigated. These chromatographic materials can be used to resolve, to a greater or lesser extent, binary mixtures of some organisms both from broth cultures and from artificially inoculated food suspensions.

The mechanism of interaction between micro-organisms and the chromatographic adsorbents, Bio-Rex 70 and Phenyl-sepharose was also investigated. The presence of specific microbial cell surface proteins i.e. K88a antigen of E.coli and protein A of Staph.aureus enhanced adsorption of the organisms to both Bio-Rex 70 and Phenyl-sepharose.

The interaction of micro-organisms with negatively charged Bio-Rex 70 may not involve significantly the opposite charges on these bodies. Several lines of evidence strongly suggest that organisms interact with the 'hydrophobic' acrylic matrix of Bio-Rex 70. For instance, the modification of carboxyl groups of Bio-Rex 70 by various treatments did not reduce adsorption of Staph.aureus compared with the non-treated control.

The adsorption of micro-organisms to Phenyl-sepharose gel involved hydrophobic interactions e.g. in distilled water ca 40% of Staph.aureus adsorbed to the gel while in presence of 1M $(\text{NH}_4)_2\text{SO}_4$ the adsorption was >99%.

Novel immunosorbent chromatographic methods were demonstrated for the enrichment of viable cells of Salm.typhimurium from mixed broth cultures containing other Enterobacteriaceae. There was 11-fold enrichment of Salm.typhimurium from a mixture with E.coli and 5-fold enrichment from a mixture with Cit.freundii using Salmonella antiserum coupled to a magnetic gel, Magnogel AcA 44. However, Salm.typhimurium was not enriched from food preenrichment broths.

A method involving separation and concentration of micro-organisms from meats was devised prior to rapid estimation of the organisms by measurement of microbial ATP. A linear relationship was found between $\log_{10}$ (total viable count) and $\log_{10}$ (microbial ATP content) for lamb ($r = 0.98$) and for beef ($r = 0.97$).

DEDICATED TO

My parents; Dahyabhai C Patel & Kantaben D Patel,
my wife; Divya and my brothers; Hemant & Shirish

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PART I

BACKGROUND

PART I

Chapter 1: GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

In the past few decades numerous biochemical and biophysical techniques have been developed for the separation and estimation of biomolecules. These techniques include many forms of chromatography (e.g. gel filtration, ion exchange and affinity chromatography), electrophoresis and immunoelectrophoresis, partition in aqueous polymer two-phase systems and density gradient centrifugation. The estimation techniques include spectrophotometric measurement, luminometry and immunological techniques such as radio immunoassays and enzyme immunoassays. These separation and estimation techniques are widely used by clinical and research biochemistry and immunology laboratories but have had very limited applications in microbiological analyses.

The traditional methods for detection and estimation of viable organisms are based upon growth of the organisms to produce a signal visible to the eye e.g. a colony or turbidity in broth. Although these cultural techniques are highly sensitive (i.e. capable of detecting 1 viable cell) and are usually not subject to interference by non-microbial components, they have several disadvantages. They are labour-intensive, tedious and most importantly they are time-consuming, requiring upto 3 days for the estimation of total viable organisms and longer periods for the detection of specific organisms e.g. at least 4 days for Salmonella (D'Aoust, 1981).

In recent years, food manufacturers have sought novel microbiological methods which can produce Quality Control information more rapidly than do the traditional cultural methods. This has resulted in the development of various instrumental techniques for the estimation of total viable micro-organisms in

foods. These include the Direct Epifluorescent Filter Technique (DEFT; Pettipher, 1981), impedimetric estimation (Hardy et al., 1977; Richards et al., 1978), luminometry (Patel & Wood, 1983; Stannard & Wood, 1983) and radiometry (Previte, 1972). These instrumental techniques are generally less sensitive than the cultural methods and may also be subject to interference from non-microbial components in the milieu e.g. ATP intrinsic to foods (Sharpe et al., 1970) or buffering constituents of medium which can depress the impedimetric response of micro-organisms (Ur & Brown, 1973). In other areas of science these problems are normally 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 micro-organisms behave as self-amplifying systems and other (non-replicating) biomaterials are normally of little significance (Wood, 1979).

In these and future instrument-based techniques the ability to manipulate (i.e. enrich, separate or concentrate) micro-organisms by physical techniques may offer advantages in preparing samples for analysis. For instance, separation and concentration of micro-organisms from other food constituents could result in greater sensitivity of detection by instrumental techniques. In addition, separation of specific groups of organisms from mixed microbial populations may lead to the more rapid selective estimation of these organisms.

The broad objective of the present study was to investigate methods for the physical manipulation of micro-organisms in order to effect

enrichment, separation or concentration of the organisms from other biological materials prior to detection or estimation. The major area chosen for investigation was the use of chromatographic materials viz. cation exchange resin, hydrophilic-hydrophobic gel and immunosorbent affinity media for the manipulation of viable micro-organisms.

PART I

Chapter 2: SURVEY OF THE LITERATURE

2. SURVEY OF THE LITERATURE

The following areas of science, aspects of all of which have an important bearing on the present study, are briefly surveyed:

Structure of the bacterial cell envelope.

Physico-chemical characteristics of micro-organisms.

Development of methods for the detection of Salmonella.

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

2.1 Structure of the bacterial cell envelope

The bacterial cell envelope represents a boundary separating the cytoplasm from the external environment. It is a specialised structure and has many functions viz: protection of the cell from toxic chemicals or changes in the external environment, allowing entry of nutrients and solutes into the cell and diffusion of waste products out of the cell.

The structure of the cell wall of Gram-positive bacteria differs markedly from that of the Gram-negative bacteria. The immediate structure responsible for containing the cytoplasmic constituents is known as the cytoplasmic or inner membrane and is basically similar in both Gram-positive and Gram-negative bacteria. It consists of a lipid bilayer with integrated transport proteins and enzymes of the oxidative phosphorylation pathway (Filip et al., 1973). In the Gram-positive bacteria, the structure separating the cytoplasmic membrane from the external environment is basically a peptidoglycan layer whereas in the Gram-negative bacteria it is a more complex structure containing a peptidoglycan (or mucopeptide) layer plus an outer membrane. The

thickness of the Gram-positive wall varies between 10 and 50 nm compared with the Gram-negative wall which varies between 10 and 15 nm (Glauert & Thornley, 1969).

Structure of Gram-positive cell wall

The Gram-positive wall consists mainly of the peptidoglycan layer with associated proteins, teichoic acids and some polysaccharide (Rogers & Perkins, 1968).

Peptidoglycan (syn. Mucopeptide)

In some strains of Staphylococcus aureus the peptidoglycan accounts for almost 80% of the weight of the cell wall (Strominger, 1962). It forms a rigid layer and consists of polymers containing repeating units of β 1-4 N-acetyl glucosamine and β 1-4 N-acetyl muramic acid which are cross-linked at intervals by short pentapeptide side chains.

Proteins

Barculis & Jones (1957) reported the presence of M protein in streptococcal cell wall while Forsgren & Sjöquist (1969) demonstrated the presence of Protein A on staphylococcal cell wall. Usually, only small amounts of protein are found in Gram-positive walls except for streptococci which contain large amounts of M, T and R proteins in the wall (Ellwood, 1978). The occurrence of protein in patches on the cell-surface of Staph.aureus was reported by James & Brewer (1968). A basic protein was produced by three strains of Staph.aureus grown in a high carbohydrate, high salt medium (Brock & Reiter, 1976). Masuda & Kawata (1979) reported the presence of regular arrays of hexagonally arranged protein (M.wt. 55,000) on the surface of Lactobacillus büchneri. Similar proteins have also been reported in L.fermentum and L.brevis.

Teichoic acids

The teichoic acids are polymers of polyol phosphates and are usually either ribitol teichoic acid or glycerol teichoic acid in the majority of Gram-positive bacteria (Archibald et al., 1973). Ribitol teichoic acid is usually associated with the cell wall whilst glycerol teichoic acid is usually considered to be a non-wall polymer and, may, occur in the cell membrane (Rogers & Perkins, 1968). However, the glycerol form has been shown to occur in walls of Staph.aureus (Reeder & Ekstedt, 1973). In the cell wall the teichoic acids are covalently attached to peptidoglycan (Rogers & Perkins, 1968).

Polysaccharides

The walls of the Gram-positive bacteria contain polysaccharides which contain sugar residues such as rhamnose, glucosamine and deoxysugars (Rogers & Perkins, 1968). The presence of galactose, glucose, glucosamine and N-acetyl-glucosamine in the walls of lactobacilli was reported by Shimohashi (1975). The polysaccharide antigen of Staph.aureus contained taurine, D-amino-galacturonic acid and fucosamine (Liau & Hash, 1977). Karakawa et al. (1974) showed that an acidic surface antigen of Staph.aureus consisted of aminouronic acid and an amino sugar.

Structure of Gram-negative cell wall

As mentioned previously, the cell envelope consists essentially of three layers i.e. the inner membrane (or cytoplasmic membrane), peptidoglycan layer and the outer membrane.

Peptidoglycan

The peptidoglycan represents about 10% by weight of the cell walls of Gram-negative bacteria (Rogers & Perkins, 1968) and is structurally related to the mucopeptide of Gram-positive species. Lugtenberg et al. (1977) reported that at least one protein (i.e. protein b or c) is associated with the peptidoglycan of various Gram-negative bacteria (e.g. Escherichia coli, Salmonella typhimurium and Klebsiella). The bound form of the lipoprotein was reported to be covalently linked to the peptidoglycan layer (Bosch & Braum, 1973).

Structure and composition of the outer membrane

This is a highly specialised and complex structure which forms an interface between the peptidoglycan layer and the external environment. It has been subjected to critical study and a number of reviews and books exist in the literature (Strominger, 1962; Salton & Owen, 1976; Di Rienzo et al., 1978; Lugtenberg, 1981).

The main components of the outer membrane are phospholipid, lipopolysaccharide (LPS) and protein (1:2:2 by weight). The lipopolysaccharide which may occupy 45% of the surface of the outer membrane is composed of three moieties; Lipid A, the core polysaccharide and the O-antigen. The core mainly contains sugars of which heptose and 3-keto-2-deoxyoctonate (KDO) are specific to LPS. The O-antigen is made up of about thirty repeating units each containing 3 to 6 sugar residues (Lugtenberg, 1981) and forms the variable saccharide portion of the LPS.

At least fifty protein species have been isolated from the outer membrane of E.coli K-12 (Sato et al., 1977). These proteins have M.wt ranging from 10,000 to 83,000 and have numerous functions e.g. transport of vitamin B₁₂ and Maltodextrin, general pores for hydrophilic solutes and receptors for phages (Osborn & Wu, 1980). A matrix protein, unlike the lipoprotein, spans the whole outer membrane from innermost murein to the cell surface where it is exposed (Braun & Hantke, 1977) unlike most of the proteins which are intrinsic to the membrane and are not exposed on the surface. A number of outer membrane proteins have been shown to be exposed in Pseudomonas facilis (Rittenhouse et al., 1973), E.coli, Salm.typhimurium (Kamio & Nikaido, 1977) and Neisseria gonorrhoeae (Heckels, 1978).

Besides the cell envelope components other cell surface structures may also be present (Lugtenberg, 1981). These include a layer of capsular material consisting of polysaccharide, flagella and pili (or fimbriae).

2.2 Physico-chemical characteristics of Micro-organisms

From the previous section it is clear that the bacterial cell walls are complex structures varying widely in organisation and composition.

Many components of the bacterial cell surface are charged e.g.

aminosugars of the polysaccharides (Sherbet & Lakshmi, 1973), proteins and teichoic acids (James & Brewer, 1968). The bacterial cell surface has been reported to bear an overall negative charge (Schott & Young, 1972; Neihof & Echols, 1973).

In this section some of the methods which are used to investigate the physico-chemical characteristics of the microbial cell surface are reviewed briefly.

Electrophoresis and Isoelectric focusing of micro-organisms

Electrophoresis

Electrophoresis has been used by many workers to investigate the nature of microbial surfaces. James & List (1966) showed that the electrophoretic mobility of E.coli and Klebsiella aerogenes was not affected by the presence or absence of fimbriae on the capsulated organisms. The presence of glucuronic acid in the capsules imparted a high negative charge to these organisms. It was reported (James & Brewer, 1968) that the negative surface charge of Staph.aureus Cowan 1 (possesses cell surface Protein A) was increased after treating the cells with trypsin while the same treatment of Staph.aureus Wood 46 (does not possess cell surface Protein A) did not result in a change of electrophoretic mobility.

Chemical groups at cell surface may be revealed by comparing the pH-mobility curves of untreated cells with curves of chemically modified cells (Gittens & James, 1963; Richmond & Fisher, 1973) e.g. a Proteus spp. treated with benzene sulphonyl chloride had a higher negative charge compared with the untreated cells and imidazole and amino groups were thought to be substituted. Similarly, Hill et al. (1963) showed that the cell surface of Strep.pyogenes had free carboxyl and amino groups. Neihof & Echols (1978) studied the electrophoresis of bacterial cell walls after chemical modification of the surface charges e.g. carboxyl groups modified by carbodiimide and amino groups by fluorodinitrobenzene. They concluded that the neutralisation of negative wall surface charge allowed the underlying amino groups to become electrokinetically effective. Abramson et al. (1942) examined the mean charge density of E.coli during the growth phase and concluded that actual changes in the physical or chemical constitution of the

bacterial surface can occur during the culture cycle. The variation in the electrophoretic mobilities of micro-organisms was reported by Ramsey et al. (1980). They exploited the differences in mobilities in order to resolve mixtures of organisms into their individual components e.g. E.coli from a mixture of E.coli and Staph.aureus.

Isoelectric focusing

In this technique, a mixture of organisms is subjected to an electric field in a supporting medium in which a pH gradient has been generated. The organisms then migrate and become "focused" at that portion of the pH gradient where the pH is equal to their isoelectric pH and form a band there. The isoelectric points (I_p) of various micro-organisms have been determined by this technique. The results indicate different proportions of charged groups at the surfaces of cells of different species e.g. E.coli has I_p of 5.6 (Sherbet & Lakshmi, 1973), Diplococcus and Brucella abortus have an $I_p < 4.6$ (Sherbet, 1978) and I_p of a strain of Streptococcus was 5.3 (Longton et al., 1975). The differences in I_p values shows potential for separating micro-organisms by this method.

Partition of micro-organisms in an aqueous two-phase polymer system

When biological macromolecules and cells are placed into an aqueous solution of two immiscible polymers i.e. dextran (D) and polyethylene glycol (PEG), they either go into the top phase (PEG) or the bottom phase (D), depending on their partition coefficients. Walter (1977) exploited this technique in order to separate or fractionate cell populations on the basis of surface properties (e.g. hydrophobicity and charge) in a wide variety of mammalian, bacterial and algal cells. Using this technique, Stendahl et al. (1977) found that the smooth form

of Salm.typhimurium exhibited less charge at neutral pH than did the rough form of this organism. Edebo et al. (1977) showed that when smooth bacteria were treated with IgG or IgA antibodies, the surface negative charge was increased and, therefore, the bacteria were more liable to interact with mammalian cells. By partition analysis, Magnusson et al. (1979) showed that Neisseria gonorrhoeae had a low negative surface charge at pH 7.2 whilst at pH 6.0 there was an increase in the negative charge whereas it was reported (Colleen et al., 1979) that Staph.saprophyticus and Staph.epidermidis were negatively charged at pH 7.2.

The techniques described above indicate variability in proportion of charges on microbial cell surfaces and the effect of various factors e.g. pH and growth phase on the physico-chemical properties of microbial cell surfaces.

Interaction of micro-organisms with solid surfaces

The terms adhesion and adsorption are used to describe the interaction between micro-organisms and solid surfaces. There is an extensive and diverse range of solid adsorbents for micro-organisms which include organic and inorganic compounds, soils and minerals, living surfaces and other materials such as charcoal and glass (Daniels, 1967).

Several books (Berkeley et al., 1980; Bitton & Marshall, 1980), review articles (Rotman, 1960; Daniels, 1972; Jones, 1977) and theses (Daniels, 1967; Wood, 1979) describe interactions of micro-organisms with solid surfaces.

Jones (1977) described various fimbrial (e.g. Type 1, 3 and 4), non-fimbrial (e.g. K99 antigen) and colonisation factor (e.g. K88 fibrillar adhesin) adhesins which may be involved in the colonisation of

intestinal mucosa. He concluded that the presence of adhesive appendages on bacterial cell surfaces reduces repulsion by lowering the surface negative charge between the organism and the animal cell. A similar conclusion was reached by Heckels et al. (1976) who studied the interaction of N.gonorrhoeae with mammalian cells. Stendahl & Magnusson (1982) studied the interaction of micro-organisms with phagocytes. They suggested that when specific adhesins (fimbriae) are present on the bacterial cell surface they may, together with more general physico-chemical properties, promote attachment. The attachment of Salm.typhimurium to Hela cells was studied recently by Jones et al. (1981) who suggested that the interaction occurred in three phases. The motility of bacteria promoted their contact with Hela cells. At this stage there was a reversible binding with weak long-range attractive forces being involved. This was followed by irreversible attachment with bacteria approaching close to Hela cells. Perers et al. (1977) studied the interaction of Salm.typhimurium and E.coli with mouse intestinal mucosa and suggested that their physico-chemical surface properties may be responsible for the association with the host cells. Cohen et al. (1981) showed that the adhesion of E.coli to human epithelioid tissue culture cells followed Michaelis-Menten enzyme kinetics i.e. it was linear upto 10^9 bacteria per ml after which saturation occurred.

The interaction of viruses with ion exchangers has been studied (Puck & Sagik, 1953; Hoyer et al., 1958). Puck & Sagik (1953) demonstrated that the adsorption of bacteriophages T1, T2 and influenza virus to strong anion exchanger was not salt-dependent whereas attachment to strong cation exchanger was salt-dependent. They suggested that the cations neutralised the repulsive electrostatic potential due to excess

of negative charges on the virus. Hoyer et al. (1958) found differences in affinity of various viruses for cellulose anion exchange resins. It was concluded that such differences may be due to virus surface components (e.g. proteins). Rotman (1960) showed that although the cell wall constituents (e.g. diaminopimelic acid and/or amino sugars) of bacteria provided cell surface charge, the differences in the adsorption of some bacterial species to ion exchange resins was much more difficult to explain. For instance, the cell wall composition and electrophoretic behaviour of cells that adsorbed and the cells that did not adsorb was similar. They concluded that the presence of charges on bacterial surfaces was not the only factor which influenced the adsorption of bacteria to ion exchange resins but that other factors may play a major role. The bacterial cell was later described as a 'macroscopic zwitterion' (Daniels, 1967). At a pH below the apparent I_p the bacterium behaved as a cation and therefore adsorbed to the cation exchange resin whereas at a pH greater than the I_p it behaved as an anion and therefore adsorbed to the anion exchange resin. This concept was based on the separation of six bacterial species (Bacillus cereus, B.subtilis, E.coli, Proteus vulgaris, Pseudomonas ovalis and Staph.aureus) from aqueous suspensions by adsorption onto strong anion and cation exchange resins. More recently, Daniels (1980) has described various attractive and repulsive forces involved in the interaction between a microbial cell and the adsorbent. For instance, the attractive forces include ion-pair formation ($-\text{NH}_3^+ \dots -\text{OOC}-$), ion-triplet formation ($-\text{COO}^- \dots \text{Ca}^{2+} \dots -\text{OOC}-$) and van der Waals forces of attraction while the repulsive forces include repulsion between surfaces of similar charge and van der Waals forces of repulsion. Moreover, the reversible sorption between micro-organisms and solid surfaces may

occur with attraction at separation distances upto the secondary minimum (10-100nm) while irreversible sorption occurs after the sorbed cell overcomes the repulsive barrier and moves still closer to the adsorbent surface i.e. in the primary minimum (<10 nm; Rutter, 1980). Recently, Kolot (1981) has described various types of microbial carriers and has suggested an electrostatic interaction between the negatively charged bacteria or yeast cells with the positively charged DEAE-sephadex gel and the AG-21K resin. In a review, Daniels (1972) had described the separation of binary mixtures of organisms into their individual components on the basis of differential adsorption to or elution from the anion and cation exchange resins. E.coli and Staph.aureus were separated from a binary mixture using a cation exchange resin column (Wood, 1979). DEAE-cellulose columns were used for the separation of Salm.typhimurium and E.coli (Hall et al., 1976), non-fimbriate and fimbriate cells of Salm.typhimurium (Hall et al., 1978) and capsulated and non-capsulated cells of Klebsiella aerogenes(Hall & Jafri, 1980).

Overall, the survey illustrates the complex nature of bacterial cell envelope. The physico-chemical properties of the microbial surface e.g. electrical charge and hydrophilic-hydrophobic balance have been established by a number of techniques and indicate the potential for the physical manipulation of microbial populations by exploitation of surface properties.

2.3 Development of methods for the detection of Salmonella

Over the last 12 years, the total number of food poisoning cases reported in England has almost doubled from ca 8000 in 1970 to 14,500 in 1982 (Sheard, 1984). Salmonellas, especially Salm.typhimurium

caused the majority of the outbreaks e.g. in 1982 Salmonella spp. accounted for 56% of the total followed by campylobacters (18%), Cl.perfringens (5.4%) and Staph.aureus (0.62%) (Sheard, 1984).

Similarly, in Canada and the United States between 1973 and 1977 the three main food-poisoning micro-organisms were Salmonella, Staph.aureus and Cl.perfringens (Todd, 1983).

Clearly, Salmonella is a major public health problem and methods for the detection of these organisms are an important facet in the Quality Control of foods.

This brief review covers the general development of methodology for detection of salmonellae in foods and/or clinical specimens.

Cultural methods

The conventional cultural procedures for the isolation and identification of Salmonella spp. in foods are tedious, time-consuming and costly requiring 4-5 days for the presumptive identification and upto 7 days for confirmation (AOAC, 1965; ICMSF, 1978). The procedures involved are:

- 1) Preenrichment in a non-selective medium for 24h.
- 2) Enrichment in selective media for 24 to 48h.
- 3) Isolation on selective differential agar media requiring upto 48h (Litchfield, 1973).

There are various types of preenrichment media (Litchfield, 1973; D'Aoust, 1981) used basically for two reasons for the detection of

salmonellae in foods. Firstly, to resuscitate salmonellae injured by various food-processing conditions e.g. low temperature, heat, drying and radiation . Secondly, very low numbers of salmonellae may be present in foods (e.g. 1 cfu/50g). In order to improve recoveries of Salmonella, a preenrichment step may be required for dehydrated foods such as dried milk products (Litchfield, 1973) but probably not required for raw foods and finished products (Fagerberg & Avens, 1976).

Among the numerous types of selective enrichment media (Fagerberg & Avens, 1976; Litchfield, 1973; D'Aoust, 1981), those based on the inhibitors selenite (Leifson, 1936) and tetrathionate (Kauffman, 1930) have been widely used to recover salmonellae from foods. The selective enrichment broths are used to increase the numbers of Salmonella present whilst suppressing the growth of other Gram-negative organisms such as coliforms, Proteus and Pseudomonas (Fagerberg & Avens, 1973). Direct selective enrichment (i.e. no preenrichment) has been used for certain raw or highly contaminated food products (APHA, 1976) as well as clinical samples where a large population of physiologically active salmonellae may be present. However, D'Aoust (1981) has shown that detection of salmonellae in raw meats was substantially greater with preenrichment than with direct selective enrichment.

Recently, Sveum & Kraft (1981) have described a combined preenrichment and selective enrichment technique in which inhibitors are added to the preenrichment broth after 4h of incubation. The value of this procedure has yet to be confirmed (van Schothorst & van Leusden, 1972).

In the conventional procedure for isolating Salmonella from foods, a portion of the enrichment broth is streaked on a selective or a differential agar medium for isolating suspect salmonellae. The common media include brilliant green agar, Xylose lysine desoxycholate agar and bismuth sulphite agar (Fagerberg & Avens, 1976) and may have one or more of the following functions; distinguish lactose fermenters from non-lactose fermenters, inhibit enteric bacteria other than salmonellae; inhibit Gram-positive organisms and suppress spreading by Proteus spp. (Litchfield, 1973; Moats, 1981).

The suspect Salmonella colony isolated on the selective agar media is then confirmed by a variety of biochemical reactions (Litchfield, 1973) of which triple sugar-iron and lysine-iron agars are the most common (Moats, 1981). Finally, antigenic analysis using both O and H antisera is necessary in order to identify a suspect Salmonella isolate (Edwards & Ewing, 1972).

Rapid methods

The preceding section dealt with the isolation and identification of salmonellae from foods by the conventional cultural procedures which are tedious, time-consuming (ca 5-7 days), costly and require considerable skill in the selection of colonies to be tested. In the food industry, the time required to obtain results is one of the most serious constraints to testing as a means of microbiological control and more rapid methods would lead to more rapid release of finished food products subject to microbiological control (Silliker, 1982). The rest of this review will deal with rapid (≤ 3 days) immunological and non-immunological techniques for detection of salmonellae in foods and/or clinical specimens.

Immunological methods

In the rapid immunological techniques, generally, the selective enrichment broth cultures are subcultured for a short time (i.e. 4-6h) in a non-selective broth (e.g. M broth; i.e. post-enriched) to permit good antigen development prior to detection of salmonellae. The various immunological techniques are described briefly.

Sperber & Deibel (1969) reported an Enrichment Serology (ES) technique in which the M broth culture was mixed with Spicer-Edwards flagellar antiserum and incubated at 50°C for 1h. Provided that sufficient numbers of salmonellae were present (i.e. 5×10^7 cells/ml), flocculation was indicative of Salmonella. Longer incubation (i.e. 24h) of the M broth was reported to increase the sensitivity of the method (Boothroyd & Baird-Parker, 1973).

The Fluorescent Antibody (FA) technique for detection of salmonellae first reported by Thomason et al. (1957) is now an official screening method (Anon, 1975). In the direct FA method, an aliquot of the enrichment or post-enrichment culture is fixed on a glass slide and the reaction between somatic and/or flagellar antigens and fluorescein conjugated antibodies is visualised by fluorescence microscopy (Gibbs et al., 1972; Insalata et al., 1972; Fantasia et al., 1975; Swaminathan et al., 1978). In the indirect FA method two steps are involved. The sample is first exposed to unlabelled specific antibody (prepared in rabbits) followed by thorough washing and then to the fluorescein-conjugated sheep antibody to rabbit immunoglobulin prior to visualisation (Wood, 1970; Gibbs et al., 1972; Svenungsson et al., 1979). The major disadvantage of the FA techniques is high incidence

of false-positive reaction (Gibbs et al., 1972; Thomason, 1981) i.e. a rate of 1-9% with even higher rates for certain foods whereas the rate of false-negative reactions is significantly lower i.e. 0-1.6% (Silliker, 1982).

The Enzyme-linked ImmunoAssay (EIA) technique for detection of salmonellae in foods was first reported by Krysinski & Heimsch (1977). In the indirect EIA technique (Krysinski & Heimsch, 1977), rabbit Salmonella H antibodies were reacted with salmonellae adsorbed to membranes. The membrane was then soaked in peroxidase-labelled goat anti-rabbit antibodies. When the membrane is immersed in substrate solution, insoluble brown product is deposited where the salmonellae are present. Minnich et al. (1982) reported an improved indirect EIA in which polyvalent Salmonella H antiserum free of 'O' antibodies was used. In addition, peroxidase, which may occur as intrinsic bacterial peroxidases, was replaced by alkaline phosphatase in the goat anti-rabbit antiserum detection system in order to reduce the false-positive reactions. In this Enzyme Linked ImmunoSorbent Assay technique (ELISA; Minnich et al., 1982), salmonellae were adsorbed to wells of a microtitration plate and reacted with rabbit polyvalent flagellar (H) antisera. After washing away unbound serum, alkaline phosphatase coupled goat anti-rabbit antiserum was added to the well. Unbound globulin conjugate was removed by washing and alkaline phosphatase substrate was added. The soluble yellow product, indicative of a positive result was measured spectrophotometrically. In the direct EIA reported by Swaminathan & Ayres (1980), fixed smears of enrichment cultures are reacted with peroxidase-coupled anti-Salmonella (H) antibody. After adding peroxidase substrate, positive samples exhibit cells with brown cell envelopes and flagella under

light microscope. Robison et al. (1983) have reported a direct EIA which utilises alkaline phosphatase labelled IgA monoclonal antibody for detection of salmonellae in food. In this EIA, heat-solubilised extracts of flagella (and not whole cells) were adsorbed to wells of a microtitration plate and reacted with the alkaline phosphatase-coupled IgA antibodies. Unbound conjugate was removed by washing and the substrate was added. The soluble yellow product was then measured spectrophotometrically. Although the sensitivity of EIA techniques is high (i.e. 10^5 salmonellae/ml; Minnich et al., 1982) and therefore applicable to preenrichment broths, the major drawback is the high proportion of false-positive reactions similar to the level found by Silliker (1982) for the FA technique (i.e. 7-10%). Although the level of false-positives is high, FA technique would clear most batches of foods much earlier than conventional technique.

The potential of radiometric techniques for detection and estimation of bacteria in foods was reported by Previte (1972). The detection system was based on the measurement of $^{14}\text{CO}_2$ evolved from the metabolism of ^{14}C -glucose. Salm.typhimurium at a concentration of 10^4 cfu/ml of trypticase soy broth were detected in 3 or 4h. Recently, a radiometric method based on evolution of $^{14}\text{CO}_2$ from ^{14}C -dulcitol was reported for the rapid detection of Salmonella in meat, eggs and coconut (Stewart et al., 1980). Although almost all Salmonella spp. and a few other Enterobacteriaceae metabolise ^{14}C -dulcitol with liberation of $^{14}\text{CO}_2$, the confirmatory test is also necessary. This is based on the inhibition of dulcitol utilisation by reaction with Salmonella poly H agglutinating antiserum. Using this method, Koning et al. (1982) have reported ca 7% false-positive reactions.

Coagglutination techniques, based on the use of cells of the protein A-bearing Cowan 1 strain of Staph.aureus, have been used in clinical laboratories for detection of salmonellae from selective enrichment broths (Goldschmidt, 1982). Protein A on the cell surface binds to the F_c portion of immunoglobulin G molecules thus leaving the two F_{ab} portions for normal immunological reactions. The coupling of the 'sensitised staphylococci' containing protein A-immunoglobulin complex with the homologous antigen (e.g. Salmonella) results in an agglutination reaction (Svenungsson & Lindberg, 1979; Sanborn et al., 1980).

Non-immunological methods

The various non-immunological rapid techniques for detection of salmonellae in foods include Hydrophobic Grid-Membrane Filter (HGMF) technique (ca 3 days), DNA-DNA hybridisation assay (<30h) and High Pressure Liquid Chromatography (HPLC; ≤ 24 h).

HGMF technique has been applied successfully for detection of Salmonella from selective enrichment broths (Entis et al., 1982). The unique property of ISO-GRID membranes is that it restricts the lateral spread of virtually all microbial colonies and therefore allows the detection and enumeration of specific micro-organisms in the presence of higher levels of background flora. The ISO-GRID HGMF method involves 24h preenrichment and a 6-8h selective enrichment of which an aliquot (10-100 μ l) is filtered through the HGMF. The filter is placed on selective lysine agar and incubated for 24h. Presumptive salmonellae are identified by a specific colour reaction. Further confirmation is accomplished by replicating on further sterile HGMFs placed on various biochemical agar media (e.g. lactose fermentation agar) and incubated for a further 24h.

Fitts et al. (1983) have reported a DNA-DNA hybridisation technique for detection of Salmonella in foods. In this technique, fragments of Salm.typhimurium DNA (a 'conserved gene') which are specific for the genus Salmonella were radio-labelled (^{32}P). These fragments were used as DNA probes to detect complementary sequences of DNA in salmonellae, concentrated and lysed, on nitrocellulose filters. The total detection time for salmonellae in foods was <30h and included a 24h preenrichment step. However, the detection of Salmonella required a concentration of $\geq 10^8$ cells/ml of the preenrichment broth; the lower limit of sensitivity for detection of salmonellae was not reported.

The HPLC method for detection of salmonellae in milk makes use of the Salmonella specific bacteriophage Felix-01 (Hirsh & Martin, 1983). The technique involves concentration of salmonellae on electropositive filter followed by elution with brain heart infusion broth pH 10. After incubation of the broth at 42°C for 4 or 16h, the cells were harvested by centrifugation and resuspended in CaCl_2 -supplemented nutrient broth. Bacteriophage (3×10^8 PFU/ml) was added and broth incubated at 37°C for 2h. Chloroform was then added and the tube shaken vigorously. After centrifugation for 15 min at 1,000g, the aqueous phase was removed and filtered (0.45 μm filter). The increase in number of bacteriophage in the filtrate (in presence of salmonellae) was detected by HPLC utilising a gel-permeation column. Five salmonellae/ml of milk could be detected within 24h of sample collection.

The future trend in the rapid detection of salmonellae in foods is towards development of novel techniques which may be applied after the preenrichment step of the conventional cultural procedure. These

techniques include the use of highly specific monoclonal antibodies and complementary DNA fragments against salmonellae in enzyme-linked immunoassays utilising the sensitivity and specificity of Biotin-Avidin systems (Kendall et al., 1983).

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

The traditional methods for the enumeration of bacterial cell numbers, little changed for more than a century, have two major disadvantages. Firstly, the methods are labour-intensive since they involve manipulations such as serial dilution of the sample and inoculation of the growth medium. Secondly, they require at least 24 to 48h for the colony forming unit to form a visible colony of cells which can be enumerated.

In the development of more rapid methods for the estimation of microbial population both of these aspects are considered. Several books (Hedén & Illeni, 1975; Johnston & Newsom, 1976) and reviews (Wood, 1979; Pettipher, 1981; Stannard et al., 1982; Wood & Gibbs, 1982) have been published on rapid microbiological methodology. The following review describes rapid methods under two main categories.

Cell and colony-counting methods, based on the principles of the traditional techniques.

Non-counting methods, based either on the estimation of microbial cell components or aspects of microbial metabolism.

Cell and colony-counting methods

Direct cell counts

The direct microscopic count of bacteria in foods such as milk is simple and rapid, involving the preparation of a milk film followed by

staining and microscopic examination. (Breed & Brew, 1916). This method is limited by low sensitivity, interference from food components and operator fatigue associated with long periods at the microscope.

These problems have generally been overcome by the development of a rapid Direct Epifluorescent Filter Technique (DEFT) for the estimation of micro-organisms in raw milk and some other food products (Pettipher et al., 1980). The method uses membrane filtration and epifluorescent microscopy. Raw milk is treated with trypsin and Triton X-100 for 10 min at 50°C in order to facilitate filtration of sufficient volume of milk through a 0.6 µm polycarbonate membrane. The bacteria retained on the filter are stained with acridine orange/Tinopal stain and counted using an epifluorescent microscope. The DEFT technique is highly sensitive, with a lower detection limit of ca 6×10^3 bacteria/ml, and rapid, taking ca 25 min to complete.

Variants of the colony counting methods

Miniaturised techniques

In these methods the time and labour required for sample preparation i.e. serial dilution and inoculation of the growth medium, have been reduced.

In the loop-dilution method (Fung, 1969) 0.025ml of the sample, delivered by a calibrated loop, was serially diluted in 0.225ml of the sterile diluent placed in wells of a microtitre tray. Sharpe & Kilsby (1971) developed an agar droplet technique in which samples were diluted in molten nutrient agar and 0.1ml aliquots of agar cast as drops in which organisms could grow to visible colonies. The problem that can occur with this technique is interference from small non-microbial particles e.g. food components.

Instrumented techniques

A saving in time and materials can also be made by eliminating the need for the dilution series in the standard plate count method and employing instruments to perform the manipulations required.

An example is the mechanisation of the loop dilution technique of Thompson et al. (1960) for the enumeration of bacteria in milk. In this technique two loops which retain 0.01ml and 0.001ml are positioned above two petri dishes and flushed with the diluent. Nutrient agar is then added and the contents of the petri dish mixed. This technique has resulted in the development of the Petri-Foss (Foss Electric, Denmark) plate loop machine which can prepare upto 300 samples per hour compared with 20-30 per hour by manual methods.

Gilchrist et al. (1973) developed a Spiral plate method which also avoids the use of a dilution series. This machine dispenses a known volume of sample on a rotating agar plate such that the volume of inoculum on any given area is known. The separated colonies in a given area are counted and the concentration of organisms is then calculated. Jarvis et al. (1977) showed a good correlation ($r = 0.96 - 0.99$) between the total microbial population of foods obtained by the Spiral plate method and four colony count techniques. The time consuming operation involved in counting colonies has also been subject to automation. The Laser Colony Counter uses a laser beam to scan a plate. When the beam is interrupted a colony is registered. Either all the colonies or colonies in an area of the plate are counted. In the latter case, the concentration of organisms is determined from a calibration curve.

Membrane filtration techniques

Fifield et al. (1957) reported a membrane filtration method for enumerating coliform bacteria in raw and pasteurised milk. In this method, the membrane filter retaining viable cells were placed on a pad of nutrient medium and incubated for 1-3 days prior to enumeration of the colonies. A variant of this method (i.e. Hydrophobic Grid Membrane Filter; HGMF) described previously (section 2.3), has been used to enumerate coliforms in foods (Sharpe et al., 1979).

Non-counting methods

Estimations based on microbial metabolism

In these methods, changes induced by microbial metabolism are measured. The measurement may then be converted into bacterial numbers by extrapolating from a calibration curve. The estimations based on microbial metabolism are generally more rapid than the conventional growth amplification methods.

Dye reduction test

The dyes, resazurin and methylene blue, change colour as a result of their reduction by growing bacteria. These tests were used in grading raw milk (Anon, 1968) but are now suspect. There may be at least two reasons why the dye reduction times and the estimates of numbers of bacteria fail to correlate (Pettipher, 1981). Firstly, the reducing activities of various bacteria are different. In this context, the change in practice of handling milk has resulted in a change from mesophilic to psychophilic flora which has lower reducing activity than the mesophilic flora. Secondly, the clumping of bacteria does not lower the reducing activity whereas the plate count is lowered. These tests can provide estimates of microbial levels of approximately

10^5 cfu and 10^7 cfu in 7 to 1h, respectively. In general, the tests are not used for quantification but rather as a semi-quantitative measure of microbial populations in milk.

Radiometric estimations

Estimation of numbers of micro-organisms in foods by radiometric determination of $^{14}\text{CO}_2$, produced by metabolism of ^{14}C -labelled glucose, was reported by Rowley et al. (1976). There was an inverse linear relationship between detection and the initial concentration of micro-organisms. Rowley et al. (1978) tested 404 samples of cooked and frozen foods of which 95% were correctly classified within 6h as having microbial populations of $\geq 1 \times 10^5$ cfu/g. Estimates of micro-organisms in frozen orange juice was reported by Hatcher et al. (1977) who showed that 10^4 cfu/ml of yeast, Leuconostoc and Lactobacillus could be detected in 6, 7 and 10h, respectively. Of 600 orange juice samples examined, 44 positives (10^4 cfu/ml) were detected in 12h, 41 of which were positive in 8h.

Microcalorimetric estimation

All organisms liberate heat during metabolism and micro-calorimetry is a sensitive technique for measurement of this heat (Mor, 1976). In addition to the study of antibacterial effects of antibiotics and identification of micro-organisms (Mårdh et al., 1976), microcalorimetry has been used to screen urine samples for bacterial content (Beezer et al., 1974). In a later study, Beezer et al. (1978) demonstrated a linear relationship between the heat output and initial concentration of organisms over the range 10^5 - 10^9 cfu/ml.

Impedimetric estimation

The impedimetric estimation of micro-organisms is based on the measurement of changes in the electrical properties of nutrient media resulting from growth of the organisms. These changes are continuously monitored by passing alternating current through the media and comparing the impedance of the inoculated media with that of uninoculated media or a preset impedance value. Cady (1975) demonstrated an inverse linear relationship between numbers of micro-organisms and the impedimetric detection time using a bactometer viz. 10^3 and 10^2 cfu/ml of E.coli were detected in 4 and 5h, respectively.

In food microbiology, impedimetric techniques have been investigated mainly for the estimation of total viable micro-organisms (Wood & Gibbs, 1982). Hardy et al. (1977) reported the impedimetric estimation of micro-organisms in frozen vegetables i.e. 10^5 cfu/g could be detected in 4.5h with >90% agreement between the impedimetric and colony count methods. With raw meats, Wood & Gibbs (1982) showed that there was >90% agreement between the impedimetric detection time and the colony count.

Estimations based upon microbial cell components

Direct estimation of the microbial surface structures or metabolic components provide the most rapid methods for estimating microbial populations than the conventional growth amplification methods. Two of these methods are considered.

Limulus Lysate Test (LLT)

The LLT was devised on the basis of an observation that Gram-negative bacterial infections of Limulus polyphemus (horseshoe crab) produced

intravascular coagulation (Bang, 1956). Levin & Bang (1968) later described the specific coagulation of a lysate of amoebocytes prepared from Limulus polyphemus in the presence of nanogram quantities of endotoxin.

The LLT has been evaluated for detecting pyrogens in clinical samples (Jorgensen & Jones, 1975) and in determining the microbial quality of foods e.g. chilled ground beef (Jay, 1977). Previously, Jorgensen et al. (1973) found an inverse relationship between the time for coagulation and the number of organisms and that 10^5 cells/ml could be estimated within 1h. Coates (1976) demonstrated that pretreatment of bacterial cells with saline/EDTA greatly improved the sensitivity of the assay with 4×10^2 cells detectable in 1h. Since the LLT is based on reaction with endotoxin of Gram-negative organisms, the test would detect both viable and non-viable cells.

Adenosine triphosphate (ATP) assay

The technique depends on the fact that all living cells contain ATP, which is an important cellular nucleotide serving as an energy source for many of the cells metabolic functions. An 'average' bacterium contains around one femtogram (10^{-15} g) of ATP and the bioluminescent assay of ATP provides estimates of the numbers of bacteria in the sample (Stanley, 1982). The bioluminescent reaction sequence is as follows:

The light produced is proportional to the ATP concentration and permits the estimation of picogram levels (10^{-12} g) of ATP which corresponds to ca 10^3 bacteria. This level will depend on the sensitivity of the luminometer used.

Levin et al. (1967) reported a rapid bioluminescent assay for quantification of microbial contamination of water, whereas its use for the estimation of micro-organisms in foods was reported by Sharpe et al. (1970) and Wood (1979). However, attempts to estimate low levels (ca $\leq 10^6$ cfu/g) of microbial populations in a range of foods were not successful due to the presence of high levels of ATP intrinsic to foods (Sharpe et al., 1970). Therefore, in order to estimate microbial populations in foods, destruction or separation of non-microbial ATP from the microbial ATP was considered necessary.

The selective extraction of ATP from the non-microbial material and the enzymatic destruction of the 'free' ATP has been reported by Vanstaen (1980) for the estimation of yeasts in orange juice. The orange juice was treated with a nucleotide releasing agent (NRS) which selectively extracted ATP from the somatic cells in the orange juice. The non-microbial ATP released was then destroyed by incubation with ATPase for 45 min at 37°C. The yeast ATP was released by treating yeast cells with a stronger nucleotide releasing agent (NRB).

The physical separation of bacteria from raw meat homogenates prior to the rapid estimation of microbial ATP has been reported (Patel & Wood, 1983; Stannard & Wood, 1983). Stannard & Wood (1983) used a three-stage process involving low speed centrifugation, cation exchange resin

and 0.22 μm filtration in order to separate and concentrate micro-organisms from the meat homogenates. A linear relationship was demonstrated between $\log_{10}$ fg ATP/g and $\log_{10}$ cfu/g of meat in the range 10^5 - 10^8 cfu/g.

PART II

EXPERIMENTAL

PART II

Chapter 3: GENERAL MATERIALS AND METHODS

3. GENERAL MATERIALS AND METHODS

Micro-organisms

The following food-associated micro-organisms were used in this study:

Escherichia coli NCTC 9001, Staphylococcus aureus NCTC 8530,

Pseudomonas fluorescens NCTC 10038, Lactobacillus plantarum NCIB 8960

and Salmonella typhimurium NCTC 74. (see also Table 3.1)

The organisms, Staphylococcus saprophyticus 2 (haemagglutinating strain) and Staphylococcus saprophyticus 11 (non-haemagglutinating strain) were supplied by Dr. P.A. Mårdh of University of Lund, Sweden. Other organisms were obtained from international culture collections.

Preparation of microbial cultures

Microbial cultures were grown in 10ml of Trypticase Soy Broth (Table 3.1) and the cells were harvested by centrifugation at 2,000g for 10 min. The cells were washed three times by centrifugation and resuspension in the appropriate buffer. These cell suspensions were used in experiments e.g. column application. In order to investigate the separation of binary mixtures of pure cultures of organisms, mixed inocula of two types of organism in the appropriate buffer was used.

Enumeration of micro-organisms

Viable counts of micro-organisms were determined using the Spiral Plate Method (Jarvis et al., 1977). All dilutions were prepared in sterile Ringers and Peptone (R & P) solution (quarter-strength, containing 0.1% Difco proteose peptone).

Viable organisms were estimated using the appropriate selective media (Table 3.1).

TABLE 3.1 Micro-organisms and cultural conditions

Culture	Temperature and time of incubation in growth medium (TSB ¹)	Selective plating media ² /temperature and time of incubation
<u>GRAM-POSITIVE ORGANISMS</u>		
<u>Staphylococcus aureus</u> (NCTC 8530 & NCTC 7121)	37°C, 16h	BP; 37°C, 24-48h
<u>Staphylococcus saprophyticus</u> (Haemagglutinating and non-haemagglutinating strains)	37°C, 16h	BP; 37°C, 48h
<u>Lactobacillus plantarum</u> (NCIB 8960)	30°C, 48h	MRS; 30°C, 48h
<u>Streptococcus lactis</u> (NCIB 10484)	30°C, 16h	MRS; 30°C, 24-48h
<u>GRAM-NEGATIVE ORGANISMS</u>		
<u>Escherichia coli</u> (NCTC 9001 & NCTC 10650)	37°C, 16h	VRBA; 37°C, 16h
<u>Pseudomonas fluorescens</u> (NCTC 10038)	25°C, 24h	CFC; 25°C, 24-48h
<u>Salmonella typhimurium</u> (NCTC 74 & Var. Copenhagen)	37°C, 16h	BGA or XLD; 37°C, 24h
<u>Citrobacter freundii</u>	37°C, 16h	XLD; 37°C, 24h

1. Trypticase Soy Broth (Gibco)

2. BP; Baird-Parker (1962) medium (Oxoid)
MRS; Bacto-Lactobacilli MRS broth (Difco; de Man et al.,1960)
VRBA; Violet Red Bile Agar (Oxoid)
CFC; Cephaloridine-Fucidin-Cetrimide Agar (Mead & Adams, 1977)
BGA; Brilliant Green Agar (Oxoid CM263; Kauffman, 1935)
XLD; Xylose Lysine Desoxycholate medium (Oxoid)

Meat and Dairy products

These were either purchased locally or obtained from the Meat and Fish Section of the Food R.A. Otherwise they were supplied by the member companies of the Food R.A.

Adsorbents

Ion exchange resins and gels

The resins used in this study were obtained from Bio-Rad laboratories (Richmond, California, U.S.A.), ion exchange gels were purchased from Pharmacia Fine Chemicals (Prince Regent Road, Hounslow, Middlesex).

<u>Resin/gel type</u>	<u>Polymer lattice</u>	<u>Exchange groups</u>
Cation exchange resin, Bio-Rex 70	acrylic polymer	$\text{R-COO}^- \text{Na}^+$
Anion exchange resin, AG3 X4A	styrene-divinylbenzene copolymer lattice	$\text{O-CH}_2\text{N}^+(\text{R})_2\text{Cl}^-$
Cation exchange gel, CM-sepharose CL-6B	agarose polymer	$\text{R-O-CH}_2\text{COO}^- \text{Na}^+$
Anion exchange gel, DEAE-sepharose CL-6B	agarose polymer	$\text{R-O-CH}_2\text{-CH}_2\text{-N}^+\text{H} \begin{array}{l} \diagup \text{C}_2\text{H}_5 \\ \diagdown \text{C}_2\text{H}_5 \end{array} \text{Cl}^-$

Further details of the particle size and degrees of cross-linking of the polymer lattice are given in the appropriate chapters.

Hydrophilic-hydrophobic gels

Phenyl-sepharose CL-4B and Octyl-sepharose CL-4B (Pharmacia Fine

Chemicals) are derivatives of the cross-linked agarose gel sepharose and contain hydrophobic phenyl and n-octyl groups, respectively. They have the following structures:

Anionic polyelectrolyte flocculant CA243

The soluble polyelectrolyte (ChemViron Ltd., Grays, Essex) is essentially a hydrolysed polyacrylamide consisting of polyacrylic backbone with negatively charged carboxyl functional groups, viz:

The flocculant solution (0.01%^m/v) was prepared in sterile distilled water by mixing vigorously using a magnetic stirrer for 30 min.

Batch equilibration of the adsorbents

The amounts of anion and cation exchange resin and the volumes of both hydrophilic-hydrophobic gels and cation exchange gel used for the preparation of large columns (1.6 x 6.5cm) was approximately 10g of resin and 20ml of gel. For preparation of small columns (0.6 x 6.5cm) the amount of resin or volume of gel were halved. Sepharose CL-6B (approximately 10ml) was used for the preparation of small columns.

For the cation exchange resin Bio-Rex 70, an appropriate amount of resin in a 250ml conical flask was shaken with approximately 50 to 200ml of 0.05M phosphate-citrate buffer pH 3.0, depending on the final

equilibration pH value required. If the pH value required was low (e.g. pH 5.0) then the volume of buffer used was large (approximately 200ml) and vice versa (with other solid adsorbents appropriate amounts of resin or volumes of gel were shaken with 100-200ml of the equilibration buffer). The pH of the suspension was noted and the flask allowed to stand until the adsorbent had settled. The supernatant liquid was decanted and the washing stage was repeated until pH of the adsorbent was close (± 0.3 pH unit) or equal to the desired equilibration pH. The adsorbent was washed a further 1 to 3 times with 0.05M phosphate-citrate buffer of the desired equilibration pH, resuspended in the same buffer and de-aerated under vacuum. During the final washing stages the pH value of the equilibrated resin remained constant. This equilibrated adsorbent was used for the preparation of both large and small columns.

Chromatographic columns

Two types of column systems were used unless specified otherwise.

Preparation of large adsorbent columns (1.6 x 6.5cm) for automatic elution and monitoring of micro-organisms (Plate 3.1)

A precision bore glass tube (1.6 x 35cm) was assembled together with the flow adaptors according to the LKB 2137 chromatography column instruction (No: 9001 0422, LKB Ltd., Bromma, Sweden), except that the cooling jacket unit was omitted. A buffer reservoir and a 3-way valve system were connected onto the top of the column. The column effluent end was connected via a peristaltic pump (Varioperpex, LKB Ltd.) and a flow-through cell in a spectrophotometer SP6-350 (Pye Unicam) to the fraction collector (Ultrorac 7000, LKB Ltd.). A recorder 2210 (LKB Ltd.) was linked to the spectrophotometer.

1. Buffer reservoir	5. Spectrophotometer
2. 3-way valve	6. Fraction collector
3. Adsorbent column	7. Chart recorder
4. Peristaltic pump	

Plate 3.1 Apparatus for the automatic elution and monitoring of micro-organisms from columns of adsorbent

The equilibrated adsorbent was packed into the column (1.6 x 6.5cm) by the procedure described for Ultrogel (LKB Ultrogel instruction manual No: 1-2204-E01, LKB Ltd.). The column packing flow rate was adjusted to 1.0ml/min and 0.3ml portion of culture was applied to the column via the 3-way valve system. Elution (flow rate approximately 1.1ml/min) was continued with the equilibration buffer from the reservoir. The column effluent was monitored at 420nm and 1 or 2ml fractions were collected.

When 20ml of the buffer was eluted from the column, distilled water was placed in the reservoir and elution continued until all fractions in the following peak were collected. The fractions comprising the first peak (buffer elution) were pooled as were the fractions in the second peak (distilled water elution) and the total viable count determined for each peak using selective media appropriate to the organisms applied to the column (Table 3.1). An experiment was normally completed within 1 hour.

Preparation of small adsorbent columns (0.6 x 6.5cm) for manual operation (Plate 3.2)

A pellet of glass wool was placed in the tapered end of a glass tube (0.6 x 12cm) in order to retain the adsorbent. A polyethylene tube (approximately 6cm) was connected to the tapered end through which the flow was adjusted by a gate clip and the whole unit was mounted vertically.

The gate clip was closed and equilibrated adsorbent was applied to the column using a pasteur pipette. When a thin layer of resin had settled, the gate clip was opened (flow rate approximately 0.35ml/min). More adsorbent was applied to the column until the bed

1. Tapered glass column	4. Gate clip
2. Adsorbent bed	5. Polyethylene tube
3. Glass wool	6. Universal bottle

Plate 3.2 Apparatus for the manual operation of adsorbent columns

height was approximately 6.5cm. A 0.1 to 1ml portion of culture was applied to column and elution (flow rate approximately 0.35ml/min) was continued with the equilibration buffer until a final eluted volume of 10ml was collected. The column elution was continued with 10ml of sterile distilled water and the effluent (10ml) was collected. Viable counts in both, the buffer and distilled water effluents were determined. An experiment was normally completed within 1 hour.

Unless specified otherwise small columns were used throughout this study.

Separation of binary mixtures of micro-organisms grown in foods

The following protocol was used:

Food	+	R & P or sterile	+	Mixture of two types
(10g)		distilled water		of organisms
		(90ml)		(2ml)

- 1) Homogenise in stomacher 400 (Seward Labs.) for 2 min* and incubate at appropriate temperature (e.g. a mixture of E.coli and Staph.aureus at 37°C for 16 h).
- 2) Filter homogenate through glass wool.

Food homogenate*

- 3) 5ml** + 25 µl calcium chloride solution (20%^m/v) + 1ml of anionic flocculant CA243 solution (0.01%^m/v). Gently shake, stand (10 min) and centrifuge (2,000g for 30s with zero holding time).

- 4) Discard deposit and centrifuge (2,000g for 15 min) 5 ml of supernatant. Wash pellet twice by centrifugation (2,000g for 10 min) and resuspension with 7ml of buffer at appropriate pH value.
- 5) Resuspend pellet in 2 or 3ml of the same buffer* and apply 1ml portion to the adsorbent column at appropriate pH value.
- 6) Elute with 9ml of the equilibration buffer*.

* Sample (1ml) for selective enumeration.

** As a control, sterile distilled water was substituted for calcium chloride and the flocculant.

Protein estimation

Protein estimations were made by the method of Lowry et al. (1951).

Standard solution (40-400 μ g/ml) of bovine serum albumin was prepared and a standard curve constructed of absorbance at 750nm against the protein concentration. The protein concentration of unknown samples was obtained by interpolation.

PART II

Chapter 4: INTERACTION OF MICRO-ORGANISMS WITH ION
EXCHANGE RESINS AND GELS

4. INTERACTION OF MICRO-ORGANISMS WITH ION EXCHANGE RESINS AND GELS

4.1 Introduction

Most conventional microbiological methods for estimation of total viable organisms in foods require 24-48h while detection of specific organisms (e.g. E.coli and fecal streptococci) may require a further 24-48h incubation.

In recent years, instrumental techniques such as the Direct Epifluorescent Filter Technique (DEFT; Pettipher, 1981), Impedimetric estimation (Hardy et al., 1977) and Luminometry (Patel & Wood, 1983; Stannard & Wood, 1983) have been developed which can reduce significantly the time required for the estimation of total microbial populations in foods. However, at present, such techniques have a limited use in the estimation of specific groups of micro-organisms e.g. Enterobacteriaceae. The potential of such methods would be enhanced if such organisms could be separated from food prior to estimation.

Several techniques (e.g. electrophoresis, partition in aqueous two-phase polymer systems and ion exchange chromatography) have been described previously (Chapter 2) for the physical manipulation of micro-organisms. The differences in the affinity of organisms for ion exchange chromatographic media has been exploited in order to resolve mixtures of pure cultures of organisms into their individual components e.g. separation of E.coli and Salm.typhimurium (Hall et al., 1976). The chromatographic columns used by many workers were large and the separation of micro-organisms was reported in terms of extinction

ratios of adsorbed to unadsorbed cells and the number of viable cells was not determined. In other cases the conditions under which organisms were eluted were lethal (Kosinkiewicz & Varanka, 1975).

The objectives of the present study were to:

- (a) Investigate the interactions of pure cultures of viable micro-organisms with columns of ion exchange resins and gels.
- (b) Exploit differences in adsorption and/or elution of micro-organisms for resolving binary mixtures of organisms into either one or both the components.
- (c) Study the involvement of microbial cell surface components (e.g. proteins) in the interaction between the organisms and the cation exchange resin Bio-Rex 70.
- (d) Apply the cation exchange resin columns for separation of specific micro-organisms from spiked foods.

4.2 Materials and Methods

Resins and Gels

Cation exchange resin Bio-Rex 70 (100/200 U.S. mesh size) and anion exchange resin AG3 X4A (100/200 U.S. mesh size) were used unless specified otherwise. Sepharose CL-4B gel and the ion exchange variants of this, CM-sepharose CL-6B and DEAE-sepharose CL-6B which possess carboxyl and diethylaminoethane groups, respectively, were also used.

Effect of various eluting agents on the viability of *Staph.aureus*

Aliquots (4 x 1ml) of a *Staph.aureus* culture were centrifuged at 2,000g for 15 min and the supernatants discarded. Each pellet was

suspended in one of the following solutions; R & P (control), sterile distilled water, 0.05M phosphate-citrate buffer pH 5.0 containing 1M NaCl and 0.5% ^m/v bovine serum albumin pH 9.5. The suspensions were incubated at ambient temperature (ca 20°C) and at various time intervals (0 to 120 min), 1ml was removed for estimation of the number of survivors.

Effect of column eluants on the recovery of *Staph.aureus*

A suspension of *Staph.aureus* (0.1ml, approximate total 1×10^8 cfu) was applied to five small columns of Bio-Rex 70 at pH 5.0 and the unadsorbed cells were removed by elution with 0.05M phosphate-citrate buffer pH 5.0. To each column one of the eluting agents (Table 4.1) was applied and the number of viable cells in the effluent was determined. The percentage elution was calculated from the total number of *Staph.aureus* added and the total number recovered in the column effluent after elution, as determined by colony counts.

Heterogeneity in a population of *E.coli* cells

A series of experiments was done in order to determine whether there was heterogeneity in adsorption characteristics within a pure culture of *E.coli*.

Experiment A

Five small columns of Bio-Rex 70 were equilibrated to pH 5.0 using 0.05M phosphate-citrate buffer pH 5.0. A series of 10-fold dilutions of *E.coli* was prepared and each dilution applied (0.15ml, ca total $\log_{10}$ 4.0-8.0 cfu) to a separate column followed by 10ml of the equilibration buffer. The effluents were collected and total viable counts determined. The proportion of viable cells adsorbing to each column was then calculated.

Experiment B

Five small columns of Bio-Rex 70 were equilibrated to pH 5.0 using 0.05M phosphate-citrate buffer pH 5.0. A 0.1ml aliquot (ca total $\log_{10}$ 8.0 cfu) of E.coli was applied to the first column followed by 10ml of the equilibration buffer. Ten ml of the effluent was collected and the number of viable E.coli was determined using 1ml of the effluent. The remaining 9ml was applied to the second column and the effluent (9ml) was collected and a further 1ml of the equilibration buffer was passed through the column to give a final effluent volume of 10ml. The number of viable E.coli was determined and this procedure was repeated until the effluent of the fifth column was collected.

The number of viable cells adsorbing to each column was determined and the proportion adsorbing calculated.

Experiment C

Three small columns and one large column of Bio-Rex 70 were prepared and equilibrated to pH 5.0 using 0.05M phosphate-citrate buffer pH 5.0. The reason for using a large column was to investigate if the fraction of the population of E.coli cells which apparently did not adsorb, depended on the capacity of the cation exchange resin.

The experiment was done exactly as described in experiment A except that the effluent of the third column was applied via a 3-way valve system into the fourth large column. Elution was continued with the equilibration buffer and transmittance of the column effluent was monitored at 280nm^{*}. The fractions showing a decrease in transmittance were pooled and the number of viable E.coli determined.

* A one off experiment; otherwise absorbance monitored at 420 nm

Investigation of interaction of microbial cell surface proteins with Bio-Rex 70 and CM-sepharose

Strains of E.coli (NCTC 10650; K88a) and Staph.aureus (NCTC 8530; Cowan 1 strain) each of which possess specific cell surface-associated proteins were applied to small and/or large columns of both resin and gel at pH 5.5 and the numbers of viable cells appearing in the effluents were determined. Strains (i.e. E.coli NCTC 9001 and Staph.aureus NCTC 7121) that did not possess the specific cell surface proteins were also tested under these conditions.

Electron microscopic analysis of E.coli

E.coli (NCTC 10650; K88a) and E.coli (NCTC 9001) were grown on nutrient agar plates at 37°C for 16h. The bacteria were suspended in distilled water at a concentration of approximately 10^8 cfu/ml. A collodion coated grid was placed face down on the bacterial suspensions for 20s then removed and immediately placed face down on a solution of 0.4% ^m/v uranyl acetate containing 40 µg/ml bacitracin. The grids were air-dried, coated with carbon in an Edwards coating unit and examined in a Jeol 1200 Ex Electron microscope operating at either 80 kV or 100 kV.

Chemical modification of carboxyl groups of Bio-Rex 70

The procedures for modification of carboxyl groups were essentially followed from Wood (1979).

Bio-Rex 70, 5g for each small column, was washed three times in distilled water. The supernatant liquid was decanted before being resuspended in the reagents described below.

Staph.aureus cultures were equilibrated to the appropriate pH before applying to Bio-Rex 70 columns.

Esterification of carboxyl groups (Zvaginstev & Gusev, 1971)

Bio-Rex 70 was suspended in 40ml of 0.1M HCl in methanol for 24h at ambient temperature (ca 20°C). The resin was then equilibrated to pH 5.5 using 0.05M phosphate-citrate buffer pH 5.5.

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

Bio-Rex 70 was suspended in 40ml of a solution containing 0.2M MgCl₂ and 0.2% ^m/v Alcian Blue (T.Gurr Ltd.) and stirred for 24h at ambient temperature. The resin was equilibrated to pH 5.5 using 0.05M phosphate-citrate buffer pH 5.5.

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

Bio-Rex 70 was suspended in 5ml of 0.8% ^m/v NaCl and 5ml of a solution containing 0.5g carbodiimide, 0.6g methylamine hydrochloride and 5g of MgCl₂ was added. The pH was adjusted to 4.5 with 1M HCl and the resin gently stirred at ambient temperature for 4h. At 30 min intervals the pH was adjusted to 4.5 with 0.1M NaOH. The resin was then equilibrated to pH 5.5 with 0.05M phosphate-citrate buffer pH 5.5.

Investigation of interaction between soluble anionic polyelectrolyte flocculant CA243 and micro-organisms

Preparation of flocculant solutions

A solution of 0.1% ^m/v flocculant was prepared in 0.05M phosphate-citrate buffer pH 5.5 and decimally diluted in the same buffer to give a range of concentrations (i.e. 0.01-0.0001% ^m/v). These solutions were used in the experiment.

Preparation of cultures

Cells of Staph.aureus (NCTC 8530, Cowan 1), L.plantarum and E.coli (NCTC 10650, K88a) were each suspended (ca 10⁸ cfu/ml) in 20ml of 0.05M phosphate-citrate buffer pH 5.5.

Flocculation test

For any one type of organism e.g. Staph.aureus, 1ml of culture was dispensed into each of 16 test tubes (10 x 100mm). A 0.5ml portion of each flocculant solution and 25 μ l of calcium chloride solution (20% m/v) was added to duplicate tubes (i.e. total of 8 tubes). To the rest of the tubes (controls), 0.5ml of 0.05M phosphate-citrate buffer pH 5.5 and 25 μ l calcium chloride solution (20% m/v) was added. The tubes were gently shaken and allowed to stand at ambient temperature for 45 min. The tubes were observed for flocculation in comparison with the controls.

4.3 Results

4.3.1 Affinity of food-associated micro-organisms for the cation exchange resin Bio-Rex 70

The absorbance trace given by micro-organisms (e.g. Staph.aureus) appearing in the effluents of large columns is shown in Fig. 4.1. Staph.aureus did not adsorb substantially at pH 8.0 as shown by the presence of peak A (Fig. 4.1) and by the viable counts of the pooled fractions comprising the peak (Fig. 4.2). A fraction (ca 8% of the viable count of the column inoculum; data not included) of Staph.aureus was eluted from the column with distilled water (peak B). However, at a column pH of 4.5, Staph.aureus was adsorbed to the resin as shown by both the disappearance of the first peak (buffer effluent; Fig. 4.1) and the reduction in viable count of the pooled fractions (Fig. 4.2).

In general, the Gram-negative bacteria studied (E.coli and Ps.fluorescens) adsorbed <90% to this resin in the pH range 4.5 to 8.0 (Fig. 4.2). The Gram-positive bacteria (Staph.aureus and L.plantarum)

Column size: 1.6 × 6.5 cm

Flow rate: 1.1 ml/min

(A) Elution of Staph. aureus with
0.05M Phosphate-citrate buffer pH 8.0

(B) Elution of Staph. aureus with
distilled water

Fig. 4.1 Elution of Staph. aureus (NCTC 8530) from a column of Bio-Rex 70 resin at (1) pH 4.5 and (2) pH 8.0

Fig. 4.2 Differences in the affinity of micro-organisms for large columns (1.6 × 6.5 cm) of cation exchange resin Bio-Rex 70

showed a greater adsorption (i.e. >99%) at low pH values (i.e. between pH 4.5 and 6.0 for Staph.aureus and between pH 4.5 and 5.5 for L.plantarum) than at higher pH values (pH >6.0).

The differences in the affinity of pure cultures of viable organisms for the large cation exchange resin columns were exploited in order to enrich or separate one or both the components from mixed suspensions of two species of organisms. The term enrichment was used when the ratio between the organisms in the mixture applied to the column and in the effluent from the column, changed by four-to ten-fold. The term separation was used when these ratios changed by more than ten-fold.

Separation of organisms by adsorption from buffer

The following organisms were either enriched or separated from a mixed culture applied to the column, on the basis of adsorption from buffer. In general, the Gram-negative/Gram-positive separation was better than either Gram-negative/Gram-negative or Gram-positive/Gram-positive, for example:

- (a) E.coli from an E.coli/Staph.aureus mixture at pH 5.0 and 5.5 (Figs. 4.3 a & b).
- (b) E.coli from E.coli/L.plantarum at pH 5.0 (Fig. 4.3 c).
- (c) Ps.fluorescens from Ps.fluorescens/L.plantarum at pH 5.0 (Fig. 4.3 d).
- (d) Ps.fluorescens from Ps.fluorescens/Staph.aureus at pH 5.0 (Fig. 4.3 e).
- (e) L.plantarum from an L.plantarum/Staph.aureus mixture at pH 5.5 (Fig. 4.3 f).

The recovery of the enriched or separated bacteria varied between 6% (Fig. 4.3 f) and 90% (Fig. 4.3 b) of the inoculum level.

Fig. 4.3a Separation of E. coli from a mixture of E. coli and Staph. aureus (Bio-Rex 70, pH 5.0)

Fig. 4.3b Separation of E. coli from a mixture of E. coli and Staph. aureus (Bio-Rex 70, pH 5.5)

Fig. 4.3c Separation of *E. coli* from a mixture of *E. coli* and *L. plantarum* (Bio-Rex 70, pH 5.5)

Fig. 4.3d Separation of *Ps. fluorescens* from a mixture of *Ps. fluorescens* and *L. plantarum* (Bio-Rex 70, pH 5.0)

☐ Ps. fluorescens
☒ Staph. aureus
 ↓ Inoculum level

Fig. 4.3e Separation of Ps. fluorescens from a mixture of Ps. fluorescens and Staph. aureus (Bio-Rex 70, pH 5.0)

☐ L. plantarum
☒ Staph. aureus
 ↓ Inoculum level

Fig. 4.3f Separation of L. plantarum from a mixture of L. plantarum and Staph. aureus (Bio-Rex 70, pH 5.5)

Separation of organisms by elution from adsorbent

The following organisms were either enriched or separated on the basis of differential elution with distilled water.

- (a) Staph.aureus from a Staph.aureus/E.coli mixture at pH 5.5
(Fig. 4.3 b).
- (b) Ps.fluorescens from Ps.fluorescens/Staph.aureus at pH 5.0
(Fig. 4.3 e).
- (c) L.plantarum from L.plantarum/Staph.aureus at pH 5.5
(Fig. 4.3 f).
- (d) L.plantarum from L.plantarum/E.coli at pH 5.5
(Fig. 4.3 c).

The recovery of the enriched or separated bacteria varied between 10% (Fig. 4.3 e) and 70% (Fig. 4.3 c) of the inoculum level.

The affinity of organisms for large (Fig. 4.2) and small columns (Fig. 4.4) of the cation exchange resin at pH 5.0 showed similar patterns. The differences in the affinity of the organisms for small columns of resin showed that an enrichment or a separation of one type of organism from a mixed culture of two types of organisms was possible (Fig. 4.5).

The affinity of some other bacterial species for the cation exchanger is shown in Fig. 4.6. The adsorption of Salm.typhimurium was ca 69% at pH values of 5.0 and 7.5. Streptococcus lactis adsorbed to the resin at pH 5.0 (ca 50%), whereas at pH 7.5 the adsorption was 20% (c.f. adsorption of Staph.aureus, Fig. 4.2).

4.3.1.1 Effect of particle size on the adsorption of Staph.aureus

The adsorption of Staph.aureus to Bio-Rex 70 resin at pH 5.0 increased with increase in the surface area of the resin (i.e. a decrease in the particle size) (Fig. 4.7).

↓ Inoculum level

Fig. 4.4 Differences in affinity of micro-organisms for small columns (0.6 × 6.5 cm) of cation exchange resin Bio-Rex 70 at pH 5.0

- ▨ E. coli NCTC 9001
- Staph. aureus NCTC 8530
- ⊥ Mean and SD are shown,
n=2 for inoculum level
n=4 for effluent level

Fig. 4.5 Separation of E. coli from a mixture of E. coli and Staph. aureus (Bio-Rex 70, pH 5.5)

□ Inoculum level
 ▨ Effluent level
 I Mean and SD are shown, n=2 for inoculum level, n=3 for effluent level

Fig. 4.6 Affinity of *Salm. typhimurium* and *Strep. lactis* for cation exchange resin
Bio - Rex 70

Fig. 4.7 Effect of resin particle size on the adsorption of Staph. aureus at pH 5.0

4.3.1.2 Effect of various eluants on the viability and recovery of *Staph.aureus*

The eluting agents (distilled water and 0.5% bovine serum albumin pH 9.5) did not reduce the viability of *Staph.aureus* during upto 2h exposure (Fig. 4.8). However, there was ca 60% reduction in the viability of *Staph.aureus* suspended in 0.05M phosphate-citrate buffer pH 5.0 containing 1M NaCl after 2h.

Almost complete recovery of *Staph.aureus*, adsorbed to the cation exchanger, was obtained by batch elution with 10ml of 0.05M phosphate-citrate buffer pH 8.0 (Table 4.1).

TABLE 4.1 Recovery of *Staph.aureus* NCTC 8530 from columns of Bio-Rex 70 resin at pH 5.0

Column No.	Elution agent*	% Elution
1	Batch elution with 0.05M phosphate-citrate buffer pH 8.0 (10ml)	98
2	Distilled water (10ml)	48
3	0.05M phosphate-citrate buffer pH 5.0 containing 0.5M NaCl (20ml)	0
4	0.05M phosphate-citrate buffer pH 5.0 containing 1M NaCl (20ml)	0
5	0.5% m/v bovine serum albumin pH 9.5 (50ml)	26

* Total volume used is indicated in brackets.

Staph.aureus NCTC 8530, adsorbed to the cation exchanger, could not be eluted by buffer containing NaCl (Table 4.1). Buffered NaCl did not have a lethal effect on *Staph.aureus* since an experiment was completed within 45 min (Fig. 4.8). Wood (1979) showed that the adsorption of

- Control (Ringers and Peptone)
- Distilled water
- △ 0.05M phosphate-citrate buffer pH5.0 (+1M NaCl)
- ▲ 0.5% m/V bovine serum albumin pH9.5

Fig. 4.8 The effect of various column eluants on the viability of *Staph. aureus*
NCTC 8530

Staph.aureus to the cation exchanger was increased from 0-20% in distilled water to 70% in the presence of 0.05M phosphate-citrate buffer pH 5.5. Puch & Sagik (1953) reported that the attachment of bacteriophages to cationic exchangers occurred only in the presence of salt.

A high concentration of bovine serum albumin at pH 9.5 only desorbed 26% of Staph.aureus after eluting with 50ml of the solution (Table 4.1).

4.3.1.3 Heterogeneity in the affinity of E.coli cells for Bio-Rex 70

The results of the experiment A, in which serial decimal dilutions of E.coli were applied to separate columns of the cation exchanger, showed that a high and approximately equal percentage of cells (i.e. >70% except at highest concentration) were adsorbed to each of the columns 2 to 5 (Fig. 4.9). At a column inoculum level of Log_{10} 6.5 cfu ca 80% of the cells were adsorbed in contrast with results of experiment B (Fig. 4.9) in which at a similar inoculum level only $\leq 13\%$ of the cells were adsorbed (by the fourth and fifth columns in the sequence).

In the experiment B where non-adsorbed cells of E.coli in the column effluent were applied to a second column of the cation exchanger, the following results were obtained. In the first three columns (Fig. 4.9) the "adsorbing" population of cells was removed, leaving a "non-adsorbing" population in the effluents of the fourth and fifth columns.

In the experiment C where non-adsorbed E.coli cells in the column effluent of the third small column in sequence were finally re-applied to a larger column, the following results were obtained (Fig. 4.9).

- Experiment A; serial decimal dilutions applied to separate columns
- Experiment B; non-adsorbed E. coli from 1st column applied to 2nd column and so on
- ▲—▲ Experiment C; same as (○—○) except that column no. 4 was large (1.6×7.0 cm)
- () total log₁₀ cfu applied to each column

Fig. 4.9 Affinity heterogeneity in a population of E. coli NCTC 9001 as shown by chromatography on Bio-Rex 70 columns

In the first three columns, the "adsorbing" population of cells was removed, leaving the "non-adsorbing" population in the effluent of the fourth (large) column. This indicated that the adsorption of the cells was independent of the capacity of the cation exchanger in the column and that there was a residual population which did not adsorb to the cation exchanger.

Overall, these results suggest that an apparently homogeneous population of a pure culture of E.coli may contain at least two types of cells, i.e. those that adsorb to the cation exchanger and those that do not. Although these experiments were performed at pH 5.0, it cannot be assumed that such a heterogeneity would also occur at other pH values (e.g. pH 8.0).

4.3.1.4 Involvement of cell surface proteins in the interaction of micro-organisms with Bio-Rex 70

Staphylococcal Protein A

Strain of Staph.aureus, i.e. Cowan 1 (possesses cell surface Protein A; James & Brewer, 1968) showed greater adsorption (ca 2 log cycles) than the Wood 46 (does not possess Protein A; James & Brewer, 1968) strain (Fig. 4.10).

E.coli fibrillar antigen (K88a)

When E.coli NCTC 10650 possessing K88a antigen (Smyth et al., 1978; Plate 4.1) was applied to the column of cation exchange resin, >99% of the cells were adsorbed to the column compared with ca 69% of the E.coli strain NCTC 9001, which does not possess K88a antigen (Plate 4.2) (Fig. 4.10). This result strongly suggests that cell surface proteins are involved in interaction with the cation exchanger and such differences in surface components may be used to separate different antigenic types of E.coli.

▨ Inoculum level
 □ Effluent level
 • Mean and SD are shown,
 n=3

Fig. 4.10 Involvement of proteins in the interaction of micro-organisms with Bio-Rex 70 resin

Plate 4.1 Fibrillar K88a antigen in E. coli NCTC 10650

Plate 4.2 Non-adhesive fimbriae in E. coli NCTC 9001

4.3.1.5 Application of Bio-Rex 70 columns for separation of groups of organisms grown in foods

In this experiment, an anionic polyelectrolyte flocculant CA 243 was used to partially clarify food homogenates without flocculating or damaging the organisms to a significant extent (Patel & Wood; 1983). Pure cultures of organisms were spiked into foods and allowed to grow in order to simulate natural growth of organisms in foods. A typical example of the growth of test organisms (Staph.aureus NCTC 8530 and L.plantarum NCIB 8960) in milk is shown in Table 4.2.

TABLE 4.2 Growth of a mixture of Staph.aureus and L.plantarum in milk

Total viable count*of milk before inoculation (Log ₁₀ total cfu)	Mixture of organisms inoculated	Log ₁₀ cfu/ml before incubation	Log ₁₀ cfu/ml after incubation (16h at 30°C)
2.3	<u>L.plantarum</u>	5.6	6.5
	<u>Staph.aureus</u>	6.8	7.9

* on Plate Count Agar (Lab M).

The organisms were then separated and concentrated from milk by centrifugation. The subsequent differential separation of L.plantarum from Staph.aureus on Bio-Rex 70 columns is shown in Table 4.3. The choice of the column pH values for resolving one type of organism from binary mixtures was derived from the adsorption characteristics of pure cultures of organisms (Fig. 4.2).

The adsorption characteristics of a mixture of E.coli and Staph.aureus separated from pork and milk not treated with the flocculant and

TABLE 4.3

A summary of the application of Bio-Rex 70 columns
for differential separation of organisms grown in
foods

Food	Mixture of organisms	pH of column	Total log ₁₀ cfu applied to column	Total log ₁₀ cfu in the buffer effluent
Raw pork (Low count)	<u>E.coli</u> (NCTC 9001)	5.5	8.3 (8.1)	8.2 (7.7)
	<u>Staph.aureus</u> (NCTC 8530)		7.2 (7.4)	< 3.6 (3.6)
Milk (pasteurised)	<u>E.coli</u> (NCTC 9001)	5.5	8.2 (8.2)	8.0 (7.9)
	<u>Staph.aureus</u> (NCTC 8530)		5.5 (5.3)	4.6 (2.6)
Milk (pasteurised)	<u>L.plantarum</u> (NCIB 8960)	6.0	6.8 (6.9)	6.2 (6.4)
	<u>Staph.aureus</u> (NCTC 8530)		7.9 (8.0)	3.9 (4.6)
Cream (pasteurised)	<u>L.plantarum</u> (NCIB 8960)	6.0	7.0 (7.0)	6.8 (6.7)
	<u>Staph.aureus</u> (NCTC 8530)		6.6 (6.6)	5.5 (5.7)

Figures in brackets represent an experiment in which the anionic flocculant was not used to clarify foods prior to application of organisms to columns.

L.plantarum and Staph.aureus separated from milk (Table 4.3) were similar to the interaction of these organisms with Bio-Rex 70 resin in pure cultures (Figs. 4.3b & f). However, the adsorption of Staph.aureus and L.plantarum separated from cream was lower than both in milk (Table 4.3) and the adsorption from pure cultures (Fig.4.3f). This suggests that components of cream (e.g. lipids) may be adsorbing to the organisms and/or resin thus reducing adsorption of the organisms. In general, the

partial clarification of foods by the flocculant did not have a significant effect on the separation of micro-organisms on Bio-Rex 70 columns (Table 4.3).

4.3.2 Affinity of micro-organisms for other adsorbents

In general, the organisms studied showed $\leq 60\%$ adsorption to small columns (0.6 x 3.0cm) of either hydrophilic matrix sepharose (Table 4.4) or the carboxyl-substituted CM-sepharose (Table 4.5) at pH

5.5. A similar result was obtained (Table 4.6) when larger columns (1.6 x 6.5cm) of CM-sepharose gel were used.

TABLE 4.4 Interaction of micro-organisms with small columns (0.6 x 3.0cm) of the hydrophilic gel Sepharose-6B at pH 5.5

Organism	Log ₁₀ total cfu:	
	Applied to column	Column effluent
<u>E.coli</u> NCTC 9001	8.2	7.9
<u>Salm.typhimurium</u> NCTC 74	7.8	7.4
<u>L.plantarum</u> NCIB 8960	6.9	6.8

TABLE 4.5

Interaction of micro-organisms with small columns
(0.6 x 3.0cm) of the cation exchange gel CM-sepharose
CL-6B at pH 5.5

Organism	Log ₁₀ total cfu:	
	Applied to column	Column effluent
<u>E.coli</u> NCTC 9001	7.9	7.8
<u>Salm.typhimurium</u> NCTC 74	8.7	8.5
<u>L.plantarum</u> NCIB 8960	8.3	8.1
<u>Staph.aureus</u> NCTC 8530	8.1	7.6

TABLE 4.6

Interaction of micro-organisms with large columns
(1.6 x 6.5cm) of CM-sepharose CL-6B at pH 5.5

Organism	Log ₁₀ total cfu:	
	Applied to column	Column effluent
<u>E.coli</u> NCTC 9001	8.2	8.2
<u>E.coli</u> NCTC 10650 (K88a)	8.0	7.9
<u>Staph.aureus</u> NCTC 8530*	8.3	8.1
<u>Staph.aureus</u> NCTC 7121**	8.5	8.6
<u>L.plantarum</u> NCIB 8960	7.6	7.6

* possesses cell surface Protein A

** does not possess Protein A

TABLE 4.7

Interaction of micro-organisms with small columns
(0.6 x 6.5cm) of the anion exchange resin
AG3 X4A at pH 5.5

Organism	Log ₁₀ total cfu:	
	Applied to column	Column effluent
<u>E.coli</u> NCTC 9001	7.9	6.6
<u>Salm.typhimurium</u> NCTC74	7.9	6.6
<u>Staph.aureus</u> NCTC 8530	7.7	6.2
<u>L.plantarum</u> NCIB 8960	6.7	3.0

TABLE 4.8

Interaction of micro-organisms with large columns
(1.6 x 6.5cm) of the anion exchange gel
DEAE-sepharose CL-4B

Organism	Log ₁₀ total cfu:			
	Applied to column	Column effluent	Dist.water elution	Elution with 0.05M phos-cit. buffer pH 5.5 (+0.5M NaCl)
<u>E.coli</u> NCTC 9001	8.1	6.4	< 3.6	7.2
<u>L.plantarum</u> NCIB 8960	8.2	< 3.6	< 3.6	6.9
<u>Staph.aureus</u> NCTC 7121	8.4	< 3.6	< 3.6	6.7

However, $\geq 95\%$ of the organisms were adsorbed to columns of both the anion exchange resin AG3 X4A (Table 4.7) and anion exchange gel DEAE-sepharose (Table 4.8) at pH 5.5. A small proportion

(ca 2-12%) of the organisms were eluted with 0.05M phosphate-citrate buffer pH 5.5 containing 0.5M NaCl, but not with distilled water.

4.3.3 Effects of modification of carboxyl groups of Bio-Rex 70 on the interaction with Staph.aureus NCTC 8530

The carboxyl groups of Bio-Rex 70 were modified as demonstrated by lack of titrability of resin after the various treatments. The treated resin showed adsorption of Staph.aureus comparable to the untreated control (Table 4.9). Approximately 25% of Staph.aureus was eluted with distilled water from treated resins except for the Alcian blue treated resin where the recovery was <0.001%.

4.3.4 Effect of anionic polyelectrolyte flocculant CA243 on micro-organisms

Cells of Staph.aureus, L.plantarum and E.coli did not flocculate compared with the controls, in which the flocculant was not added, at any of the flocculant concentrations tested.

TABLE 4.9 Effect of modification of carboxyl groups of Bio-Rex 70 on the adsorption of Staph.aureus

Treatment	Log ₁₀ total cfu:		
	Applied to column	Column ¹ effluent	Elution with ¹ distilled water
Methanol/HCl	8.6	5.4	8.0
Alcian blue ²	8.9	2.7	3.6
Carbodiimide	8.8	5.7	8.2
Non-treated control ³	8.8	4.3	8.4

1, represents a mean of between 3 and 7 results.

2, In a separate experiment (not reported) it was shown that viability of Staph.aureus in Alcian blue was not reduced for upto 120 min.

3, represents a mean of 5 results.

4.4 Discussion

The physico-chemical characteristics of the microbial cell surface can be exploited in order to resolve binary mixtures of organisms both from pure cultures and from foods into one or both components using columns of the cation exchange resin Bio-Rex 70. There are several lines of evidence which strongly suggest that the interaction of micro-organisms with the cation exchanger may not involve to a significant extent the charge-charge interaction between the two bodies.

Firstly, it has been reported that micro-organisms usually have a low apparent isoelectric point ($I_p \leq 5.0$; Daniels, 1967; Neihof & Echols, 1973; Sherbet, 1978). Therefore, at pH values higher than the I_p , micro-organisms bear an overall negative surface charge, while at a pH lower than I_p , they bear an overall positive charge (Daniels & Kempe, 1966; Daniels, 1980). The results showed that the Gram-negative bacteria adsorbed <90% to the cation exchanger in the pH range 4.5 to 8.0. This may be related to their low apparent I_p value. However, the Gram-positive bacteria (e.g. Staph.aureus) showed a greater adsorption (i.e. >90%) even at pH 7.0, at which pH Staph.aureus would not be expected to adsorb (Fig. 4.2).

Secondly, neither Gram-positive nor Gram-negative organisms adsorbed substantially to either Sepharose-6B (Table 4.4) or the carboxyl-substituted gel CM-sepharose-6B at a pH value of 5.5 (Tables 4.5 & 4.6). This showed that the carboxyl functional groups of the gel were not involved to any significant extent in the specific interaction with microbial cell-surface.

The difference between CM-sepharose gel and Bio-Rex 70 resin is the matrix supporting the carboxyl functional groups. In the former case it is hydrophilic polysaccharide while in the latter case it is hydrocarbon ("hydrophobic") acrylic polymer. Since the Gram-negative organisms are negatively charged (Neihof & Echols, 1973) and are essentially hydrophilic due to the presence of large amounts of polysaccharides (in the form of lipopolysaccharides) on the cell surface (Inouye, 1979) they would not be expected to adsorb substantially to either hydrophilic or the hydrophobic surface. However, an E.coli strain possessing fibrillar K88a proteinaceous antigen adsorbed >99% to Bio-Rex 70 resin (Fig. 4.10) compared with ca 20% to CM-sepharose gel (Table 4.6). The same strain of E.coli also showed >99% adsorption to the hydrophilic-hydrophobic gel, Phenyl-sepharose CL-4B (Fig.5.9; Chapter 5). Furthermore, Gram-positive organisms (e.g. Staph.aureus) showed significant adsorption to both, Bio-Rex 70 in the pH range 4.5 to 7.0 (Fig.4.2) and to Phenyl-sepharose gel (Fig.5.4; Chapter 5) but not to CM-sepharose gel (Tables 4.5 and 4.6).

Thirdly, the modification of carboxyl groups of Bio-Rex 70 resin by various treatments did not reduce the adsorption of Staph.aureus (Table 4.9). Approximately 25% of Staph.aureus which were adsorbed to either methanol/HCl and carbodiimide treated resin or the untreated control were eluted with distilled water. However, <0.01% of Staph.aureus was eluted from Alcian blue treated columns (Table 4.9). This may be due to adsorption of Staph.aureus to the strongly hydrophobic Alcian blue dye.

Finally, cells of Staph.aureus which were adsorbed to the cation exchanger could not be eluted with buffered sodium chloride (Table 4.1). If this interaction were based entirely on the charged groups of both the surfaces, a greater recovery of Staph.aureus would be expected on elution with the buffered sodium chloride. For instance, between 20 and 80% of staphylococcal enterotoxins (as an example of a zwitterion) are recovered from a column of cation exchanger with buffered sodium chloride (Holbrook & Baird-Parker, 1975).

Overall, these results strongly suggest that micro-organisms interact with the hydrophobic acrylic (Gerson & Scheer, 1980) backbone of Bio-Rex 70 resin through a combination of physical forces including hydrophobic interactions.

The cells of Staph.aureus NCTC 8530 and E.coli NCTC 10650 adsorb >99% to the Bio-Rex 70 resin (Fig.4.10) but do not flocculate in the presence of the soluble anionic flocculant CA243 which is essentially a soluble Bio-Rex 70. This suggests that a solid surface (i.e. Bio-Rex 70) is essential in the interaction of these organisms. (The physico-chemical aspects of the interaction between charged particles and macroscopic surface are described in the General Discussion, Chapter 8).

Both Gram-positive and Gram-negative organisms showed significant adsorption ($\geq 95\%$) to the anion exchange resin (Table 4.7) and anion exchange gel (Table 4.8). This suggested that charge-charge interaction (i.e. negatively charged organisms and positively

charged ion exchanger) was involved in the adsorption of organisms to the anion exchangers. However, the recovery of micro-organisms from the columns after elution with buffered sodium chloride was low (e.g. 2% in the case of Staph.aureus). It is feasible that when micro-organisms approach the surface of anion exchanger, the initial adsorption is based on charge-charge interaction. When salt is introduced in the medium the energy barrier (Fig.8.1, Chapter 8) between organisms and the macroscopic surface may be reduced (Tadros, 1980) thus causing cells to approach closer to the surface resulting in stronger adhesion probably due to short-range forces (e.g. hydrophobic interactions). This may account for the low recoveries observed after elution with buffered NaCl. Wood (1979) reported a recovery of ca 5% Staph.aureus after elution from the anion exchange resin AG1X8 with 0.05M phosphate buffer pH 7.0, ca 50% with 0.5M NaCl & ca 5% with 0.8M NaCl.

An apparently homogeneous population of a pure culture of E.coli was in fact heterogeneous with respect to the interaction with Bio-Rex 70 resin. Techniques which have shown or indicated heterogeneity in an apparently homogeneous population of E.coli include ion exchange chromatography (Rotman, 1960; Kolot, 1981), electrophoresis (Abramson, et al., 1942; Ramsey et al., 1980), and density gradient centrifugation (Maruyama & Yamigita, 1956; Poole, 1977). Such results may have practical disadvantages. For instance, the efficiency of separation (or enrichment) of an organism of interest (e.g. Salm.typhimurium) from other types of organisms (e.g. E.coli) may be reduced because a proportion of the E.coli may be eluted along with Salm.typhimurium.

The feasibility of manipulating micro-organisms has been demonstrated both from pure cultures and from spiked foods by using the cation exchange resin Bio-Rex 70. However, the physical separation/enrichment of organisms in naturally contaminated foods may present several problems. Firstly, in general, low numbers of organisms of significance (e.g. E.coli) are present in food and therefore a growth enrichment stage may be required prior to separation using the cation exchanger. Secondly, a heterogeneous population of micro-organisms may be present in foods along with the organism of interest (e.g. E.coli & Salm.typhimurium). In this case selective enrichment of the organism of interest may be required prior to physical separation. On the other hand both growth enrichment and selective enrichment of particular organisms may be required. Thirdly, food constituents (e.g. lipids, proteins, inorganic ions etc.) may interfere with adsorption of organisms to the cation exchanger by altering either the cell surface properties of organisms or the cation exchanger. Provided that these problems are considered, the cation exchanger may be used for physical separation and concentration of micro-organisms of significance in foods as a preliminary step prior to rapid detection and estimation by the various unconventional techniques (see Introduction). The traditional method for the detection of specific organisms (e.g. E.coli & Salm.typhimurium) in foods is usually tedious and time-consuming ($\geq 48h$). However, the use of the cation exchanger in conjunction with rapid detection techniques (e.g. impedance) could significantly reduce the analysis time ($\leq 24h$). Several applications of this study are listed:

1. Enrichment or separation of food-spoilage organisms (e.g. Lactobacillus spp. and Pseudomonas spp.) or food-poisoning organisms (e.g. staphylococci, Salmonella spp. and E.coli) from a mixed population of organisms in foods and enrichment broths.
2. Possible separation of yeasts from other organisms from foods.
3. Immobilisation of viable micro-organisms on ion exchangers for biotechnological applications.
4. Academic study of the growth kinetics (including sporulation and conjugation) of immobilised viable cells.

4.5 Summary

In this study, interaction of food-associated micro-organisms with ion exchange resins has been investigated. The results show that the physico-chemical differences in adsorption between viable micro-organisms and the cation exchange resin Bio-Rex 70 can be exploited in order to resolve, to a greater or lesser extent, binary mixtures of some organisms both from pure cultures and from artificially inoculated food suspensions.

An apparently homogeneous population of a pure culture of E.coli was found to be heterogeneous on the basis of the interaction observed with the cation exchange resin. It was shown that the presence of certain protein components on the microbial cell surface enhanced the adsorption of microbial cells to the cation exchanger, e.g. K88a antigen of E.coli.

There are several lines of evidence which indicate that the interaction of micro-organisms with the negatively charged resin may not involve significantly the opposite charges on both the bodies.

Instead, the organisms might interact with the matrix of the resin i.e. 'hydrophobic' acrylic polymer probably through a combination of physical forces including hydrophobic interactions.

Finally, several potential applications of this study are described, particularly with reference to the separation of micro-organisms from foods.

PART II

Chapter 5: INTERACTION OF MICRO-ORGANISMS WITH HYDROPHILIC-HYDROPHOBIC GELS

5. INTERACTION OF MICRO-ORGANISMS WITH HYDROPHILIC-HYDROPHOBIC GELS

5.1 Introduction

Several workers have exploited the electrical properties of microbial cell surfaces in order to manipulate micro-organisms by techniques such as ion exchange chromatography (Daniels, 1972; Wood, 1979), electrophoresis (Ramsey et al., 1980) and partition in aqueous polymer two-phase systems (Stendahl et al., 1977) (see Chapter 2.2).

Micro-organisms also possess cell-surface properties which confer hydrophobicity (Wadström et al., 1980). Several approaches have been used to examine the hydrophobicity of microbial cell surfaces. These include the adherence of bacteria to hydrocarbons e.g. hexadecane (Weiss et al., 1982) and plastics such as polystyrene (Rosenberg, 1981), interaction of micro-organisms with hydrophobic fluorescent probes e.g. 2-p-toluidinyl naphthalene does not fluoresce in aqueous solution but when it binds to hydrophobic components of microbial cell envelopes such as with E.coli the fluorescence is markedly increased (Neiva-Gomez & Gennis, 1977), measurement of contact angles (Gerson & Akit, 1980) and 'Salting-out' of micro-organisms from aqueous suspensions (Lindahl et al., 1981). The two techniques most commonly used for studying the surface hydrophobicity of micro-organisms are partition in aqueous polymer two-phase system incorporating polymers with covalently linked hydrophobic groups (e.g. palmitoyl group) (Edebo et al., 1980) and hydrophobic interaction chromatography using Phenyl and Octyl-sepharose gels (Wadström et al., 1981).

Many of these workers have reported the adsorption of micro-organisms to hydrophobic gels in terms of extinction ratio of adsorbed to unadsorbed cells i.e. the number of viable cells was not determined (Wadström et al., 1981). Moreover, the conditions under which the organisms were eluted from the columns were lethal e.g. elution with 3% Triton X-100 (Wadström et al., 1981). There have been no reports on the physical separation of micro-organisms from mixed cultures using the hydrophilic-hydrophobic gels. The objectives of this study were to:

1. Investigate the interaction of pure cultures of viable micro-organisms with Phenyl-sepharose (and Octyl-sepharose) gel.
2. Exploit differences in hydrophobicity of micro-organisms for resolving binary mixtures of organisms into one or both components.
3. Investigate the involvement of microbial cell-surface components (e.g. proteins) in the interaction between organisms and hydrophilic-hydrophobic gels.
4. Apply Phenyl-sepharose columns for the separation of micro-organisms grown in foods.

5.2 Materials and methods

Hydrophobic Interaction Chromatography (HIC)

Except where otherwise stated, Phenyl-sepharose CL-4B and Octyl-sepharose CL-4B gels were used and HIC in small columns (0.6 x 6.5cm) was performed using 0.01M phosphate buffer pH 6.5 containing 1M ammonium sulphate.

Effect of ammonium sulphate on the viability of food-associated micro-organisms

Two aliquots (1ml) of a culture were centrifuged at 2,000g for 15 min and the supernatants discarded (see Chapter 3). One of the pellets was resuspended in 10ml of 0.01M phosphate buffer pH 6.5 whilst the other pellet was resuspended in 10ml of 0.01M phosphate buffer pH 6.5 containing 1M ammonium sulphate. At various time intervals (15 to 180 min) 1ml of the suspension was removed and the number of viable organisms determined (see Chapter 3).

Effect of 1M ammonium sulphate on the interaction of micro-organisms with hydrophilic-hydrophobic gels

Adsorption of micro-organisms to large columns (1.6 x 6.5cm) of both the hydrophilic-hydrophobic gels, equilibrated with 0.01M phosphate buffer pH 6.5 and gels equilibrated with the same buffer but containing 1M ammonium sulphate, were compared.

Effect of ammonium sulphate (upto 1M) on the interaction of *Staph.aureus* and *L.plantarum* with Phenyl-sepharose gel

Aliquots (5 x 0.5ml) of the culture were centrifuged at 2,000g for 15 min and the supernatants discarded. Each pellet was washed twice by centrifugation and resuspension in 2ml of one of the following solutions; sterile distilled water, 0.01M phosphate buffer pH 6.5 and same buffer but containing 0.25M, 0.5M and 0.75M ammonium sulphate and then resuspended in 2ml of the same solution.

To each of five Phenyl-sepharose columns equilibrated with the above solutions, an aliquot (0.5ml) of the corresponding cell suspension was applied. The number of viable cells appearing in the column effluent on elution with the equilibrating solution was then determined.

Electron microscopy of *Staph.aureus* and *L.plantarum* adsorption to Phenyl-sepharose gel

Staph.aureus and *L.plantarum* suspensions in both 0.01M phosphate buffer pH 6.5 containing 1M ammonium sulphate and in distilled water (control) were applied to the corresponding Phenyl-sepharose columns equilibrated with 0.01M phosphate buffer pH 6.5 containing 1M ammonium sulphate and with distilled water. Unadsorbed cells were removed by passing 20ml of the equilibration solutions.

An aliquot (1ml) of each gel was fixed in 2.5% v/v glutaraldehyde (EM grade) for 1h. The gels were washed three times in distilled water and then dehydrated with ethanol (AR grade). The beads were then transferred to iso-amyl acetate and vacuum dried. Dried beads were fixed onto pieces of copper which had been precoated with Temfix adhesive and coated with carbon in an Edwards Coating Unit prior to examination in a Jeol 1200 Ex electron microscope operating at 10kV.

Interaction between phenyl groups of Phenyl-sepharose gel and microbial cell surfaces

Both sepharose CL-4B and Phenyl-sepharose CL-4B gels were equilibrated with 0.01M phosphate buffer pH 6.5 containing 1M ammonium sulphate. Microbial cell suspensions in the same buffer were applied to both the columns and the number of viable cells in the column effluent was determined.

5.3 Results

5.3.1 Interaction of micro-organisms with Phenyl-sepharose and Octyl-sepharose gels

5.3.1.1 Effect of ammonium sulphate on the viability and hydrophobicity of food-associated micro-organisms

Viability of Staph.aureus, E.coli and L.plantarum was not reduced in the presence of 1M ammonium sulphate for upto 3h (Figs. 5.1 a, b & d). With Ps.fluorescens, suspended in ammonium sulphate for upto 1h, the difference in viable count compared with the control was probably due to loss in viability rather than reproducibility of the method. However, there was a marked decrease (approximately 2 log cycles) in viability of Ps.fluorescens after 2h.

In general, the adsorption of both Staph.aureus (Fig. 5.2) and L.plantarum (Fig. 5.3) increased with an increase in ammonium sulphate concentration from 0 to 1M. The organisms did not adsorb to Phenyl-sepharose gel equilibrated with distilled water as shown by both viable counts and by electron microscopy (Plate 5.1). However, in the presence of 1M ammonium sulphate both Staph.aureus (Plate 5.2) and L.plantarum (Plate 5.3) were adsorbed to the beads.

The adsorption of L.plantarum, Staph.aureus and Ps.fluorescens (but not E.coli) to large columns of Phenyl-sepharose was markedly increased (i.e. >90%) in the presence of 1M ammonium sulphate (Fig. 5.4). However, for Octyl-sepharose only L.plantarum showed marked adsorption (Fig. 5.5). Although the aliphatic octyl group is more hydrophobic than the aromatic phenyl group (Pharmacia Fine Chemicals, 1979) the organisms showed greater affinity for the

Fig. 5.1 Effect of 1M ammonium sulphate on the viability of food-associated micro-organisms

↓ Inoculum level
 * Control column equilibrated with distilled water
 ** Result from Fig.5.8 included

Fig. 5.2 Effect of various concentrations of ammonium sulphate on the adsorption of Staph. aureus NCTC 8530 to Phenyl-sepharose gel

↓ Inoculum level
 * Control column equilibrated with distilled water

Fig. 5.3 Effect of various concentrations of ammonium sulphate on the adsorption of L. plantarum to Phenyl-sepharose gel

Plate 5.1 Phenyl-sepharose beads equilibrated with distilled water. No adsorption of either Staph. aureus or L. plantarum

Plate 5.2 Adsorption of Staph. aureus to Phenyl-sepharose beads equilibrated with 1M ammonium sulphate

Plate 5.3 Adsorption of *L. plantarum* to Phenyl-sepharose beads equilibrated with 1M ammonium sulphate

Fig. 5.4 Effect of ammonium sulphate (1M) on hydrophobicity of micro-organisms using large columns (1.6 x 6.5 cm) of Phenyl-sepharose gel

Fig. 5.5 Effect of ammonium sulphate (1M) on the hydrophobicity of micro-organisms using large columns (1.6 x 6.5 cm) of Octyl-sepharose gel

Phenyl-sepharose than for Octyl-sepharose gel in the presence of ammonium sulphate but little difference in the absence of ammonium sulphate.

The differences in affinity of organisms for Phenyl-sepharose gel were exploited in order to enrich or separate one of the components from binary mixtures of organisms. The term 'enrichment' was used when the ratio between the organisms in the mixture applied to the column and in the effluent from the column changed by 4- to 10-fold. The term 'separation' was used when these ratios changed by more than 10-fold. The following organisms were separated from a mixed culture on the basis of adsorption from buffer.

1. Ps.fluorescens from Ps.fluorescens-L.plantarum mixture by ca 200-fold (Fig. 5.6a).
2. E.coli from E.coli-Staph.aureus by ca 500-fold (Fig. 5.6b).
3. E.coli from E.coli-L.plantarum mixture by ca 2000-fold (Fig. 5.6c).

The recovery of the enriched or separated bacteria varied from 2% (Fig. 5.6a) to 36% (Fig. 5.6c) of those applied to the column.

The same organisms were either enriched or separated on the basis of differential elution with distilled water. In this case the recovery varied from 14% (Fig. 5.6c) to 49% (Fig. 5.6a).

The affinity of Staph.saprophyticus strains and Strep.lactis for Phenyl-sepharose gel is shown in Fig. 5.7. The hydrophobicity of both Staph.saprophyticus strains was similar to that of Staph.aureus (Fig. 5.8) i.e. $\geq 99\%$ adsorption. However, ca 65% of Strep.lactis was adsorbed to this gel (Fig. 5.7).

a. Separation of *Ps. fluorescens* from a mixture of *Ps. fluorescens* and *L. plantarum*

□ *Ps. fluorescens*
 ▨ *L. plantarum*
 ↓ Inoculum level

b. Separation of *E. coli* from a mixture of *E. coli* and *Staph. aureus*

□ *E. coli* NCTC 9001
 ▨ *Staph. aureus* NCTC 8530
 ↓ Inoculum level

Fig. 5.6 Separation of binary mixtures of organisms into one of the components on Phenyl-sepharose columns

c. Separation of *E. coli* from a mixture of *E. coli* and *L. plantarum*

- *E. coli* NCTC 9001
 ▨ *L. plantarum*
 ↓ Inoculum level

Fig. 5.6 Separation of binary mixtures of organisms into one of the components on Phenyl-sepharose columns

- Effluent count
- ▨ Distilled water elution count
- ↓ Inoculum level

Fig. 5.7 Affinity of *Staph. saprophyticus* and *Strep. lactis* for Phenyl-sepharose gel

5.3.1.2 Effect of column size on the adsorption of micro-organisms

The affinity of organisms for large and small columns of Phenyl-sepharose gels was similar (compare Figs 5.4 and 5.8).

5.3.1.3 Interaction of Phenyl groups with microbial cell surfaces

Fig. 5.9 shows that adsorption of L.plantarum, Staph.aureus, E.coli NCTC 10650 and Ps.fluorescens was markedly increased in the presence of the hydrophobic phenyl ligand. However, E.coli strain NCTC 9001 showed much lesser affinity for the Phenyl-sepharose gel.

5.3.1.4 Involvement of microbial cell surface proteins in the interaction with Phenyl-sepharose gel

Staphylococcal Protein A

The Staph.aureus strain Cowan 1 (possesses cell surface protein A) showed greater adsorption to the Phenyl-sepharose gel than did the Wood 46 strain which does not possess cell surface protein A (Fig. 5.9).

E.coli fibrillar K88a antigen

When E.coli NCTC 10650 possessing fibrillar (Wadström et al., 1978) K88a antigen (Plate 4.1) was applied to the Phenyl-sepharose column (Fig. 5.9) <0.1% of cells were recovered in the column effluent compared with ca >10% of E.coli NCTC 9001 strain (does not possess fibrillar K88a antigen but possesses non-adhesive fimbriae, Plate 4.2).

5.3.1.5 Application of Phenyl-sepharose columns for separating groups of organisms grown in milk

The anionic flocculant CA243 described earlier (Chapter 4), was used to partially clarify food homogenates without flocculating or

↓ Inoculum level

Fig. 5.8 Affinity of micro-organisms for small columns ($0.6 \times 6.5 \text{ cm}$) of Phenyl-sepharose gel

↓ Inoculum level
 ▨ Phenyl-sepharose CL-4B
 □ Sepharose CL-4B
 ± Mean and SD are shown, n=2 or 3 for inoculum level, n=3 or 4 for effluent count

Fig. 5.9 Effect of phenyl groups on the adsorption of micro-organisms to Phenyl-sepharose gel

damaging the organisms to a significant extent (Patel & Wood, 1983). Binary mixtures of test organisms, grown in milk, were separated and concentrated by centrifugation (see Chapter 3) prior to application to Phenyl-sepharose columns. The adsorptive behaviour of a mixture of E.coli/Staph.aureus, E.coli/Ps.fluorescens and E.coli/L.plantarum recovered from milk (Table 5.1) was similar to that observed for mixtures of pure cultures of the organisms (Figs. 5.6a-c). In general, the partial clarification of milk did not affect the separation of micro-organisms on Phenyl-sepharose columns i.e. suspended particles in food did not interfere with the separation process (Table 5.1).

TABLE 5.1 A summary of the application of Phenyl-sepharose columns for separation of organisms grown in milk

Mixture of Organisms	Total Log ₁₀ cfu:	
	Applied to column	Recovered in the effluent
<u>E.coli</u> NCTC 9001 &	7.2 (7.4)	6.6 (6.8)
<u>Staph.aureus</u> NCTC 8530	6.5 (6.7)	4.1 (3.6)
<u>E.coli</u> NCTC 9001 &	8.4 (8.2)	7.3 (7.1)
<u>Ps.fluorescens</u> NCTC 10038	7.0 (6.7)	3.6 (3.6)
<u>E.coli</u> NCTC 9001 &	8.6 (8.5)	8.2 (8.2)
<u>L.plantarum</u> NCIB 8960	6.6 (6.5)	< 3.6 (3.9)

Figures in brackets represent an experiment in which the anionic flocculant was not used to clarify milk prior to application of organisms to columns.

5.4 Discussion

The surface hydrophobicity of food-associated micro-organisms was studied by HIC using Phenyl-sepharose and Octyl-sepharose gels. There are several lines of evidence which suggest that hydrophobic interaction promotes adsorption of organisms to hydrophilic-hydrophobic gels.

Firstly, micro-organisms, e.g. Staph.aureus, adsorb > 99% to Phenyl-sepharose gel (Fig. 5.4) and this effect is not due to loss in viability of organisms in 1M ammonium sulphate (Fig. 5.1a, b & d). However, the sensitivity of Ps.fluorescens to 1M ammonium sulphate probably reflects its physiology as a fresh water organism (Fig. 5.1c). The differences in hydrophobicity of micro-organisms (Fig. 5.4) have been exploited in order to resolve binary mixtures of organisms into one of the components either from pure cultures (Fig. 5.6a-c) or from milk inoculated with the test organisms (Table 5.1).

Secondly, in distilled water neither Staph.aureus (Fig. 5.2) nor L.plantarum (Fig. 5.3) adsorbed to Phenyl-sepharose gel. However, Staph.aureus was adsorbed (ca 97%) in the presence of 0.01M phosphate buffer pH 6.5, whereas L.plantarum did not adsorb. Tylewska et al. (1979) showed that strains of streptococci, possessing protein M, were significantly hydrophobic since they adsorbed to Phenyl-sepharose gel in the presence of 0.01M phosphate buffer pH 6.5. In general, Staph.aureus has a greater affinity for Phenyl-sepharose gel than L.plantarum and adsorption of both the organisms is enhanced with an increase in concentration of ammonium sulphate from 0 to 1M.

These results may be explained by reference to the mechanism of hydrophobic interaction. Ochoa (1978) reported that when a hydrophobic colloid is introduced in water there is a negative change in entropy ($-\Delta S$), which means an overall increase in the degree of order of water molecules around the hydrophobic groups. However, when hydrophobic groups come in contact, the ordered water molecules are displaced and therefore there is an increase in the degree of disorder ($+\Delta S$) of water molecules. Thus, the water molecules are largely excluded in the region of contact. On the other hand, Lewin (1974) has shown hydration of both anions (e.g. SO_4^{2-}) and cations (e.g. Ca^{2+}) in water, as a result of which the availability of the bulk water is reduced.

The affinity of micro-organisms for the Phenyl-sepharose gel may be enhanced in at least two ways. The hydration of salt (i.e. ammonium sulphate) in aqueous buffer would reduce the availability of bulk water and thus increase the degree of disorder ($+\Delta S$) of water molecules surrounding hydrophobic components (i.e. phenyl groups and microbial cell surface). The removal of free water molecules in the vicinity of hydrophobic components of the microbial cell surface, may expose these components resulting in hydrophobic interaction between organisms and the gel. When the salt concentration is sufficiently high (i.e. $> 1.0\text{M}$) organisms may be precipitated out of suspension, a phenomenon known as 'salting-out' (Lindahl et al., 1981). Additionally, the contact between micro-organisms and the gel may result in an increase in the degree of disorder of water molecules surrounding the hydrophobic components further increasing the hydrophobic interaction between the two bodies.

Thirdly, the phenyl ligand of Phenyl-sepharose gel, which has greater hydrophobicity at high salt concentrations (Pharmacia Fine Chemicals, 1979) was specifically involved in the interaction with micro-organisms at high salt concentration (Fig. 5.9). However, the matrix sepharose gel may also retain a fraction of the organisms e.g. Olsson & Westergren (1972) showed 50% adsorption of streptococci to sepharose gel compared with 80% for Octyl-sepharose gel. Ochoa (1978) has reported that non-polar 3,6-methylene diether bridges present in agarose (a polysaccharide) exhibit hydrophobic properties at high salt concentrations. Therefore, these groups may contribute to the hydrophobic interaction of micro-organisms with the matrix.

Finally, the presence of certain cell surface proteins e.g. K88a antigen in E.coli, which consists of a high proportion of non-polar amino acids (Stirm et al., 1967), enhanced adsorption of the organism to Phenyl-sepharose gel (Fig. 5.9). The involvement of K88 antigen (Smyth et al., 1978; Lindahl et al., 1981), K99 antigen (De Graaf et al., 1980) of E.coli and surface protein A (Wadström et al., 1981) of Staph.aureus in the interaction of these organisms with Phenyl-sepharose gel has been reported previously. Protein M on the surface of Strep.pyogenes contributed to adsorption of the organisms to hydrophilic-hydrophobic gels (Tylewska et al., 1979) whereas Miörner et al. (1983) showed that lipoteichoic acid on the surface of Group A streptococci was the major component responsible for surface hydrophobicity.

Low recoveries of micro-organisms adsorbed to Phenyl-sepharose columns were obtained on elution with distilled water e.g. < 0.1% of Staph.aureus. Similar low recoveries have been reported for organisms adsorbed to Phenyl-sepharose gel e.g. E.coli (Smyth et al., 1978) and N.gonorrhoeae (Magnusson et al., 1979). Tylewska et al. (1979) showed that the streptococci possessing protein M could not be desorbed from a Phenyl-sepharose column with distilled water, 0.5-3% v/v Triton X-100 or 0.5-3% v/v Tween 80. If the adsorption of organisms to the gel were based entirely on hydrophobic interactions then a greater recovery of organisms in the column effluent would be expected on elution with these reagents (Pharmacia Fine Chemicals, 1979). Therefore other physical forces may also be involved in conjunction with the hydrophobic interactions (see General Discussion, Chapter 8).

Although octyl groups are more hydrophobic than phenyl groups (Pharmacia Fine Chemicals, 1979), the organisms showed a greater affinity for Phenyl-sepharose (Fig. 5.4) than for Octyl-sepharose gel (Fig. 5.5). There may be two explanations for this. Firstly, since equal concentrations of both phenyl or octyl groups are coupled to the sepharose matrix (i.e. 40 μ moles/ml gel; Pharmacia Fine Chemicals, 1979), the aromatic phenyl groups on the sepharose gel may represent a greater concentrated hydrophobic core compared with the flexible aliphatic chains of the octyl group. Secondly, there may be more specific aromatic interactions (π - π interactions) between phenyl groups on the gel and aromatic groups (e.g. phenyl

and tyrosine residues of proteins) present on microbial cell surfaces. Such interactions have been suggested as a mechanism in the purification of β -amylase on phenyl and Octyl-sepharose gels equilibrated with 0.01M phosphate buffer pH 6.8 containing 1M ammonium sulphate (Pharmacia Fine Chemicals, 1979).

Colleen et al. (1979) reported that Staph.saprophyticus (strains 2 and 11) had a low surface hydrophobicity as shown by partitioning in an aqueous polymer two-phase system. However, the same strains (kindly supplied by the authors) were strongly hydrophobic (i.e. $\geq 99\%$ adsorption, Fig. 5.7) as judged by HIC. Miörner et al. (1982) showed that staphylococci were not hydrophobic when judged by partition in aqueous polymer two-phase systems and reported that the results obtained by this method did not always correlate with HIC results. It was suggested by Miörner et al. (1982) that partition experiments measure the overall quantitative expression of hydrophobic and hydrophilic sites whereas in HIC only a few strongly hydrophobic sites on the surface will cause bacteria to adsorb. On the other hand, Olsson & Westergren (1972) showed good agreement between adsorption of organisms (Strep.sanguis, S.salivarius, and S.miteor) to hydrocarbons and behaviour in HIC. Overall, these data suggest that the expression of hydrophobicity by micro-organisms depends on the type of method used for the study.

In this study, we have demonstrated the feasibility of manipulating micro-organisms from pure cultures and from food (i.e. milk) by hydrophobic interaction chromatography. The results show the

potential of hydrophilic-hydrophobic gels for separation and concentration of micro-organisms from foods as a novel method of preparing samples for analysis by the rapid instrumental techniques now emerging in microbiology. The potential applications of the results of this study are similar to those listed in Chapter 4.

5.5 Summary

Interaction of various bacteria with the hydrophilic-hydrophobic gels Phenyl-sepharose and Octyl-sepharose has been investigated. The results showed that differences in the affinity of micro-organisms for Phenyl-sepharose gel can be exploited in order to resolve, to a greater or lesser extent, binary mixtures of organisms into one of the components both from pure cultures and from inoculated foods.

In the presence of ammonium sulphate, the hydrophobic interaction between micro-organisms and Phenyl-sepharose gel was markedly increased. The order of hydrophobicity of micro-organisms was Staph.aureus > L.plantarum > Ps.fluorescens > E.coli and presence of the phenyl ligand was essential for the interaction of organisms with the gel.

Certain protein components on the cell surface enhanced the adsorption of microbial cells to the Phenyl-sepharose gel e.g. K88a antigen of E.coli.

PART II

Chapter 6: INTERACTION OF SALMONELLAE AND OTHER ENTEROBACTERIACEAE WITH IMMUNOSORBENT CHROMATOGRAPHIC MATERIALS

6. INTERACTION OF SALMONELLAE AND OTHER ENTEROBACTERIACEAE WITH IMMUNOSORBENT CHROMATOGRAPHIC MATERIALS

6.1 Introduction

The conventional method for detection of salmonellae in foods is both tedious and time consuming requiring 4-5 days for the presumptive identification (D'Aoust, 1981) and upto 7 days for the confirmation of salmonellae (Koning et al., 1982). Efforts to reduce the detection time for salmonellae have led to the development of a combined enrichment technique (Sveum & Kraft, 1981).

In the more rapid methods for salmonellae, the selective cultures are generally subcultured for a short time (i.e. 4-6 h) in a non-selective broth prior to rapid detection by techniques such as fluorescent antibody technique (FAT; Thomason, 1981), enrichment serology (E/S; Sperber & Deibel, 1969) and enzyme immunoassay (EIA; Swaminathan & Ayres, 1980). By these methods, the total detection time for salmonellae is reduced to approximately 3 days compared with 7 days by the conventional method.

There are several problems associated with the use of these rapid techniques. They are all serological techniques and the lack of commercially available high titre specific antisera against salmonellae gives rise to cross-reactions with antigenically closely related species such as Citrobacter and E. coli (Fantasia et al., 1975). This results in high levels of false-positive reactions in techniques such as FAT (Thomason, 1981) and EIA (Krysinski & Heimsch, 1977). Furthermore, food debris can give rise to high background staining reactions especially in FAT (Anon, 1975) and EIA (Krysinski & Heimsch, 1977).

Finally, a high concentration of salmonellae (i.e. 5×10^7 cfu/ml; Sperber & Deibel, 1969) is required for detection by the FAT or E/S, although with EIA, 1×10^5 cfu/ml of salmonellae can be readily detected (Krysinski & Heimsch, 1977).

The major objective of this study was to devise immunosorbent chromatographic methods for the physical manipulation (i.e. separation and concentration) of salmonellae from mixed cultures with other Enterobacteriaceae and possibly from food preenrichment broths. The ultimate aim was to obtain a clear and concentrated preparation of salmonellae from food preenrichment broths for presentation to the instrumental techniques already available e.g. FAT and EIA.

6.2 Materials and Methods

Antisera against *Salmonella* flagellar antigens

Spicer-Edwards antiserum

Spicer-Edwards antiserum (Difco), specific for a range of *Salmonella* flagellar antigens, was pooled according to Boothroyd & Baird-Parker (1973). The antiserum was dialysed at 4°C for 16 h against 1 litre of either 0.1M phosphate buffer pH 7.0 or 0.05M phosphate buffer pH 6.0. The dialysed antiserum was sterilized by filtration (0.22 µm filter; Millipore) and stored at 4°C. For column application, 0.3ml to 0.5ml of the antiserum was used. Otherwise, 1ml of the antiserum was used.

Polyvalent H antiserum

A 1ml aliquot of polyvalent H, phase 1 and 2 antiserum (Wellcome Research Laboratories, Beckenham), was dialysed at 4°C for 16 h against 1 litre of 0.1M phosphate buffer pH 7.0 and used where appropriate.

Absorbent materials

Protein A-sepharose CL-4B gel

Protein A, isolated from Cowan 1 strain of Staph. aureus, binds specifically with IgG-type antibodies. It interacts with the F_c portion of IgG molecules, thus leaving two F_{ab} portions for normal immunological reactions. Protein A-sepharose CL-4B (Sigma Chemical Co.), is the gel sepharose CL-4B to which protein A has been covalently coupled by the cyanogen bromide method.

Staph.aureus-adsorbed Bio-Rex 70

Ten ml of formalin fixed cells of Staph.aureus (10% w/v; Bethesda Research Laboratories) possessing cell-surface protein A were dialysed at 4°C for 16 h against 1 litre of 0.05M phosphate buffer pH 6.0. After dialysis, the clumped cells were removed by centrifugation (2,000g for 30s) and an aliquot (1ml) of the supernatant containing homogeneously suspended cells (approximately $10^8 - 10^9$ cells/ml) was used for adsorption to the cation exchange resin Bio-Rex 70.

Act-Magnogel AcA 44

Act-Magnogel AcA-44 (LKB Ltd., Croydon, Surrey) consists of 4% polyacrylamide and 4% agarose in a bead form with 7% iron oxide (Fe_3O_4) in the matrix, enabling it to be attracted by a magnetic field. The beads are activated by glutaraldehyde and therefore free amino groups (e.g. in proteins) can be immobilised to the gel.

Immobilisation of proteins to Act-Magnogel AcA-44

The following solutions of proteins were coupled to the Act-Magnogel; whole Spicer-Edwards antiserum, IgG-type antibodies

purified from Spicer-Edwards antiserum by affinity chromatography (i.e. Protein A-sepharose chromatography; described later) and whole Salmonella polyvalent H, phase 1 and 2 antiserum.

The procedure for immobilisation of proteins was essentially similar to that described by the manufacturer (Product Information No. 201103, Reaéctifs IBF, supplied by LKB Ltd.). The following steps were involved:

- a) Act-Magnogel (1ml) was washed twice by suspension in ca 25ml of de-ionised water. After each washing stage the gel was separated from the liquid phase by a magnet (12 x 9 x 42mm, LKB Ltd.). The supernatant liquid was discarded.
- b) The gel was washed twice with ca 25ml of 0.1M phosphate buffer pH 7.0, resuspended in 1ml of the protein solution and mixed (RM/54 Rolamix; Luckham Ltd.) at 4°C for 16-20 h.
- c) If clumps of Magnogel were present either in the initial gel suspension or any subsequent steps, it was thoroughly mixed (Whirlimixer; Fisons Ltd.) to form a homogeneous suspension.
- d) The protein-coupled Magnogel was separated using a magnet and the supernatant liquid was tested for residual immunological activity by the slide agglutination technique.
- e) The Magnogel complex was washed with ca 25ml of 0.1M phosphate buffer pH 7.0, resuspended in 1ml of 0.1M phosphate buffer pH 7.4 containing 0.1M glycineamide and mixed at 4°C for 3h to block remaining glutaraldehyde groups.
- f) The Magnogel complex was washed twice with ca 25ml of 0.05M phosphate buffer pH 7.0 and resuspended in 4ml of the same buffer. This protein-coupled Magnogel was used.

Immunosorbent separation systems

Two types of systems were investigated.

1. Column chromatography

Two types of columns were used; Protein A-sepharose and

Staph.aureus-adsorbed Bio-Rex 70.

a) Protein A-sepharose column

Preparation of the column

Protein A-sepharose gel (250mg) was reconstituted in 5ml of 0.1M phosphate buffer pH 7.0 and allowed to swell for 15 min. The gel was de-aerated under vacuum and equilibrated to pH 7.0 with 50ml of 0.1M phosphate buffer pH 7.0 prior to preparation of the column (0.6 x 2cm bed) as described in Chapter 3.

Purification of IgG-type antibodies from Spicer-Edwards antiserum

An aliquot of Spicer-Edwards antiserum was drained into the column until the level was just below the bed. The space above the bed was filled with 1ml of the equilibration buffer and the column incubated at ambient temperature for 30 min to allow reaction of IgG-type antibodies with protein A. The column was connected via polyethylene tube to peristaltic pump, Uvicord S (model 2138; LKB Ltd.) and fraction collector.

The IgG-sensitised column was eluted with the equilibration buffer at a flow rate of approximately 1ml/min. The absorbance of the effluent, monitored at 280nm by the Uvicord S, was recorded on the chart recorder (model 2210; LKB Ltd.) and 2ml fractions were collected.

After the excess proteins (including unbound immunoglobulins) were eluted (i.e. peak A; Fig. 6.1), IgG bound to the column was eluted with 0.1M glycine-HCl (0.2M) buffer pH 3.5 (peak B; Fig. 6.1). The fractions comprising each peak were pooled separately and protein concentration of each pool determined. The pooled fractions were concentrated by dialysis against 40% m/v polyethylene glycol at 4°C for 16h. The concentrated proteins were reconstituted in 0.2ml of 0.1M phosphate buffer pH 7.0 and tested for immunological activity by the slide agglutination technique.

In other experiments (i.e. batch system) the concentrated IgG-type antibodies (peak B; Fig. 6.1) were reconstituted in 1ml of 0.1M phosphate buffer pH 7.0 for coupling to Act-Magnogel AcA-44.

Interaction of micro-organisms with IgG-sensitised Protein A-sepharose

The following modifications to the above procedure were made in order to investigate the affinity of Salm.typhimurium and E.coli NCTC 9001 for the IgG-sensitised column:

- i) The tapered end of the column was connected by a polyethylene tube with attached gate clip (as shown in Plate 3.2) and the excess antiserum proteins were eluted with 15ml of 0.1M phosphate buffer pH 7.0.
- ii) An aliquot (0.1ml; ca total 10^5 to 10^7 cfu) of the organisms was drained into the IgG-sensitised column and the column incubated at 37°C for 30 min. Non-adsorbed cells were removed by elution with 15ml of 0.1M phosphate buffer pH 7.0.

iii) The cells, adsorbed to the column, were eluted by removing the gel from the column into 5ml of 0.05M phosphate buffer pH 5.0 (containing 0.1M glycyl-tyrosine) and mixing on a whirlimixer for 1 min. The gel was separated from the aqueous phase by centrifugation (2,000g for 30s).

b) Staph.aureus-adsorbed Bio-Rex 70 column

Preparation of the column

Approximately 3g of the cation exchange resin Bio-Rex 70 was batch-equilibrated to pH 6.0 using ca 300ml of 0.05M phosphate buffer pH 6.0 as described in Chapter 3. A 1ml aliquot of the homogeneous formalised Staph.aureus cells was drained into the column (0.6 x 2.5cm bed) until the level was just above the bed and the column was incubated at ambient temperature for 15 min. Non-adsorbed cells were removed by elution with 15ml of the equilibration buffer. The total number of cells both before and after the column application was determined using a haemocytometer (Weber Scientific International Ltd.) and the proportion adsorbed calculated.

Sensitisation of Staph.aureus-Bio-Rex 70 column

A Staph.aureus-adsorbed Bio-Rex 70 column was sensitised with Spicer-Edwards antiserum as follows: An aliquot of the antiserum was drained into the column and the column was incubated at ambient temperature for 30 min to allow reaction of IgG-type antibodies with the cell surface protein A. Excess antiserum proteins were eluted with 15ml of the equilibration buffer.

In order to demonstrate adsorption of immunoglobulins to the Staph.aureus-Bio-Rex 70 column, the adsorbed proteins were batch-eluted by removing the Staph.aureus-Bio-Rex 70 into 5ml of 0.1M glycine-HCl (0.2M) buffer pH 3.5 and mixing on a whirlimixer for 1 min. Staph.aureus cells and Bio-Rex 70 resin were separated by centrifugation (2,000g for 10 min). The supernatant containing proteins was sterilised by filtration (0.22 μ m filter). Both, initial effluent (i.e. excess proteins) and the batch-eluted proteins were concentrated by dialysis at 4°C for 16h against 10%*m/v* polyethylene glycol. The concentrated proteins were each reconstituted in 1ml of 0.1M phosphate buffer pH 7.0 and tested for immunological activity by the slide agglutination technique.

Interaction of micro-organisms with IgG-sensitised Staph.aureus-Bio-Rex 70

The affinity of Salm.typhimurium, E.coli NCTC 9001 and Cit.freundii for the IgG-sensitised column was investigated as follows: An aliquot (0.1ml; ca total 10^5 to 10^7 cfu) of the culture was drained into the column bed and the column incubated at 37°C for 30 min. Non-adsorbed cells were removed by elution with 20ml of the equilibration buffer. The adsorbed cells were eluted by removing Staph.aureus-Bio-Rex 70 into 5ml of 0.05M phosphate buffer pH 8.0 containing 0.1M glycyl-tyrosine and mixing on a whirlimixer for 1 min. The resin was separated by centrifugation (2,000g for 30s).

Effect of different column treatments on the enrichment of *Salm.typhimurium* from mixed cultures

In these experiments, the IgG-sensitised Staph.aureus-Bio-Rex 70 columns were pre-treated with the following solutions prior to application of mixed organisms.

- i) 1ml of 5%_{m/v} bovine serum albumin in 0.05M phosphate buffer pH 6.0 followed by elution of the excess proteins with 15ml of the equilibration buffer.
- ii) 1ml of E.coli outer membrane preparation (see below) in 0.05M phosphate buffer pH 6.0 followed by elution with 15ml of the equilibration buffer.

2. Batch system

Salmonella flagellar antibodies coupled to Magnogel

The protein-coupled Magnogel complex was separated from 1ml of the gel suspension and the supernatant liquid discarded. The gel was suspended in 2ml of the test organisms (ca total 10^6 to 10^8 cfu) and mixed on the Rolamix at 37°C for ca 1.5 h. Magnogel complex was separated and 1ml of the supernatant was removed for viable counts on the appropriate media. The rest of the supernatant was discarded and the gel was gently washed with 20ml of 0.05M phosphate buffer pH 7.0 in order to remove unbound cells. Magnogel complex was suspended in 5ml of 0.05M phosphate buffer pH 8.0 and mixed on a whirlimixer for 1 min. The gel was separated and 1ml of the supernatant was removed for estimation of the viable organisms.

Method for the physical enrichment of *Salm.typhimurium* from food preenrichment broths

1. To 500ml of buffered peptone water, 50g of food and 1ml of *Salm.typhimurium* (ca $<10^2$ cfu) was added. The mixture was incubated at 37°C for 24 h.

2. Centrifuge 10ml of broth (upto 2,000g for 30s with zero holding time at 2,000g). Discard pellet and centrifuge the supernatant (2,000g for 10 min).
3. Discard supernatant and resuspend pellet in 2ml of 0.05M phosphate buffer pH 7.0. Remove 1ml for viable counts on XLD plates.
4. Suspend Spicer-Edwards antiserum-coupled Magnogel in 1ml of the supernatant and incubate at 37°C for 1.5 h on a Rolamix.
5. Gently wash Magnogel twice with 0.05M phosphate buffer pH 7.0 and resuspend in 1ml of 0.05M phosphate buffer pH 8.0. Shake the Magnogel vigorously for 1 min using a whirlimixer in order to desorb organisms from the gel. Remove 1ml of supernatant for counts on XLD plates.

Preparation of outer membranes of *E.coli* NCTC 9001

The procedure was followed essentially as described by Lambert & Booth (1982). *E.coli* cells were lysed by sonication and the cell

envelopes were treated with N-lauryl sarcosine in order to solubilise the cytoplasmic membrane. The outer membranes were then sedimented by centrifugation.

Slide agglutination technique

The procedure was followed essentially as described in the leaflet 'Salmonella agglutinating sera' (Wellcome Research Laboratories, Beckenham).

6.3 Results

6.3.1 Interaction of Salm.typhimurium and E.coli with IgG-sensitised Protein A-sepharose gel

6.3.1.1 Purification of Salmonella-specific IgG-type immunoglobulins

A typical affinity purification of IgG-type antibodies from pooled Spicer-Edwards antiserum is shown in Fig. 6.1. Protein estimations showed that ca 85% of the serum proteins were recovered in the initial column effluent (peak A) and ca 17% of protein was recovered as the IgG-type antibodies (peak B). The concentrated protein comprising peak B showed greater immunological activity than peak A in the slide agglutination test.

6.3.1.2 Affinity of Salm.typhimurium and E.coli for IgG-sensitised Protein A-sepharose gel

Salm.typhimurium showed greater adsorption than E.coli when both the organisms were applied separately to the affinity gel columns (Table 6.1).

Fig. 6.1 Purification of IgG-type antibodies from pooled Spicer-Edwards *Salmonella* antiserum on a column of Protein A-sepharose CL-4B

TABLE 6.1 Adsorption of *Salm.typhimurium* and *E.coli* to IgG-sensitised Protein A-sepharose column

Organism	Total cfu:		% adsorption
	Applied to column	Column effluent	
<u><i>Salm.typhimurium</i></u>	2.5×10^7	9.5×10^5	96
<u><i>E.coli</i></u>	8.5×10^7	7.5×10^7	12

When a mixture of *Salm.typhimurium* and *E.coli* was applied to the affinity column, *Salm.typhimurium* was adsorbed to the column whereas *E.coli* was considerably enriched in initial column effluent (Table 6.2). Batch elution of the adsorbed organisms resulted in ca 2.5-fold enrichment of *Salm.typhimurium* as shown by the reduction in ratios of *E.coli* to *Salm.typhimurium* applied to the column and elution of the adsorbed organisms.

TABLE 6.2 Interaction of mixed culture of *Salm.typhimurium* and *E.coli* with IgG-sensitised Protein A-sepharose column

Ratio of <u><i>E.coli</i></u> : <u><i>Salm.typhimurium</i></u>		
Applied to column	Column effluent	After batch elution
8:1	235:1	3:1

6.3.2 Interaction of Enterobacteriaceae with Spicer-Edwards antiserum-sensitised *Staph.aureus*-Bio-Rex 70

When formalised *Staph.aureus* cells were applied to Bio-Rex 70 at pH 6.0, approximately 43% of the cells were adsorbed to the resin.

6.3.2.1 Adsorption of antibodies to *Staph.aureus*-Bio-Rex 70 column

The slide agglutination test of the concentrated protein representing initial column effluent showed slight agglutination while the protein batch-eluted from the resin showed heavy agglutination when tested with *Salm.typhimurium*. This qualitative test confirmed the adsorption of antibodies from pooled Spicer-Edwards antiserum to *Staph.aureus*-Bio-Rex 70 column.

6.3.2.2 Affinity of Enterobacteriaceae for the antiserum-sensitised *Staph.aureus*-Bio-Rex 70

When a mixture of *E.coli* and *Salm.typhimurium* was applied to the antiserum-sensitised column (Table 6.3; Plate 6.1A), *Salm.typhimurium* was adsorbed whereas *E.coli* was enriched considerably in the initial column effluent (Plate 6.1B) as shown by high ratio (i.e. 452:1) of *E.coli* to *Salm.typhimurium* (Table 6.3). Batch elution of the adsorbed organisms showed a significant enrichment of *Salm.typhimurium* (Plate 6.1C) i.e. there was a reduction in the ratio of *E.coli* to *Salm.typhimurium* applied to the column and the batch-eluted organisms by ca 5-fold.

TABLE 6.3 Interaction of Enterobacteriaceae with Spicer-Edwards antiserum-sensitised *Staph.aureus*-Bio-Rex 70 columns

Mixture of Organisms	Ratio of <u><i>E.coli</i></u> or <u><i>Cit.freundii</i></u> : <u><i>Salm.typhimurium</i></u>		
	Applied to column	Column effluent	After batch elution
<u><i>E.coli</i></u> & <u><i>Salm.typhimurium</i></u> *	11:1	452:1	2:1
<u><i>Cit.freundii</i></u> & <u><i>Salm.typhimurium</i></u>	11:1	7:1	14:1

* represents a mean of 4 separate experiments.

(A)

(B)

(C)

Plate 6.1 XLD plates containing a mixture of Salm. typhimurium (black colonies) and E. coli (grey colonies) before application to Staph. aureus - Bio-Rex 70 column (A), recovery of E. coli in the column effluent (B) and batch elution of the adsorbed organisms (C)

Salm.typhimurium was not enriched from a mixture with Cit.freundii as shown by similar ratios of Cit.freundii to Salm.typhimurium applied to and batch-eluted from the column (Table 6.3).

6.3.2.3 Effect of various column treatments on the enrichment of Salm.typhimurium from mixed cultures

Treatment of the antiserum-sensitised column with either bovine serum albumin or E.coli outer membrane preparation did not increase the enrichment of Salm.typhimurium (Table 6.4) compared with the untreated column (Table 6.3).

TABLE 6.4 Effect of various column treatments on the enrichment of Salm.typhimurium from mixed cultures

Mixture of organisms	Column treatment	Ratio of <u>E.coli</u> or <u>Cit.freundii</u> : <u>Salm.typhimurium</u>		
		Applied to column	Column effluent	After batch elution
<u>E.coli</u> & <u>Salm.typhimurium</u>	Bovine serum albumin	15:1	630:1	3:1
<u>E.coli</u> &* <u>Salm.typhimurium</u>	<u>E.coli</u> outer membrane preparation	10:1	284:1	2:1
<u>Cit.freundii</u> &* <u>Salm.typhimurium</u>	<u>E.coli</u> outer membrane preparation	7:1	54:1	14:1

- * 1. Represents a mean of 2 separate experiments.
 2. Approximately 20µg protein/ml of the outer membrane preparation was obtained from ca 1.68g of E.coli cells. The viable count of the preparation was $\leq 1.3 \times 10^3$ cfu/ml as estimated using Plate Count Agar (Oxoid).

6.3.3 Interaction of Enterobacteriaceae with the immunosorbent Magnetic gel

6.3.3.1 Immobilization of antibodies to Act-Magnogel

Spicer-Edwards antiserum and the affinity purified IgG showed heavy agglutination by the slide agglutination test before coupling to Act-Magnogel. However, the supernatant after coupling to Act-Magnogel showed slight or no agglutination indicating immobilization of protein to the gel.

6.3.3.2 Effect of various types of coupled Magnogel on the adsorption of Salm.typhimurium and E.coli

The adsorption of Salm.typhimurium and E.coli to glycinamide-coupled Magnogel was similar as shown by little difference in the ratio of organisms before Magnogel treatment and after batch elution of the Magnogel (Table 6.5). However, with whole antiserum and the purified IgG-coupled Magnogel the adsorption of Salm.typhimurium was greater than E.coli by 29- and 65-fold, respectively.

TABLE 6.5 Adsorption of Salm.typhimurium and E.coli separately to various types of coupled Magnogel

Ligand coupled to Mangnogel	Ratio of <u>Salm.typhimurium</u> : <u>E.coli</u>		
	Before Magnogel treatment	Supernatant after Magnogel treatment	After batch elution of Magnogel
Glycinamide*	1:1	0.3:1	2:1
Spicer-Edwards antiserum	1:1	0.4:1	29:1
Affinity purified IgG	2:1	1:1	13:1

* represents a mean of triplicate results.

The maximum adsorption of Salm.typhimurium to Spicer-Edwards antiserum-coupled Magnogel occurred after >1h of reaction time (Fig. 6.2).

6.3.3.3 Affinity of mixed cultures of Enterobacteriaceae for various proteins coupled to Magnogel

Salm.typhimurium was enriched by 11-fold and by 5-fold from a mixture with E.coli and Cit.freundii, respectively, using the Spicer-Edwards antiserum-coupled Magnogel (Table 6.6). This was calculated from reduction in the ratios of organisms before Magnogel treatment and after batch elution of the Magnogel.

TABLE 6.6 Interaction of mixtures of Enterobacteriaceae with Spicer-Edwards antiserum-coupled Magnogel

Mixture of* Organisms	Ratio of <u>E.coli</u> or <u>Cit.freundii</u> : <u>Salm.typhimurium</u>		
	Before Magnogel treatment	Supernatant after Magnogel treatment	After batch elution of Magnogel
<u>E.coli</u> & <u>Salm.typhimurium</u>	11:1	26:1	1:1
<u>Cit.freundii</u> & <u>Salm.typhimurium</u>	15:1	30:1	3:1

* represents a mean of 3 separate experiments.

There was ca 13-fold enrichment of Salm.typhimurium from a mixture with E.coli when IgG-coupled Magnogel was used (Table 6.7) and 5-fold enrichment of Salm.typhimurium when polyvalent H antiserum-coupled Magnogel was used (Table 6.8). However,

● Salm. typhimurium

○ E. coli

E_0 Final extinction at 420 nm

E Initial extinction at 420 nm

Fig. 6.2 Specific adsorption of Salm. typhimurium to Spicer-Edwards Salmonella antiserum coupled Magnogel

Salm.typhimurium was not enriched from a mixture with Cit.freundii using either IgG-coupled Magnogel or the polyvalent H antiserum-coupled Magnogel.

TABLE 6.7 Interaction of mixtures of Enterobacteriaceae with purified IgG-coupled magnogel

Mixture of* Organisms	Ratio of <u>E.coli</u> or <u>Cit.freundii</u> : <u>Salm.typhimurium</u>		
	Before Magnogel treatment	Supernatant after Magnogel treatment	After batch elution of Magnogel
<u>E.coli</u> & <u>Salm.typhimurium</u>	80:1	357:1	6:1
<u>Cit.freundii</u> & <u>Salm.typhimurium</u>	10:1	36:1	40:1

* represents a mean of between 2 and 4 replicates.

TABLE 6.8 Interaction of mixtures of Enterobacteriaceae with polyvalent H antiserum-coupled Magnogel

Mixture of* Organisms	Ratio of <u>E.coli</u> or <u>Cit.freundii</u> : <u>Salm.typhimurium</u>		
	Before Magnogel treatment	Supernatant after Magnogel treatment	After batch elution of Magnogel
<u>E.coli</u> & <u>Salm.typhimurium</u>	10:1	24:1	2:1
<u>Cit.freundii</u> & <u>Salm.typhimurium</u>	12:1	29:1	15:1

* represents a mean of 3 separate experiments.

6.3.3.4 Application of Spicer-Edwards antiserum-coupled Magnogel for separation of salmonellae from food preenrichment broths

Table 6.9 shows a typical result of the application of antiserum-coupled Magnogel to a cocoa preenrichment broth. The ratios of non-salmonellae to salmonellae before and after the Magnogel treatment were similar. With chicken preenrichment broth (unreported data), it was found that ca total 10^7 cfu of non-salmonellae were adsorbed to the antiserum-coupled Magnogel.

TABLE 6.9 Affinity of organisms from cocoa preenrichment for the Spicer-Edwards antiserum-coupled Magnogel

Mixture of organisms	Before Magnogel treatment (total cfu)	Organisms eluted from Magnogel (total cfu)
<u>Salm.typhimurium*</u>	4.8×10^7	5.6×10^5
<u>Non-Salmonella</u>	5.9×10^8	8×10^6

- * 1. Biochemical confirmation using the Api 10 system (API System S.A., France).
 2. Salmonella was also detected by the conventional cultural procedure.

6.4 Discussion

Staphylococcal protein A binds to the F_c portion of γ -globulin molecules leaving the two F_{ab} portions for normal immunological reaction (Kronvall & Frommel, 1970). Immobilized protein A e.g. Protein A-sepharose gel (Patel, 1984) and Protein A-bearing Cowan 1 cells of Staph.aureus (Goding, 1978) have therefore been widely used as immunological reagents for the purification of IgG-type antibodies or immune complexes from sera. Recently, Protein A-

Staph.aureus cells have been used in the coagglutination techniques for detection of various clinically important organisms (Goldschmidt, 1982) including Salmonella (Sanborn et al., 1980).

In this study, novel immunosorbent chromatographic techniques have been devised for the physical enrichment of viable cells of Salm.typhimurium from mixed broth cultures with other Enterobacteriaceae but not from food preenrichment broths.

There was ca 2.5-fold (Table 6.2) and 5.5-fold (Table 6.3) enrichment of Salm.typhimurium from a mixture with E.coli using the column chromatographic techniques; Protein A-sepharose and the antiserum-sensitised Staph.aureus-Bio-Rex 70, respectively. The effect of this apparently low enrichment factor e.g. 5.5-fold using the sensitised Staph.aureus-Bio-Rex 70, appears dramatic when aliquots of a mixture of Salm.typhimurium and E.coli before (Plate 6.1A) and after (Plate 6.1C) the column treatment are cultured on XLD plates. Salm.typhimurium was not enriched from a mixture with Cit.freundii (Table 6.3). There may be at least two explanations for the incomplete resolution of Salm.typhimurium from the mixed cultures. Firstly, the non-salmonellae may adsorb to the matrix of chromatographic material because of greater number of effective collisions between the organisms and the column chromatographic material. In this context, solid separation materials may be screened initially in order to establish a surface which exhibits low non-specific binding capability. Secondly, somatic "O" antibodies which cross-react strongly with other Enterobacteriaceae

(Aleksić, 1982) may be present in the Spicer-Edwards antiserum and therefore increase adsorption of non-salmonellae to the immunosorbent chromatographic materials.

Pre-treatment of the Staph.aureus-Bio-Rex 70 resin with either bovine serum albumin or E.coli outer membrane preparation did not enhance the enrichment of Salm.typhimurium from mixed cultures (Table 6.4). These results indicate that non-salmonellae may adsorb to the column by non-specific interactions (e.g. hydrophobic interactions) between the particle (i.e. organisms) and macroscopic surface (i.e. Bio-Rex 70) rather than specific interactions between microbial surface components (e.g. proteins) and the chromatographic material.

The adsorbent, Protein A-sepharose gel was substituted by Staph.aureus-Bio-Rex 70 resin for two reasons. Protein A-sepharose is expensive and because of the hydrophilic gel matrix it packs tightly into the glass column resulting in reduced flow rates. Practically, a batch adsorption/elution system would be simpler than a column system. However, Staph.aureus-Bio-Rex 70 resin could not be used in a batch system since agitation during batch adsorption may result in desorption of the Staph.aureus.

Recently, Magnetic Enzyme ImmunoAssay (MEIA) techniques have been developed for the rapid estimation of staphylococcal enterotoxin B (Patel & Gibbs, 1984) and staphylococcal enterotoxin B IgG-type antibodies (Patel et al., 1984). In these techniques, an immunosorbent magnetic gel is used for the separation of unbound

enzyme conjugate from the bound material. A magnetic gel, to which Salmonella-specific antibodies were coupled, was used in a batch system to investigate the separation of salmonellae from mixed broth cultures and from food preenrichment broths.

The immunological specificity of adsorption of Salm.typhimurium to the protein-coupled Magnogel is shown in Table 6.5 and Fig. 6.2.

The enrichment of Salm.typhimurium from a mixture with E.coli was as follows; 11-fold using the Spicer-Edwards antiserum-coupled Magnogel (Table 6.6), ca 13-fold using the purified IgG-coupled Magnogel (Table 6.7) and 5-fold using the polyvalent H antiserum-coupled Magnogel (Table 6.8). However, Salm.typhimurium was not enriched from a mixture with Cit.freundii except where Spicer-Edwards antiserum-coupled Magnogel was used (i.e. 5-fold enrichment, Table 6.6). It is possible that the polyvalent H antiserum may contain a higher concentration of the cross-reacting somatic "O" antibodies than does the Spicer-Edwards antiserum. This may explain the greater enrichment of Salm.typhimurium when Spicer-Edwards antiserum-Magnogel was used.

Salm.typhimurium was not enriched from the cocoa preenrichment broth using the Spicer-Edwards antiserum-coupled Magnogel (Table 6.9).

The reasons for this may be similar to those described for the mixed broth cultures in the earlier part of this Discussion with the added problem of food material. Food constituents such as proteins and salts may interfere with adsorption of Salmonella and enhance adsorption of non-salmonellae to the gel. The results obtained suggest that low levels of salmonellae normally present in

preenrichment broths (D'Aoust, 1981) of Salmonella-contaminated foods may not be easily separated using the Spicer-Edwards antiserum-coupled Magnogel.

The use of adsorbents to separate salmonellae from other Enterobacteriaceae may be improved provided that highly specific antibodies (e.g. monoclonal antibodies) with high avidity against salmonellae (Robison et al., 1983) are commercially available. In addition, a monoclonal antibody coupled to Magnogel might be used to estimate components of Salmonella e.g. flagella (instead of whole cells) by an MEIA technique. Since the sensitivity of MEIA technique is comparable to radioimmunoassays (Patel et al., 1984), the proposed technique may be able to detect and also estimate salmonellae in preenrichment broths.

6.5 Summary

Novel immunosorbent chromatographic methods have been devised for the physical enrichment of viable cells of Salm.typhimurium from mixed broth cultures containing other Enterobacteriaceae although not from food preenrichment broths.

The enrichment of Salm.typhimurium from a mixture with E.coli was ca 2.5-fold using Protein A-sepharose gel and ca 5.5 fold using Staph.aureus adsorbed to Bio-Rex 70 resin. However, Salm.typhimurium was not enriched from a mixed culture containing Cit.freundii.

There was 11-fold enrichment of Salm.typhimurium from a mixture containing E.coli and 5-fold enrichment from a mixture containing Cit.freundii when Spicer-Edwards antiserum-coupled Magnogel was used.

The problems associated with the use of immunosorbent chromatographic materials are discussed and some alternative approaches are suggested.

PART II

Chapter 7: THE SEPARATION OF MICRO-ORGANISMS FROM RAW MEATS AND THEIR RAPID ESTIMATION BY MEASUREMENT OF MICROBIAL ADENOSINE TRIPHOSPHATE (ATP)

7. THE SEPARATION OF MICRO-ORGANISMS FROM RAW MEATS AND THEIR RAPID ESTIMATION BY MEASUREMENT OF MICROBIAL ADENOSINE TRIPHOSPHATE (ATP)

7.1 Introduction

ATP assay techniques have been investigated for the estimation of viable micro-organisms in sewage sludge (Vanstaen, 1979), dental plaque (Distler et al., 1980), mineral water (Baumgart et al., 1980a) and intravenous fluids (Anderson et al., 1981). Sharpe et al. (1970) investigated such a technique for the estimation of micro-organisms in foods and concluded that it was necessary to distinguish between microbial ATP and ATP intrinsic to the food. Recently, Stannard & Wood (1983) made this distinction by physical separation of micro-organisms from meat using a three stage method involving centrifugation, cation exchange resin and filtration.

The broad objective of the present study was to extend our knowledge of the ways in which micro-organisms could be separated from meats. Specifically, we wished to determine whether flocculation and centrifugation could be used to separate micro-organisms from the intrinsic ATP in raw meats. It was also of interest to determine whether intracellular ATP levels could be enhanced by supplying a readily metabolised energy source i.e. glucose.

7.2 Materials and Methods

Meat (Beef and Lamb)

The meat used in this study was stored at 4°C for upto one week in order to increase the microbial count. A 10g sample was aseptically excised from the meats and cut into pieces (approximately 0.5g) prior to step 1 (Fig. 7.1).

Methods for separation of micro-organisms from meat

Three methods were investigated (Fig. 7.1). These were:

flocculation, flocculation-centrifugation and centrifugation.

Fig.7.1 Three methods for the separation of micro-organisms from meat

1. Treat 10g of meat in a Colworth stomacher with 90ml of phosphate buffer (0.1M, pH 5.5) for 2 min and filter through glass wool. Remove 1ml for colony count.

2. To 10ml filtered meat homogenate* add 2ml flocculant solution and 0.05ml calcium chloride solution (20%^m/v). Shake gently and let stand for 10 min.

3. Centrifuge (2,000g, 30 s) to remove flocs.

FLOCCULATION

FLOCCULATION-
CENTRIFUGATION

CENTRIFUGATION

A. Retain supernatant and treat with further flocculant as in steps 2 and 3. Remove 1ml for colony count.

Retain supernatant.
B. Centrifuge (2,000g, 20 min) to sediment bacteria.

Treat 5ml meat homogenate as in B, C and D. Estimate ATP.

C. Discard supernatant liquid and resuspend pellet in 6ml of sterile R & P solution. Remove 1ml for colony count and repeat centrifugation (2,000g, 10 min).

D. Discard supernatant liquid and resuspend pellet either in 0.9ml 0.1M tris-EDTA (2mM) buffer, pH 7.75, or nutrient broth + 1% glucose. (The latter step is followed by brief incubation at 37°C for 15 min). Estimate ATP.

*In the flocculation-centrifugation method, the volumes of meat homogenate, flocculant solution and calcium chloride solution were halved.

Measurement of turbidity

The turbidity of meat homogenates both before and after repeated flocculation (step A, Fig. 7.1) was measured in an EEL Nephelometer (Evans Electroselenium Ltd., Essex).

Reagents and instruments for ATP analysis

A solution of 'Extralight' (Demco Ltd., Camden Drive, Birmingham) was used to extract ATP from microbial cells. The quantitative analysis of ATP was done by a bioluminescence technique using luciferin-luciferase mixture (monitoring reagent, LKB Ltd.). Light output of this reaction was measured in a Luminometer Model 1250 (LKB Ltd.) and recorded as mV response on the chart recorder Model 2210 (LKB Ltd.).

Measurement of ATP content

A bioluminescence technique (Thore, 1979) was used to estimate ATP. An aliquot (0.1ml) of sample was mixed with 0.1M tris-EDTA buffer pH 7.75 (0.6ml) and the monitoring reagent (0.2ml) in a cuvette. The mixture was allowed to stabilise (ca 10 min) before the cuvette was placed in the luminometer. When a steady base-line (initial mV reading) was achieved on the chart recorder, ATP was extracted from the viable cells in the mixture by adding 0.1ml of 'Extralight' reagent. The reaction was continuously monitored in the luminometer until a peak in mV output was reached. The amount of ATP in the sample was calculated from the difference between the final and initial mV reading by interpolation from a standard curve of ATP concentration (Calbiochem Ltd.) versus mV output (Fig. 7.2).

Fig. 7.2 A typical standard curve of ATP vs. mV Response obtained on LKB Luminometer model 1250

Estimation of total and microbial ATP

Measurement of total (meat + microbial) ATP in meat

The glass wool filtered meat homogenate (1ml, Fig. 7.1) was mixed with 1ml of hot (ca 70°C) 0.1M tris-EDTA buffer pH 7.75, containing Triton X-100 (0.1% V/V) in a centrifuge tube. The tube was placed in a boiling water bath (2 min) and then cooled in ice. The resulting precipitates were removed by centrifugation (2,000g for 5 min) and the supernatant liquid (0.1ml) was assayed for ATP.

Measurement of microbial ATP

The flocculation-centrifugation technique (Fig. 7.1) was used for the separation and concentration of micro-organisms from meat prior to estimation of microbial ATP.

Ratios of total or microbial ATP (fg) to the viable count (cfu/g) of raw meat were used as a measure of the efficiency of separation of microbial from non-microbial ATP.

7.3 Results

7.3.1 Clarification of meat homogenates by use of the polyelectrolyte anionic flocculant

The results (Table 7.1) demonstrate that the flocculation procedure (step A, Fig. 7.1) results in partial clarification of meat homogenates without apparent loss to the microbial population within limits of colony count which can be upto $0.42 \log_{10}$ cfu/ml (Stannard & Wood, 1983).

TABLE 7.1 Clarification of minced beef homogenates by the flocculation procedure (step A, Fig. 7.1)

Meat storage time(h)	Decrease in turbidity after flocculation (%)	Colony count of homogenate (cfu/ml):	
		Before flocculation	After flocculation
24	38	5×10^6	4×10^6
48	54	4×10^7	2×10^7
72	43	5×10^7	4×10^7

7.3.2 Recovery of micro-organisms from raw meat

The number of micro-organisms in meat homogenate both before and after flocculation-centrifugation or centrifugation procedures were similar (Tables 7.2 & 7.3), within the limits of the colony count method for raw meat (Stannard & Wood, 1983).

TABLE 7.2 Recovery of micro-organisms from raw lamb, following the flocculation-centrifugation and centrifugation procedures (step B, Fig. 7.1)

Meat Storage time (h)	Colony count (cfu/g of meat):		
	Homogenate	After flocculation-centrifugation	After centrifugation
0	6×10^4	9×10^4	2×10^4
24	3×10^5	4×10^5	3×10^5
48	9×10^7	7×10^7	9×10^7
72	2×10^8	2×10^8	2×10^8
96	1×10^9	8×10^8	1×10^9

TABLE 7.3 Recovery of micro-organisms from raw beef following the flocculation-centrifugation and centrifugation procedures (step B, Fig. 7.1)

Meat storage time(h)	Colony count (cfu/g of meat):		
	Homogenate	After flocculation-centrifugation	After centrifugation
0	2×10^5	9×10^4	9×10^4
24	3×10^6	3×10^6	3×10^6
48	6×10^7	6×10^7	6×10^7
72	6×10^8	6×10^8	6×10^8

7.3.3 Separation and estimation of microbial ATP from meat

The flocculation-centrifugation procedure removed a considerable amount ($\geq 90\%$) of non-microbial ATP from lamb (Table 7.4) and beef (Table 7.5) homogenates. For example, in lamb the ratio of total ATP to colony count (B/A) was greater and also showed a greater variation with storage time of meat than did the ratio of microbial ATP to colony count (C/A). The ratio B/A for lamb decreased with increase of storage time from 0 to 72h by 1,500-fold (i.e. meat ATP was decreasing and microbial ATP was increasing). The ratio for beef decreased by 1,000-fold (Table 7.5). The values obtained for the ratio C/A were close to those quoted in the literature (Baumgart et al., 1980 a,b) for the ATP/cfu content of micro-organisms whereas the values of B/A were always greater.

TABLE 7.4 Separation of microbial ATP from total ATP in raw lamb by the flocculation-centrifugation procedure

Meat storage time(h)	Colony count of homogenate (cfu/g of meat) (A)	ATP (fg/g* of meat):		Ratio of:	
		Total (B)	Microbial (C)	B/A	C/A
0	2×10^4	9×10^7	4×10^4	4500	2.0
24	1×10^5	3×10^8	2×10^5	3000	2.0
48	3×10^7	1×10^9	6×10^6	33	0.2
72	1×10^8	3×10^8	6×10^7	3	0.6
96	4×10^8	3×10^9	3×10^8	7.5	0.8

* femtogram = 10^{-15} g

TABLE 7.5 Separation of microbial ATP from total ATP in raw beef by the flocculation-centrifugation procedure

Meat storage time (h)	Colony count of homogenate (cfu/g of meat) (A)	ATP (fg/g of meat):		Ratio of:	
		Total (B)	Microbial (C)	B/A	C/A
0	1×10^5	5×10^7	1×10^5	500	1.0
24	2×10^6	1×10^8	4×10^6	50	2.0
48	4×10^7	1×10^8	7×10^7	2.5	1.8
72	4×10^8	2×10^8	2×10^8	0.5	0.5

7.3.4 Relationship between colony count and microbial ATP content of meat and effect of incubation in nutrient medium on the relationship

The relationship between $\log_{10}$ fg ATP/g meat and $\log_{10}$ cfu/g meat, obtained following either the centrifugation or the flocculation-centrifugation procedure, was essentially linear with a combined correlation value of $r = 0.98$ for lamb (Fig. 7.3) and $r = 0.97$ for beef (Fig. 7.4) and either method would permit estimates of the microbial contamination of meat. A brief incubation of micro-organisms (psychrotrophs) separated from meat raised the ATP/cfu (Figs. 7.3 & 7.4) by between 2- and 15-fold (Tables 7.8 & 7.9). The ATP/cfu of the psychrotrophs may be increased by incubating at 25° or 30°C, but at 37°C the multiplication of the organisms is likely to be restricted, yet metabolism continues for this short period.

7.3.5 ATP content of micro-organisms separated from meat

The amount of ATP/cfu recovered after both the flocculation-centrifugation and the centrifugation procedure was similar (Tables 7.6 & 7.7) but not constant over the range of cfu/g of meat examined. At low levels of microbial contamination (10^4 - 10^5 cfu/g), the amount of ATP/cfu was higher than at high levels of contamination (10^9 - 10^{10} cfu/g; Tables 7.8 & 7.9).

Fig. 7.3 Relationship between total viable count and the microbial ATP content of lamb and the effect of nutrient medium on this relationship.

- Flocculation - centrifugation &
- △ Centrifugation procedures, solid regression line,
 $y = 2.2 + 0.66x$ ($r = 0.97$)
- After incubation in nutrient medium, broken
regression line, $y = 2.8 + 0.65x$ ($r = 0.98$)

Fig. 7.4 Relationship between total viable count and the microbial ATP content of beef and the effect of nutrient medium on this relationship.

TABLE 7.6 Comparison of ATP/cfu separated by the flocculation-
centrifugation and centrifugation procedures (Fig. 7.1)
for lamb

Range of colony count (log ₁₀ cfu/g of meat)	Mean fg ATP/cfu produced by:	
	Flocculation- centrifugation*	Centrifugation ⁺
4-5	1.19	2.1
5-6	0.71	0.85
6-7	0.22	0.2
7-8	0.14	0.05
8-9	0.27	0.22

* No. of estimates in range varied between 2 and 6

+ No. of estimates in range varied between 1 and 3

TABLE 7.7 Comparison of ATP/cfu separated by the flocculation-
centrifugation and centrifugation procedures (Fig. 7.1)
for beef

Range of colony count (log ₁₀ cfu/g of meat)	Mean fg ATP/cfu produced by:	
	Flocculation- centrifugation*	Centrifugation ⁺
4-5	1.65	1.95
6-7	1.25	1.0
7-8	0.78	0.5
8-9	0.22	0.19
9-10	0.04	0.04

* No. of estimates in range varied between 2 and 6

+ No. of estimates in range was either 1 or 2

TABLE 7.8 Mean ATP content of micro-organisms separated from lamb by the flocculation-centrifugation and centrifugation procedures (Fig. 7.1) and the effect on ATP levels after resuscitation in nutrient broth with glucose

Range of colony count (log ₁₀ cfu/g of meat)	No. of estimates in range	Mean fg ATP/cfu after suspending in:	
		0.1M tris-EDTA buffer, pH 7.75	Nutrient broth + glucose*
4-5	5	1.4	5.4
5-6	3	0.75	ND
6-7	3	0.22	1.9
7-8	6	0.11	1.7
8-9	9	0.25	0.6

* Incubated at 37°C, 15 min

ND = not done

TABLE 7.9 Mean ATP content of micro-organisms separated from beef by the flocculation-centrifugation and centrifugation procedures (Fig. 7.1) and the effect on ATP levels after resuscitation in nutrient broth with glucose

Range of colony count (log ₁₀ cfu/g of meat)	No. of estimates in range	Mean fg ATP/cfu after suspending in:	
		0.1M tris-EDTA buffer, pH 7.75	Nutrient broth + glucose*
4-5	6	1.75	7
6-7	3	1.17	ND
7-8	6	0.69	2.4
8-9	6	0.21	0.4
9-10	3	0.04	0.23

* Incubated at 37°C, 15 min

ND = not done

7.4 Discussion

The bioluminescent assay of ATP is a well-established method for the estimation of micro-organisms suspended in buffer solutions or culture media (Baumgart et al., 1980b). Early attempts to apply this method to the estimation of micro-organisms in foods were frustrated by interference from large amounts of non-microbial ATP intrinsic to the foods (Sharpe et al., 1970). More recently, Baumgart et al. (1980b) have also encountered this problem.

In order to estimate numbers of colony forming units of micro-organisms in foods by measurement of microbial ATP, two conditions must be fulfilled. First, it is essential to distinguish microbial ATP from ATP intrinsic to the food. Second, the amount of ATP per colony forming unit (ATP/cfu) should not be subject to excessive variation. The first of these requirements may be approached either by enzymic destruction of non-microbial ATP in the food, prior to release and estimation of microbial ATP from the cells (Baumgart et al., 1980b; Bossuyt, 1981) or by the physical separation of micro-organisms from the food prior to the measurement of microbial ATP (Stannard & Wood, 1983).

In the present study, a new method of separating micro-organisms from raw meat was evaluated for distinguishing microbial from intrinsic (meat) ATP. Comparison of the ratios of total ATP and of microbial ATP to numbers of cfu in the meats (B/A and C/A, respectively in Tables 7.4 and 7.5) suggest that the flocculation-centrifugation procedure is an efficient separation process. The ratio of microbial ATP to cfu in these meats (C/A) was not constant

but the range of values obtained over the 72h storage period of the meats was relatively narrow, i.e. 0.2 to 2.0 compared with a range of 0.5 to 4,500 for total ATP to cfu.

The second requirement of the method, i.e. that there should not be excessive variation in levels of ATP/cfu, is examined in Tables 7.8 and 7.9. For organisms in tris-EDTA buffer there is a considerable variation in this value and a distinct tendency for ATP/cfu to decrease with increase in colony count. This may be due to subtle changes in the microflora of meats as the microbial count increases with increase in the meat storage time. Furthermore, competition among the organisms in meat may result in domination of certain organisms while repressing the growth of other organisms. Brief incubation in nutrient media raised the level of ATP/cfu in all cases and marginally decreased the range of values (12-fold in 0.1M tris-EDTA to 9-fold in nutrient broth + glucose, Table 7.8; 44-fold in 0.1M tris-EDTA to 30-fold in nutrient broth + glucose, Table 7.9). It has also been demonstrated that the ATP pool of the bacterial cell may be greatly diminished under various conditions of nutrient limitations (Forrest, 1965; Bachi & Ettlinger, 1973; Leps & Ensign, 1979) and it seems possible that such limitations are progressively imposed during storage of the meat. Another factor which may contribute to the reduction of the ATP pool is the increased 'stress' imposed on the microbial cells during separation and concentration by the centrifugation procedures. The values obtained after incubation in a nutrient medium still show the trend of decrease of ATP/cfu with increase in colony count although most of the values are within the range of those reported in the

literature for pure cultures of bacteria (Baumgart et al., 1980b) and those reported by Stannard & Wood (1983) for micro-organisms separated from meat by the resin/filtration method. However, in the lowest range of colony count examined, the values of ATP/cfu were higher than for any other range and are higher than reported in the literature (Baumgart et al., 1980a). This suggests that a small number of meat cells and/or membrane fragments containing active enzyme systems is separated with the micro-organisms and that these produce ATP in the nutrient medium. Such an effect would cause a large distortion in the apparent value of ATP/cfu at the lowest levels of micro-organisms but would be less obvious as the number of organisms increased, especially as the meat of low microbial count would be expected to be fresher and therefore more enzymically active.

It would be of interest to compare the efficiency of physical separation of micro-organisms from ATP in food with that achieved by the enzymic destruction of ATP. Unfortunately, the workers who have used this method (Baumgart et al., 1980b; Bossuyt, 1981) have not measured total ATP. Nor is it possible to calculate ATP/cfu from their published data since the results are presented only as plots of 'relative light units' against cfu. Both publications show linear $\log_{10}/\log_{10}$ plots for these values and comment upon the difficulties of removing non-microbial ATP.

The results of the present study suggest that the flocculation-centrifugation technique produces an efficient but not complete separation of non-microbial ATP from micro-organisms in raw meats. Consideration of Figs. 7.3 & 7.4 show that there is a linear

$\log_{10}/\log_{10}$ relationship with $r = 0.98$ for lamb and $r = 0.97$ for beef between the amount of microbial ATP separated from the meat and the colony forming units. The practical advantage of this relationship is that it permits estimates of colony counts in meat to be obtained within 45 min by the centrifugation method or 60 min by the flocculation-centrifugation method. The major practical question which arises from the proposed use of such a technique in the quality control (QC) of food is whether it produces the same or equivalent information as the colony count for QC purposes. Stannard & Wood (1983) found by statistical analysis that the colony count was a more discriminatory measure than ATP measurement, but that the two measures were well correlated and showed very good agreement in the classification of meat samples in terms of colony count. Overall, it appears that decisions made concerning the microbiological status of meat would be substantially the same whichever of these methods was used.

At a more fundamental level, the estimation of ATP would not be expected to correlate perfectly with estimates of cfu because the cfu is not a standard quantity, i.e. the number of microbial cells in a colony forming unit can vary. In this respect, the measurement of microbial ATP may represent a better basis for the measurement of total microbial contamination of food than does the colony count. The cfu is the preferred measurement at present because most of our experience of 'good manufacturing practice' and product stability is in terms of this unit, as are many microbiological guidelines and specifications. At present, therefore, the measurement of microbial ATP will most usefully serve as a rapid means of estimating colony

count in meat. However, in the future we might consider microbial biomass measured as microbial ATP (Sharpe et al., 1970) as a more standardised measure of total microbiological contamination than the colony count. Microbiological status, as an index of keeping quality, might be more readily estimated either by direct measurement of microbial ATP or by its rate of increase over a short period of product storage time.

The present investigation introduces an alternative method of physical separation of micro-organisms from raw meat to that reported by Stannard & Wood (1983) and it is of interest to compare these methods. Stannard & Wood (1983) used brief centrifugation of the meat homogenate (30 s) to remove gross particles, followed by treatment with an acrylic-carboxylic resin (Bio-Rex 70) to remove sufficient colloidal and other meat constituents to permit the collection and concentration of micro-organisms by membrane filtration. In the present flocculation-centrifugation method, filtration through glass wool removed gross particles, and other meat particles were removed by flocculation. The flocculant is essentially a soluble form of the acrylic-carboxylic resin used by Stannard & Wood (1983). The organisms were then collected and concentrated by centrifugation for 30 min. The major difference in the techniques lies in the treatment of the suspension to remove colloidal and other meat constituents. The use of resin involves prior equilibration of the ion exchange resin and more manipulation of the sample, i.e. stirring with resin and filtration, but these latter stages required only about 10 min to complete. The flocculant is less expensive than the resin; it requires no prior

treatment and the manipulations in the flocculation-centrifugation method are fewer. However, the centrifugation steps (30 min in total) increase the overall test time to 60 min and, if incubation in nutrient medium is included, produces a test time of about 75 min compared with about 25 min for the resin/filtration procedure and 45 min for the centrifugation method. All these techniques appear to be suitable for the rapid estimation of micro-organisms in meat and the choice of which to use may depend upon the availability of equipment and personal preference.

In this study, we have shown that flocculation followed by centrifugation produces a less turbid suspension for ATP analysis than centrifugation alone. However, particles in the suspensions produced by either method contain similar amounts of ATP (Tables 7.6 & 7.7). Therefore, the turbidity of the suspension produced by centrifugation alone does not reflect inadequate separation of micro-organisms from non-microbial ATP. While centrifugation alone is satisfactory for meat cuts, for mechanically recovered pork and beef meats, Stannard & Smith (1982) found that neither centrifugation alone or cation exchange resin clarify the suspension sufficiently to permit estimation of microbial ATP. However, Stannard & Smith (1982) have successfully used the anionic flocculant to clarify homogenates of mechanically recovered meats.

These physical separation methods in conjunction with estimation by the bioluminescence technique may be used by QC laboratories especially when small numbers of samples e.g. raw materials such as meats and finished products, need to be analysed quickly (i.e. ca 1h)

for the total viable count. No other technique except Direct Epifluorescent Filter Technique (DEFT) can give a result so quickly. Automation of physical separation, concentration and final estimation of micro-organisms may be required for analysis of greater numbers of samples.

7.5 Summary

An anionic polyelectrolyte flocculant was demonstrated to clarify homogenates of raw meats without causing precipitation of the microbial flora or affecting their viability. Subsequently, a method involving flocculation and centrifugation was developed for the separation and concentration of micro-organisms from lamb and beef prior to estimation of the microbial ATP content.

A linear relationship was found between $\log_{10}$ (total viable count) and $\log_{10}$ (microbial ATP content) of meat, obtained by the flocculation-centrifugation procedure. Such a relationship was also demonstrated for a centrifugation procedure in which the flocculant was not used. The combined correlation coefficients when both the methods are considered was $r = 0.98$ for lamb and $r = 0.97$ for beef. The lower limit of detection of the ATP assay technique ranged between 10^4 and 10^5 cfu/g of raw meat.

Further investigations were carried out to determine the efficiency of the flocculation-centrifugation procedure in the separation of microbial ATP from non-microbial ATP in meat.

The flocculation-centrifugation (and centrifugation) procedures can

be used to analyse several samples for total viable count in
ca 1h. These physical methods offer an alternative which may be
preferable, in some circumstances, to the recently reported batch
resin-filtration system for separation and concentration of viable
organisms from raw meats (Stannard & Wood, 1983).

8. GENERAL DISCUSSION

The broad objective of the present study was to investigate the potential of various chromatographic materials for manipulating viable micro-organisms. The two major topics considered in this work comprised the development and evaluation of simple practical systems and at a more fundamental level, mechanisms involved in the adhesion of micro-organisms to chromatographic materials. Although the two areas are closely related, they are better considered separately.

Development and evaluation of simple practical systems for manipulating micro-organisms

An extensive range of techniques is used for the separation and purification of biological molecules on the basis of size, density, charge, hydrophobicity and immunological specificity of these molecules. Since microbial cell surfaces are highly charged and may also possess hydrophobic and immunological properties, physical techniques may, potentially, be used for the manipulation of micro-organisms.

The interaction of micro-organisms with both ionogenic and non-ionogenic chromatographic materials has been studied (Chapters 2.2 and 5.4). However, the ionogenic chromatographic materials used by many researchers were unwieldly (Hall et al., 1978) and therefore not suited for large numbers of analyses normally required in routine microbiology. Another important aspect not considered by previous workers was the viability of micro-organisms recovered from the resins. Estimations based on optical absorbance (Daniels, 1967) or microscopy (Zvaginsev & Gusev, 1971) do not discriminate

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between viable and non-viable cells. Additionally, some of the conditions under which the organisms were eluted from the columns were lethal (Wadström et al., 1981).

In this study, the potential of ionogenic and non-ionogenic chromatographic materials for manipulating viable micro-organisms by simple practical systems, has been investigated. The results demonstrate that physico-chemical and immunological characteristics of the cell surface may be exploited to separate or enrich, considerably, one species from binary mixtures of organisms in broth or artificially contaminated food (Chapters 4, 5 and 6).

Although the recoveries of micro-organisms in column effluents may be low (e.g. Table 5.1), the separation or enrichment of one species over another by as much as ca 2000:1 (E.coli over L.plantarum, Fig.5.6c) would be an advantage in preparing samples for further analyses e.g. selective estimation of specific micro-organisms.

The observation that Gram-negative organisms did not adsorb significantly to the cation exchanger Bio-Rex 70 (Fig. 4.2), was exploited to recover micro-organisms from meat suspensions prior to concentration and estimation of the microbial ATP, using a bioluminescence technique (unreported data; also Wood, 1979).

An important aspect of the work was the demonstration that micro-organisms, grown in foods, could be quantitatively recovered by differential centrifugation without apparent loss in viability (Tables 4.2 and 4.3). Moreover an anionic flocculant, basically a soluble form of Bio-Rex 70, was shown to partially clarify meat

homogenates without apparent loss in viability of the indigenous flora. These simple methods were exploited in order to recover micro-organisms from meat homogenates prior to their rapid estimation by the bioluminescence technique (Chapter 7). Although the separation of micro-organisms from meat was considered in the context of a specific method i.e. estimation of microbial ATP, separation should be considered as part of a broader approach to the preparation of samples for presentation to other analytical instruments used in microbiological analyses e.g. DEFT and impedimetric estimation.

From this study, there arises a question of whether complete resolution of mixed cultures of micro-organisms can be achieved. It may be that the surface properties which we wish to exploit for separation, overlap the divisions of our conventional classification systems or are distributed unevenly within populations regarded as homogeneous (Chapter 4; also Kolot, 1981) by other criteria. However, we know that groups of organisms classified by conventional methods have different cell wall compositions and structures e.g. moulds, yeasts and bacteria. Within bacteria, the Gram reaction is aligned with cell wall structure and within these major groupings smaller groups e.g. streptococci and staphylococci are known to differ in wall composition and structure.

The diversity of surface properties contributed to micro-organisms by the composition and structure of their cell envelopes combined with large numbers of solid surfaces and physical conditions such as pH and ionic strength (Wood, 1980) may, by empirical investigation, yield systems for the complete resolution of groups of organisms.

This might involve massive experimentation and a considerable cost. An alternative approach would be to gain a better knowledge of the mechanisms involved in the adhesion which might then permit better prediction of useful systems.

Mechanism of adhesion between micro-organisms and the cation exchange resin Bio-Rex 70

For adsorption to ion exchange resins, Daniels (1967) proposed a theory in which micro-organisms were considered as 'macroscopic zwitterions' and adsorption/desorption was attributed to electrostatic forces. Daniels (1967) suggested that at $\text{pH} > \text{Ip}$, micro-organisms bear an overall negative surface charge and adsorb to anion exchangers, while at $\text{pH} < \text{Ip}$ they bear an overall positive charge and adsorb to cation exchangers. However, the results of the current study showed that the Gram-positive bacteria (e.g. Staph.aureus) adsorbed $> 90\%$ to the cation exchanger Bio-Rex 70 at pH 7.0 at which pH Staph.aureus (Ip 3.4, Daniels, 1967) would not be expected to adsorb.

Daniels (1967) theory could not explain the salt-dependent adhesion of Staph.aureus to Bio-Rex 70 resin at $\text{pH} > \text{Ip}$ (Wood, 1979). From the data of Nelhof & Echols (1978), who suggested that the microbial cell surface carried a dominant exterior negative charge which overlays amino groups, Wood (1979) proposed that these amino groups might be involved in specific interaction with Bio-Rex 70 resin and that the presence of salt accentuated the interaction.

However, the present investigation of the interaction of Staph.aureus with both carboxyl-modified Bio-Rex 70 (Table 4.9) and CM-sepharose gel (Table 4.6) suggests that negative charges on the cation

exchangers are not involved, significantly, in the adhesion process. Therefore, simple electrostatic mechanisms proposed by Daniels (1967) and Wood (1979) appear inadequate to explain this adhesion of micro-organisms to the cation exchanger Bio-Rex 70. An explanation of this phenomenon must be sought elsewhere.

Much work on the interaction of micro-organisms with solid surfaces has been concerned with cell-cell interactions in which both particles are assumed to be negatively charged. Many researchers have sought to explain these interactions in terms of the theory of colloid stability (Derjaguin, 1934; Verway & Overbeek, 1948). In this theory, adhesion of colloid particles depends upon balance of two forces i.e. van der Waals forces and overlap of electrical double layers due to charged groups present on both surfaces. These forces are also known as long-range forces.

The shape of potential energy-distance curve for interaction between particle and macroscopic surface, recently described by Tadros (1980), is shown in Fig. 8.1. The primary minimum and the secondary minimum occur at distances of approximately $<10\text{nm}$ and $10\text{--}100\text{nm}$, respectively, from the surface. The long-range forces operate at separation distances of $>2\text{nm}$ while the short-range forces, which include electrostatic and hydrogen bonds, dipole interactions and hydrophobic interactions, operate at separation distances of $<1\text{nm}$. The adhesion of particles to a macroscopic surface may be either reversible or irreversible depending on the concentration of electrolytes used (Rutter, 1980). At a high electrolyte concentration the energy barrier is eliminated thus resulting in a

Fig. 8.1 Potential energy (PE) distance curves for interaction between particle and macroscopic surface (from Tadros, 1980)

strong net attraction and an irreversible adhesion. However, at a low electrolyte concentration the large energy barrier prevents close contact (i.e. <10nm) of a particle to the surface and it may, therefore, be repelled or held at the secondary minimum and later repelled (Fletcher et al., 1980) when conditions are changed i.e. reversible adhesion.

The salt-dependent adhesion of negatively charged Staph.aureus to Bio-Rex 70 observed by Wood (1979), may be explained by referring to the energy diagram (Fig. 8.1). In the presence of salt the high energy barrier may be reduced thus allowing the cells to localise, probably, at the secondary minimum due to long-range forces. Table 4.1 shows that 98% of Staph.aureus was eluted from the resin in 0.05M phosphate-citrate buffer pH 8.0 when shearing force was applied. This result suggests that the cells are not adsorbed irreversibly (i.e. in the primary minimum).

The results of the present study showed that hydrophobic interactions (i.e. van der Waals forces) may be involved in the adhesion of micro-organisms to ionogenic and non-ionogenic surfaces e.g. modification of carboxyl groups of Bio-Rex 70 resin does not, significantly, reduce the adsorption of Staph.aureus compared with the untreated resin (Table 4.9) and the adsorption of Staph.aureus to hydrophobic Phenyl-sepharose gel is markedly increased in the presence of ammonium sulphate (Fig. 5.2). Additionally, the adsorption of Staph.aureus to the strongly hydrophobic Alcian blue-modified Bio-Rex 70 is greater compared either with the untreated resin or with the other chemically-modified Bio-Rex 70 (Table 4.9).

The mechanisms involving attachment of a negatively charged bacterium to a negatively charged animal cell was recently proposed by Ofek & Beachey (1980). The energy barrier (Fig. 8.1) between the negatively charged surfaces was overcome by interaction of hydrophobic molecules on the bacterium and the hydrophobic portion of phospholipids on the animal cell membrane. Watt & Ward (1980) suggested a similar mechanism in the attachment of gonococci to mammalian cells with gonococcal fimbriae acting as a bridge between both the negatively charged surfaces.

Although Bio-Rex 70 resin represents a large and a highly rigid structure in comparison with an animal cell, a certain analogy may be drawn between the interaction of micro-organisms and the macroscopic surfaces. It is conceivable that hydrophobic constituents such as the fibrillar K88a antigen, having a low radius of curvature (Tadros, 1980), on the surface of highly negatively charged E.coli (Neihof & Echols, 1973) interacts with the hydrophobic acrylic matrix of the negatively charged Bio-Rex 70 (Fig. 4.10) thus overcoming the energy barrier between the repulsive negative surface charges.

The above discussions indicate that the adsorption of micro-organisms to ionogenic chromatographic materials, may not necessarily, be explained in terms of simple electrostatic forces and that other forces e.g. hydrophobic interactions should also be considered.

In future, dimensions and surface properties e.g. hydrophilicity (i.e. surface charge density) and hydrophobicity, of both

chromatographic materials and micro-organisms should be considered in order to predict possible interactions and help to limit the extent of empirical investigation.

Overall, the results of this study demonstrate the potential of chromatographic materials as a basis for sample preparation as new instrumental methods become established and signal to noise ratio becomes an important consideration in microbiological analyses.

At a more fundamental level, the study of the interaction between micro-organisms and both cation exchanger Bio-Rex 70 and hydrophobic Phenyl-sepharose gel has demonstrated similarities in the mechanism of adhesion of organisms to these materials. The adhesion of organisms to the Bio-Rex 70 supports the theory of colloid stability rather than Daniels (1967) theory of 'macroscopic zwitterion' for the adhesion of micro-organisms to cation exchange resins. In view of this, chromatographic materials may provide model systems in the study of cell-cell interactions.

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Letter to the Editor

During the past few years, many food microbiologists have shown increasing interest in rapid microbiological methods. These are seen as alternatives to traditional viable counts of one type or another. Evidence for this increased interest is seen in the number of formal and informal meetings which have been held, advertisements by instrument manufacturers and many papers in the scientific press. At the Leatherhead Food Research Association, we also have received many enquiries for general information on rapid methods, for specific individual training and for method and machine evaluations.

Almost all the new alternative methods are still evaluated by direct reference to traditional methods, i.e. viable counts. These are designed to establish one of three microbiological criteria for foods:

- (a) Overall production hygiene,
- (b) Safety of the food with respect to food poisoning organisms or their toxins, and
- (c) Likely shelf-life of the food.

In the past, the determination of presence or absence of food poisoning organisms (or their toxins) has understandably received considerable emphasis in spite of frequent difficulties with some of the methodology. Similarly, methods for estimating 'total viable microbes' in foods also possess their pitfalls (viable counts of moulds, for example, are notoriously unreliable). Shelf-life estimations using viable counts can only be made in very general terms, since;

- (a) Only a specific part of the viable population may be responsible for spoilage, and
- (b) rates of growth of organisms in foods and relationships between growth rates at different or fluctuating

temperatures are not well understood.

Before food microbiologists become too entrenched in comparing new and traditional methods, we should surely re-examine the microbiological criteria by which we judge a batch of food and use rapid or alternative methods, as appropriate, to obtain better estimates of these criteria. It may be more valuable, for example, to relate detection times from electrical monitoring of food micro-organisms directly to actual shelf-life. Media and monitoring techniques should be engineered to simulate those characteristics of a foodstuff which are related to spoilage. Similarly, measurements of microbial ATP, endotoxins etc, should be corrected directly with shelf-life.

These considerations pose the question, 'what developments in rapid/alternative methods will we see over the next decade?'. Should we not be examining foods for those characteristics which render it spoiled or hazardous? Counts of organisms can still inform us of production hygiene, and thus a rapid method such as the Direct Epifluorescent Technique (DEFT) could be very suitable. Specific chemical, physico-chemical or immunological methods are, however, more likely to provide direct information about the shelf-life or safety of a food. Considerable research effort is needed in order to recognize those components of a food which lead to rejection as spoiled. Rapid, sensitive methods must be developed to enable measurement of absolute levels of such components and also of the rate at which they are formed under various storage conditions.

Much work is also necessary in order to apply sensitive immunochemical techniques for the detection in foods of food-poisoning organisms and their toxins. Considerable progress has already been made in radio- and enzyme-linked

immunoassay techniques for both soluble and particulate antigens. It may well prove possible to combine the selectivity and sensitivity of immunological reactions in order to concentrate or purify antigens from mixtures (e.g. using immobilized antibodies) as well as to either detect or estimate their concentrations.

Before we, as food microbiologists, proceed too far along the road to automation, data accumulation and data processing, we should examine the value of such methods and decide whether or not we should be accumulating more relevant data. We should appreciate other microbiologists' views on this important subject.

Paul A. Gibbs

Catherine J. Stannard

Pradip D. Patel

Anthony P. Williams

*Leatherhead Food Research Association,
Microbiology Section, Randalls Road,
Leatherhead, Surrey KT22 7RY, UK.*

1. Immobilization stage

Act-Magnogel AcA-44 + Enterotoxin → Magnogel-Ent
(Magnogel) (Ent) (a)

2. Assay stages

(i) Anti-(enterotoxin B) serum + Ent → Ab-Ent + Ab(excess)
or purified immunoglobulin G (sample) (b)

(ii) (a) + (b) → Magnogel-Ent-Ab(excess) + Ab-Ent
(c)

(iii) (c) + anti-rabbit immunoglobulin G-peroxidase conjugate
(Anti-Ab-Enz)
→ Magnogel-Ent-Ab-anti-Ab-Enz complex

Scheme 1. Principle of inhibition MEIA for the determination of staphylococcal enterotoxin B

Enzyme activity of the complex is proportional to the antibody excess (i.e. total antibody minus enterotoxin in sample).

of the chromogenic product was mixed with 100 µl of distilled water in another micro-titre plate. The absorbance was measured at 490 nm on a MicroELISA Mini-reader MR590; Dynatech Laboratories).

The enzyme activity of the Magnogel complex was inversely proportional over a 10-fold range to the initial toxin concentration (Fig. 1). The inhibition MEIA for determining enterotoxin B is rapid (<1 day) and has a sensitivity of approx. 200 ng of toxin/ml, which is 20-fold less than that of radioimmunoassay or enzyme-linked immunosorbent assay but comparable with that of the conventional double-immunodiffusion technique.

The major advantage of magnetic enzyme immunoassays over enzyme-linked immunosorbent assays or radioimmunoassays is the simple and rapid magnetic separation of bound from unbound enzyme label. With further development, enzyme-linked immunosorbent assays (and inhibition MEIA) may become a rapid simple alternative to double-immunodiffusion for measuring staphylococcal enterotoxins in foods.

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Fig. 1. Titrations of various concentrations of enterotoxin B against a constant concentration (1:500 dilution) of anti-(enterotoxin B) immunoglobulin G-type antibodies

○, 1:100 dilution (a) or 1:50 dilution (b) of enterotoxin-coupled Magnogel used; ●, 1:50 dilution of enterotoxin-coupled Magnogel, 1:500 dilution of Spicer-Edwards serum versus various concentrations of enterotoxin B (negative serum control). The arrows (←) indicate enterotoxin B-coupled Magnogel [1:100 dilution for (a) and 1:50 dilution for (b)] and 1:500 anti-(enterotoxin B) immunoglobulin G-type antibodies, enterotoxin B not used (control).

Effect of *Clostridium botulinum* toxin on neuroblastoma cells

PAUL NEAVES, LEONARD F. J. WOODS, PRADIP D. PATEL and PAUL A. GIBBS

Microbiology Section, Leatherhead Food R.A., Leatherhead, Surrey KT22 7RY, U.K.

The detection and identification of *Clostridium botulinum* in foods and pure culture is dependent on detection of its potent protein neurotoxin (Hobbs *et al.*, 1982). The only specific and sensitive assay for toxin is the mouse injection test, presumptive positive reactions being confirmed by immunological protection of further mice injected with

mixtures of sample and antiserum. Since there is a strong desire to decrease the numbers of experimental animals, an alternative method is required.

We studied the effects of botulinum toxin on nucleic acid and choline content of cells of a mammalian neuroblastoma line cultured *in vitro*. Cells of strain NB41A3 (obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) were grown at 37°C in an appropriate medium as described in the Flow Laboratories Tissue Culture catalogue. Cultures were incubated until confluent either as monolayers in culture flasks (75 cm² area) containing 50 ml of growth medium or

in suspended culture by attachment to micro carrier beads (0.5 g dry wt. of Cytodex 1; Pharmacia) in flasks containing 500 ml of medium in stirred suspension.

For nucleic acid staining experiments, cells were subcultured by preparing a suspension in trypsin/EDTA (Cumming, 1970), inoculating into sterile Leighton tubes containing coverslips and growth medium (1 ml) and incubating overnight at 37°C. Cells were then exposed to purified type A neurotoxin, to type A crystalline toxin-haemagglutinin complex or to filtered, toxic, culture supernatant of *Cl. Botulinum* type A (N.C.T.C. 7272). Toxin samples were added to the culture medium at 200 mouse LD₅₀/ml and re-incubated at 37°C for 1 to 6 days. The monolayers on coverslips were then fixed and stained with the nucleic acid-specific fluorescent dye Acridine Orange by the method of Cumming (1970), and the preparations were viewed by u.v. microscopy. Monolayers were also incubated for 7 days in culture flasks containing growth medium and in the presence (200 mouse LD₅₀/ml) or in the absence of toxin, after which the nucleic acid composition was determined quantitatively. Cell suspensions were prepared by trypsinization, and nucleic acids were extracted by the method of Volkin & Cohn (1954). Nucleic acid concentrations were determined by colorimetric assay, by the methods of Burton (1956) for DNA and Dische (1955) for RNA. Choline concentrations in extracellular medium of Cytodex cultures exposed to crystalline toxin (1000 mouse LD₅₀/ml) overnight were determined by a luminometric technique based on that of Israël & Lesbats (1980). Samples for assay were placed into luminometer cuvettes containing 0.2 M-Tris/HCl buffer, pH 8.6, luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) and horseradish peroxidase. After stabilization, choline oxidase was added and peak light output was recorded.

Monolayers incubated for up to 6 days in the absence of botulin toxin and stained with Acridine Orange appeared as small discrete cells comprising bright yellow-to-orange cytoplasm and small distinct green-to-yellow nuclei. This indicated the presence of RNA in the cytoplasm (orange) and DNA in the nucleus (green). Monolayers incubated for 1–24 h in the presence of native neurotoxin, crystalline toxin or a toxic culture supernatant were markedly different and seen as enlarged cells with pale non-fluorescent staining. Cytoplasm appeared dark green and nuclei were enlarged. After 48–72 h incubation, the effect was less marked, monolayers often containing a mixture of normal and enlarged cells. Monolayers incubated for up to 72 h in the presence of diluent alone, boiled (i.e. inactivated) toxin or *Clostridium sporogenes* culture supernatant stained similarly to the medium control monolayers.

Quantitative changes in nucleic acid concentrations were observed when cells were incubated in the presence of neurotoxin (200 mouse LD₅₀/ml). In the absence of toxin, cells contained 34.4 pg of RNA/cell and 140 pg of DNA/cell, whereas those incubated in the presence of toxin possessed

22.7 pg of RNA/cell and 180 pg of DNA/cell. The results match those from the staining experiments, suggesting that exposure of cells to toxin resulted in a decrease in RNA content whereas the DNA concentration was slightly increased.

In an investigation of the effect of crystalline toxin on the extracellular choline concentration of neuroblastoma cultures, cells (2.6 × 10⁷ cells/test) were incubated overnight at 37°C in the presence of toxin (1000 mouse LD₅₀/ml). Control cultures showed a small increase in choline from 79 ng/ml to 111 ng/ml (41%), whereas the choline concentration of toxin-treated cultures increased by 109%. In a second experiment at a lower toxin concentration (500 mouse LD₅₀/ml), the extracellular choline concentration of control cultures decreased by 38% but that of toxin-treated cultures increased by 12%. These results suggest that toxin affects the choline balance of the neuroblastoma cells either by increasing the rate of choline release or inhibiting the rate of choline transport into the cells.

Our studies indicate that methods of detection based on a physiological effect of *Cl. botulinum* toxin appear to offer potential for the development of an alternative to the mouse bioassay. Whereas toxin can be detected immunologically, the cross-reactions and variable sensitivity observed with most immunological methods (e.g. Evancho *et al.*, 1973; Kozaki *et al.*, 1979; Guilfoyle & Mestrandrea, 1980) are difficult problems to overcome. The development of tissue-culture techniques may therefore provide a sensitive and specific method of detection as well as providing a means of studying the mode of action of botulin toxin at the molecular level. However, at this stage, tissue-culture techniques do not have the same degree of sensitivity as the mouse bioassay, and further work is required to make the technique of practical value.

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Development of a Magnetic Enzyme ImmunoAssay (MEIA) technique for determination of anti-(staphylococcal enterotoxin) immunoglobulin G-type antibodies

PRADIP D. PATEL, JOHN M. WOOD and PAUL A. GIBBS

Microbiology Section, Leatherhead Food R.A., Leatherhead, Surrey KT22 7RY, U.K.

Conventional single- and double-immunodiffusion techniques for determining immunoglobulin G-type antibodies

(Hudson & Hay, 1980) are tedious and lengthy (≥16 h). Enzyme-linked immunosorbent assays (Engvall & Perlmann, 1971; Kan *et al.*, 1983) and radioimmunoassays (Cripps *et al.*, 1983) have shortened the analysis time to <7 h. In addition, novel techniques known as Magnetic Enzyme ImmunoAssay (MEIA) techniques for determining immunoglobulins (Guesdon & Avrameas, 1977; Guesdon *et*

1. Immobilization stage

Act-Magnogel AcA-44 + Enterotoxin → Magnogel-Ent
(Magnogel) (Ent) (a)

2. Assay stages

(i) (a) + Anti-(enterotoxin B) serum → Magnogel-Ent-Ab
or purified immunoglobulin G (b)
(Ab)

(ii) (b) + Anti-rabbit immunoglobulin G-peroxidase conjugate
(Anti-Ab-Enz)
→ Magnogel-Ent-Ab-Anti-Ab-Enz complex

Scheme 1. Principle of MEIA technique for the determination of anti-(staphylococcal enterotoxin B) immunoglobulin G-type antibodies

The enzyme activity of the complex is directly proportional to the amount of anti-enterotoxin immunoglobulin G antibodies.

al., 1978*a,b*) and fractionation of lymphoid cells (Antoine *et al.*, 1978) have been reported.

We have developed a rapid (i.e. <7h) MEIA (Scheme 1) for measuring anti-(staphylococcal enterotoxin B) immunoglobulin G-type antibodies in both normal immune serum and an affinity-purified immunoglobulin G fraction.

Immunoglobulin G-type antibodies were purified from anti-(staphylococcal enterotoxin B) serum (Centre for Applied Microbiological Research, Porton Down, Wilts., U.K.) by affinity chromatography on protein A-Sepharose CL-4B gel, in accordance with the instruction manual *Affinity Chromatography* (Pharmacia Fine Chemicals). Immunological activity of purified immunoglobulin G was confirmed by the double-immudiffusion technique (Patel & Wood, 1980).

Staphylococcal enterotoxin B (875 µg) was immobilized to 1 ml of act-Magnogel AcA-44 in accordance with the manufacturer's instructions (*Product Information no. 201103*; Réactifs IBF; supplied by LKB, Bromma, Sweden). Coupling efficiency of act-Magnogel was determined by quantifying by the capillary-tube assay technique (Fung & Wagner, 1971), the unbound enterotoxin B remaining in solution after coupling to Magnogel.

Anti-(staphylococcal enterotoxin B) immunoglobulin G-type antibodies in both antiserum and affinity-purified immunoglobulin G fractions were determined in accordance with the principle outlined in Scheme 1. Phosphate-buffered saline/Tween 20 (Voller *et al.*, 1979) was used unless specified otherwise. Wells in a micro-titre plate (M129A; Dynatech Laboratories) were filled with 300 µl of 1% (w/v) proteose peptone no. 3 (Difco Laboratories), and the plate was stored at 4°C for 16h. The wells were washed three times by emptying and refilling with phosphate-buffered saline/Tween, and 100 µl of enterotoxin-coupled Magnogel (1:50 and 1:100 dilution) was added to appropriate wells. A 100 µl sample of anti-(enterotoxin B) serum diluted 1:5000 to 1:100000 in phosphate-buffered saline/Tween was added to wells containing enterotoxin-coupled Magnogel. In another experiment, purified immunoglobulin G fraction (dilution range 1:1000 to 1:100000) was used instead of antiserum. The plate, with a lid, was incubated at 37°C for 2h on a shaker (Varishaker; Dynatech Laboratories) at a speed such that the gel remained suspended. The Magnogel complex was separated and concentrated at the side of wells by placing a magnet (12 mm × 9 mm × 42 mm; LKB) beneath the wells. The liquid phase was removed from wells by suction with a Pasteur pipette connected to a vacuum source. At this and subsequent stages, it was important to prevent loss of Magnogel. The Magnogel

Fig. 1. Titration of various concentrations of anti-(enterotoxin B) serum (a) or affinity-purified immunoglobulin G (b) against a constant concentration of enterotoxin B-coupled Magnogel

The results in (a) or (b) represent averages for two separate experiments, in each of which assays were done either in duplicate or in triplicate; mean and s.d. are shown. ○, Experimental results obtained with a 1:100 dilution of enterotoxin-coupled Magnogel; ●, 1:50 dilution of enterotoxin-coupled Magnogel versus Spicer-Edwards serum (control).

complex was washed three times with phosphate-buffered saline/Tween, each time by resuspension and concentration as described. To each well, 200 µl of 1:500 dilution of anti-rabbit immunoglobulin G-peroxidase conjugate (Sigma Chemical Co.) was added, and the plate was incubated at 37°C for 2h with shaking. The Magnogel complex was washed three times before the addition of 200 µl of the peroxidase-substrate solution (Voller *et al.*, 1979) to each well. The plate was incubated at ambient temperature for 30 min with shaking, followed by addition of 50 µl of 2.5M- H_2SO_4 . The Magnogel complex was separated, and 200 µl of chromogenic product was diluted to 2 ml with distilled water before measurement of absorbance (492 nm) in a spectrophotometer (SP-6 550; Pye-Unicam). Pooled Spicer-Edwards serum (Boothroyd & Baird-Parker, 1973), substituted for anti-enterotoxin serum, was used as a negative antiserum control.

After coupling of the enterotoxin to Magnogel and separation of the Magnogel complex, the supernatant liquid contained less than 25 µg of enterotoxin/ml, or over 95% of the toxin was coupled to 1 ml of act-Magnogel.

A linear relationship was obtained between the enzyme activity and dilution of either anti-enterotoxin serum (Fig. 1a) or affinity-purified immunoglobulin G fraction (Fig. 1b) in an approximate dilution range 1:2000 to 1:100000.

The major advantage of MEIA over enzyme-linked immunosorbent assay or radioimmunoassay is the simple and rapid separation of the bound from unbound radio/enzyme label and a lower limit of sensitivity that is comparable with that obtained by radioimmunoassay (Guesdon *et al.*, 1978b).

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The effect of chemical modification on the activity of heat stable enterotoxin from *Escherichia Coli*

B. R. BOOTH, E. J. WOLFFE and M. A. SOWA

Microbiology Division, Glaxo Group Research Ltd.,
Greenford Road, Greenford, Middlesex, U.K.

Enterotoxigenic strains of *E. coli* produce a cholera-toxin-like heat labile enterotoxin and/or a heat stable enterotoxin (ST). ST has been isolated by a number of groups (Staples *et al.*, 1980; Alderete & Robertson, 1978; Mullan *et al.*, 1978; Aimoto *et al.*, 1982) and its structure appears to vary depending on the bacterial strain from which it is isolated. The structure of two ST types, STa1 and STa2, have been determined by amino acid sequencing of the purified toxin and by nucleotide sequencing of the genes coding for these toxins (Aimoto *et al.*, 1982; Moseley *et al.*, 1983; So & McCarthy, 1980; Chan & Giannella, 1981). They are polypeptides 18 or 19 amino acids in length, molecular weight about 2000, are soluble in methanol and promote fluid secretion in the small intestine of infant mice probably by stimulation of the guanylate cyclase of the epithelial cells (Field *et al.*, 1978; Giannella & Drake, 1979). The amino acid sequence of these toxins varies to some extent, but there is a sequence of 14 amino acids which is common to both toxins and varies in only one or perhaps two residues. There are six cysteine residues in this sequence, none of which appear to react with 5,5'-dithiobis-(2-nitrobenzoic acid) suggesting that they are involved in disulphide bridges. This could account for the stability of the toxins to heat, acid pH and proteinases and also their lability to alkaline pH. Another characteristic of these toxins is that they do not contain any basic amino acids. Apart from the N- and C-terminal residues, the only charged residue is a glutamic acid.

At present it is not known how these toxins stimulate guanylate cyclase. We have tested the effect of various amino-acid-modifying reagents in an attempt to determine which properties of toxin STa2 are important for its activity.

The source of STa2 for this work was a strain of *E. coli* K12 into which the *est* A2 gene has been cloned. It was kindly given to us by H. R. Smith of the Central Public Health Laboratory, Colindale. The toxin was purified by

the method of Staples *et al.* (1980) and its activity assayed in the infant mouse model (Deans *et al.*, 1972). The effect of various modifying reagents on the activity of the toxin was tested as follows. Toxin (17 µg in a volume of 10 µl) was mixed with 90 µl of a 10 mM solution of reagent in the appropriate buffer (see Table 1) and the reaction was allowed to proceed for 4 h at room temperature. Toxin was separated from excess reagent on Bio-Gel P-2 columns (8 cm × 0.7 cm) and the fractions containing STa2 were pooled. Residual activity of the pooled material was measured by the infant mouse assay. The results are shown in Table 1.

N-Bromosuccinimide, a reagent which is known to modify aromatic amino acids (Ramachandran & Witkop, 1967) reduced toxin activity by 90%. It also reduced the activity of a purified preparation of STa1 by 80%. However N-bromosuccinimide may also break disulphide bridges between cysteine residues and then react with a cysteine to give a modified residue. It is quite possible that it is this property of the reagent which is responsible for toxin inactivation. Dithiothreitol, a reducing reagent which breaks disulphide bridges, also reduces the activity of the toxin (Staples *et al.*, 1980). The lack of effect with N-ethylmaleimide is not surprising if, as seems to be the case, none of the cysteine residues has a free thiol group. 1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide, a reagent which modifies free carboxyl groups (Hoare & Koshland, 1966) reduces toxin activity by about 80%. There are two free carboxyl groups in STa2, a glutamic acid residue and the C-terminal carboxyl group. At physiological pH both these groups will be ionized and therefore the toxin will have a net negative charge as the only positively charged residue is the N-terminal amino acid. Reaction of the toxin with the carbodi-imide will reduce the negative charge and it could be this event which results in the loss of activity. Alternatively it could be argued that charge is not an important factor but that merely modifying the structure of the glutamic acid and/or the structure of the C-terminal tyrosine is enough to inactivate the toxin. N-Acetyl-imidazole, a reagent which acetylates tyrosine, does not have any appreciable effect on the activity of the toxin.

In summary, our results suggest that modification of free carboxyl groups and breakage of one or more disulphide

* Abbreviation used: ST, heat-stable enterotoxin.

Evaluation of a homogeneous enzyme-linked immunoassay for staphylococcal enterotoxin B

Pradip D Patel

Certain strains of *Staphylococcus aureus* produce extracellular soluble proteins known as enterotoxins which on consumption produce food poisoning symptoms including nausea, diarrhoea and abdominal cramping. The enterotoxins occur in the molecular weight range 30,000 to 35,000 and are differentiated into six types based on their specific immunological properties.¹

The various methods used for detection of the enterotoxins^{2,3} include heamagglutination, radio-immunoassay, fluorescent antibody and double-immuno-diffusion techniques. Double-immunodiffusion is the procedure most frequently used for the detection and estimation of enterotoxins in foods; although it involves complex and lengthy extraction and purification of the sample, it has been demonstrated to perform satisfactorily in many laboratories.⁴ A result may be obtained within a week, but it frequently takes longer.

A more rapid method has been proposed recently⁵ in which both the extraction and detection of the enterotoxins are greatly simplified by use of a novel procedure, an enzyme-multiplied immunoassay technique (EMIT). Enzyme-linked immunoassays have been widely used for antigen and antibody determinations,⁶⁻⁹ in cellular preparations,¹⁰ in disease conditions^{11,12} and in the analysis of toxins.¹³⁻¹⁷ Many of the enzyme-linked assays require a solid phase to which antibodies are attached; these are known as enzyme-linked immunosorbent assays (ELISA).

In the EMIT, the antigen (morphine) assay¹⁸ was carried out in free solution and did not use a solid phase to which antibodies were absorbed. The basis of EMIT is the inhibition of an enzyme coupled to an antigen by

complexing with the homologous antibody in a balanced reaction; this balance is altered by the presence of native antigen. These free solution assays were found to work well with low molecular weight ligands such as drugs, small peptides and steroid hormones.^{19,20} The principle of this technique (Table 1) was exploited by Morita and Woodburn⁵ who devised an EMIT for staphylococcal enterotoxin B. They reported that their technique would detect as little as 5ng of enterotoxin B in foods in only 30h. This represented a considerable advance over the currently used procedures and would be of great advantage in food analysis. The objective of the current work was to repeat the observations of Morita and Woodburn.⁵

Table 1 Principle of EMIT for assay of Staphylococcal enterotoxin B

1 Preparation of conjugate

B-amylase (Enz) + Enterotoxin (Tox) $\xrightarrow{\text{glutaraldehyde}}$ Enz-Tox conjugate
Active* Active*

2 Standard 'balanced' system

Amounts of antibody (Ab) and conjugate are titrated such that after reaction there is virtually no residual enzymic activity in solution.

Enz-Tox + Ab $\xrightarrow{\quad}$ Enz-Tox-Ab
Enz-Tox + Ab $\xrightarrow{\quad}$ Enz-Tox-Ab
Active* Inactive (steric inhibition)

These amounts of antiserum and conjugate are then considered as standards.

3 Assay stages

(a) Unknown sample containing free toxin (Tox⁺) is preincubated with the standard amount of antiserum.

Tox⁺ + Ab $\xrightarrow{\quad}$ Tox⁺-Ab
Ab Ab

(b) The mixture from (a) is incubated with the standard amount of conjugate.

Tox⁺-Ab + Enz-Tox $\xrightarrow{\quad}$ Enz-Tox + Enz-Tox-Ab + Tox⁺-Ab
Ab + Enz-Tox $\xrightarrow{\quad}$ (Activity $\propto$ Tox⁺) Inactive
Active*

The amount of enzyme-toxin remaining in solution is directly proportional to toxin concentration originally present in the sample.

*Exhibiting enzymic activity

Mr Patel is a senior research scientist in the Microbiology Section at the Leatherhead Food Research Association, Randalls Road, Leatherhead, Surrey KT22 7RY.

Fig 1 Zymogram of conjugate and standard β -amylase in agarose gel

We found that the results obtained by the originators⁵ of the enzyme-immunoassay could not be reproduced and non-specific responses were encountered frequently. A detailed investigation into the basis of EMIT (Table 1) was carried out by employing a range of electrophoretic and immuno-electrophoretic techniques. The purpose was to establish whether the enzyme activity of the enzyme-enterotoxin conjugate was inhibited by the anti-enterotoxin antiserum.

Preparation of β -amylase-enterotoxin conjugate

The glutaraldehyde procedure used for the preparation of antigen-enzyme conjugates results in the formation of heterogeneous high molecular weight complexes having both immunological and non-immunological properties.^{8,10} We obtained similar results with the products of β -amylase-enterotoxin conjugation following the electrophoresis of the conjugate-containing fraction on agarose gel. The β -amylase activity in the gel was located by an enzyme-staining technique²¹ (Fig 1). The heterogeneous high molecular weight complexes were observed as the 'tailing' of the β -amylase activity in the gel.

Analysis of the conjugate

The β -amylase activity of the conjugate was confirmed by a colorimetric method,²² while its ability to combine with the antibody was demonstrated by double-immunodiffusion method.²³ The precipitin line of pure enterotoxin standard formed in the agarose gel, coalesced with the precipitin line of the conjugate fraction, thus showing antigenic identity (Fig 2). This evidence demonstrated that the conjugate had enzymic as well as immunological activity.

Fig 2 Double-immunodiffusion of conjugate and standard enterotoxin B in agarose gel

Fig 3 Crossed-over immunoelectrophoresis of conjugate and standard β -amylase on agarose gel; (a) slide before staining for β -amylase activity (W_1 = well containing conjugate; W_2 = well containing anti-enterotoxin B-serum; W_3 = well containing standard β -amylase); (b) slide after staining for β -amylase activity

Immunoelectrophoretic analysis of the reaction between conjugate and anti-enterotoxin serum

Crossed-over immunoelectrophoresis on agarose gel Using this technique, we initially confirmed that the conjugate had immunological activity (Fig 3a). However, more important was the fact that binding of antibody to the toxin moiety of the conjugate did not result in inhibition of the β -amylase (Figs 3b and 4).

Two-dimensional electrophoresis: electrophoresis linked to crossed-over immunoelectrophoresis By this technique (Figs 5 and 6), it was shown that:

- The immunological activity was confined to the heterogeneous high molecular weight complexes which were described earlier.
- The enzyme activity of the enzyme-toxin conjugate was not inhibited by enterotoxin antibody, confirming the previous observation.

In the original EMIT¹⁸ for estimation of morphine (m.wt ca. 300), the drug was conjugated to a low molecular weight

Fig 4 Crossed-over immunoelectrophoresis of conjugate and standard β -amylase in agarose gel

Fig 5 Two-dimensional electrophoresis on agarose gel: electrophoresis linked to crossed-over immunoelectrophoresis

enzyme, lysozyme (m.wt14,000). In their method, Morita and Woodburn⁵ conjugated enterotoxin (m.wt30,000) to a high molecular weight enzyme, β -amylase (m.wt152,000). The basis of these assays is that antibodies binding with the conjugated antigens i.e. morphine or enterotoxin, should block the active centre of the conjugated enzyme and therefore prevent the access of polymeric substrate molecules. It is this basic requirement which we were unable to demonstrate.

The precise relationship between the antigen and the enzyme in the conjugate is of critical importance in the operation of EMIT assays. It has been shown²⁰ that the insertion of a small spacer — a six carbon chain — between an antigen and enzyme can cause a marked diminution in the inhibition of enzyme activity of conjugate by homologous antibody. In the original EMIT¹⁸ assay for morphine, the antigen (actually a hapten) -enzyme conjugate (m.wt ca. 14,500) is relatively small compared with the antibody molecule (gamma-globulin m.wt160,000). There is therefore a high probability of the active centre of the enzyme becoming blocked when the antibody binds with

the conjugate. In the EMIT for enterotoxin, the antigen-enzyme complex is about the same size as the antibody molecule and there is a greater chance that the antibody binding site may be more remote from the active centre of the enzyme than in the case of the morphine-lysozyme system.

Conclusions

Although the data reported by Morita and Woodburn⁵ appear self-consistent and are unlikely to have occurred by chance, we have studied their experimental system in considerable depth and cannot substantiate the basis of EMIT, i.e., that the enzyme activity of an enterotoxin- β -amylase conjugate is blocked by anti-enterotoxin serum. It is possible that the reason for this difference lies in as yet unknown properties of the particular reagents used. However, while the difference remains unresolved it is not possible to recommend this EMIT as a rapid alternative to the conventional technique for the detection of staphylococcal enterotoxins.

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Fig 6 Two-dimensional electrophoresis: electrophoresis linked to crossed-over immunoelectrophoresis in agarose gel

Rapid Microbiological Methodology

by C. J. Stanndard, P. D. Patel, Dr. J. M. Wood, Dr. P. A. Gibbs

Leatherhead Food Research Association, Leatherhead, Surrey.

The detection and enumeration of particular organisms in foods is often used as an indication of food quality. Traditional techniques, basically unchanged for about 150 years, are labour-intensive and require at least 24-48 hours for completion. At present, therefore, most microbiological testing in the food industry is retrospective. Consequently there is considerable interest in the newer, more rapid techniques which can provide a result within the working day or even in under one hour. This enables immediate grading of raw materials on their microbiological condition, resulting in reduced storage space and lower labour costs. The Leatherhead Food Research Association was one of the pioneers in assessing and developing rapid methods for the microbiological testing of foods and, over a decade later, a number of the instrumental techniques evaluated are being accepted as alternatives to the traditional methods by the food industry.

Rapid methods in microbiology can be divided broadly into four main categories:

1. cell and colony-counting methods, based on the principles involved in the traditional techniques;
2. non-counting methods, based on aspects of microbial metabolism;
3. identification of organisms;
4. detection of important metabolites e.g. toxin.

In order to optimise any of these techniques, the physical separation of organisms or their metabolites from food is essential and forms an important part of the research programme at the Food RA.

Cell and colony-counting methods

There are three distinct types of method which fall within this category. The first type is labour-saving but does not decrease the incubation time required for traditional methods. However, it reduces the time and labour required for sample preparation, plate inoculation or counting. These can be minimised in manual methods

by using miniaturised techniques, such as the loop-dilution method, which require very little medium, diluent, etc. as all operations involved in a normal plate count are scaled down in size.

The Leatherhead Food RA has evaluated the Spiral Plate Maker¹ and the Laser Bacterial Colony Counter² (Don Whitley Scientific, Shipley, W Yorks) and they are now well-established methods within the food industry as alternatives to the traditional techniques.

A more automated machine than the Spiral Plate Maker, the Petrifoss (Foss Electric UK Ltd, York), is currently being installed in many dairies for the routine microbiological analysis of raw milk. It mixes inoculum with agar, pours and labels plates and delivers them ready for incubation at a rate of 600 per hour.

The second type of method reduces the time required for incubation of a sample by counting colonies earlier. The Droplette Technique³ is based on this principle. A sample is prepared in molten agar and one drop allowed to fall into a petri-dish. After incubation (24 hours rather than the usual 48 hours for pour plates) colonies may be counted with the aid of a magnifying

lens. Another approach to this type of method is the Hydrophobic Grid-Membrane Filter (HGMF) technique⁴ which the Food RA has recently evaluated in worldwide collaboration with a number of other laboratories. Microorganisms are isolated on a filter printed with a hydrophobic grid. Colonies developing in 24 hours are prevented from spreading by the grid. This facilitates counting, which can be done by an automated procedure. This method has been used with a variety of foodstuffs to detect specific groups of organisms as well as to obtain a total viable count.

The third type of method is direct microscopy, which has always been an option for a rapid total count, and a result can be obtained within a few minutes. Recently, an automated direct microscopy technique, the Direct Epifluorescence Filter Technique (DEFT)⁵, has been developed for raw milk. It has also been applied to other dairy foods, e.g. heat-treated milk, cream and butter. Samples of food are treated with an enzyme and surfactant to facilitate membrane filtration and the membrane is then stained with a dye which causes living organisms to fluoresce orange. The number of clumps or organisms, each of which might be expected to form a colony on agar, can then be counted automatically. There is good agreement between the DEFT results and the

Plate 1: The Bactomatic M120

traditional colony count for all these dairy foods.

Non-counting methods

These methods are based on some aspect of microbial metabolism. Thus radiometry depends on the sensing and measurement of the quantity of $^{14}\text{CO}_2$ produced for labelled substrates during growth of microorganisms. The commercially available instrument, the Bactec (UK suppliers: Laboratory Impex Ltd, Teddington, Middx), was primarily developed for the analysis of blood samples but has been used for the detection of microorganisms in fruit juice⁶ and salmonellae in food samples⁷. Good agreement is claimed between radiometric results and the traditional techniques.

However, in the food industry radioisotopes are not generally acceptable. An alternative is the measurement of the changes in the electrical characteristics produced by microbial metabolism in a medium. During growth, organisms utilise certain nutrients and produce waste products resulting in changes in electrical impedance (conductance and capacitance). Instruments which measure these changes have been evaluated by the Food RA and include the Bactometer B32 and currently the M120 (Plate 1., Bactomatic Inc., Marlow, Bucks) and also the Malthus 8 and 128 (Malthus Instruments Ltd, Stoke-on-Trent, Staffs). The time taken for a culture to reach a threshold detection level of impedance or conductance is inversely proportional to the number of organisms in the initial inoculum. A linear relationship between total viable count (colony count) and impedance/conductance detection time can be shown for a

variety of foods⁸. An example of the data obtained at the Food RA is shown in Fig. 1.

Radiometry and electrical measurements take a few hours for an estimate of total viable organisms to be obtained. Using specially designed media it is possible also to detect specific groups of organisms with these instruments. A radiometric method has been reported for the detection of salmonellae in which $^{14}\text{CO}_2$ production from ^{14}C -dulcitol is prevented by polyvalent H *Salmonella* antiserum⁷. Work at the Food RA suggests that it may be possible to adapt this technique for impedance/conductance instruments. We have also shown that it is possible to differentiate moulds from bacteria using impedance measurement, where the signal for moulds is optimised by careful choice of medium and the bacterial signal is inhibited by antibiotics.

For rapid results

For a very rapid result — within one hour — methods are available which measure some component of the microbial cell. The *Limulus* amoebocyte lysate assay detects endotoxin produced by Gram-negative bacteria, by coagulation. It has been used successfully to determine the microbial load on chilled meat⁹.

Adenosine triphosphate (ATP) is an important component of all living cells which can be measured quickly and easily using the luciferin-luciferase enzyme system of the firefly. The intensity of the light emitted during the reaction is directly proportional to ATP concentration. Several sensitive luminometers (e.g. LKB 1250, LKB Instruments Ltd, Croydon, Surrey; Lumac Biocounter M2010, Sterilin

Fig 1: Relationship between impedance detection time (Bactometer B32) and colony count for 43 raw meat samples

Ltd, Teddington, Middx) are now available commercially, as are reagents for carrying out the assay. Measuring microbial concentration in terms of ATP in pure cultures is a simple matter, and the relationship between ATP and colony count is surprisingly similar between species of bacteria and of yeasts. However, problems are encountered when the method is applied to foods as there are large quantities of ATP intrinsic to the food which may entirely swamp the signal for any microbial ATP. Therefore if the ATP assay technique is to be applied to foods, it is essential that microorganisms are separated from the food, or that non-microbial ATP is selectively destroyed prior to extraction and assay of microbial ATP.

At the Food RA we have developed two approaches to the separation of

Fig 3: Relationship between ATP & colony count for two types of yeast in fruit juice. Spoilage Isolate Y57; o, *Saccharomyces cerevisiae*

Fig 2: Relationship between colony count of raw beef homogenate and microbial ATP content

microbes from food ATP. For raw meat, organisms are separated from the meat homogenate by a simple, three-stage process¹⁰. Centrifugation removes coarse meat particles; stirring with cation-exchange resin removes smaller particles, and filtration through a membrane filter (0.22 μ m pore size) removes soluble materials. By this process, 70-80% of the microbial populations of meats can be isolated consistently on the filters and their ATP extracted and assayed. The entire extraction and assay procedure takes only 20-25 minutes and there is a linear relationship between $\log_{10}$ microbial ATP and $\log_{10}$ colony count (Fig. 2). In contrast, for fruit juices, a partial separation of yeasts by centrifugation is used in conjunction with enzymic degradation of non-microbial ATP. Using this technique, a result is obtained in 75 minutes. Again, clear relationships are obtained between $\log_{10}$ ATP and $\log_{10}$ colony count (Fig. 3).

Physical separation of microorganisms

If we are to take full advantage of the speed and sensitivity of the new instrumental techniques, presenting an optimal sample for analysis will become increasingly important. Unlike other sciences, microbiology employs relatively few techniques for the physical separation and concentration of microorganisms. Our interest in separation has chiefly been the separation of microorganisms by ion-exchange and hydrophobic chromatography.

Cation-exchange resin treatment has been used to separate total microorganisms from meat as described above, exploiting the fact that organisms found on meats (mainly Gram-negative) do not absorb to the resin whereas meat proteins do.

We have also shown that mixtures of

two pure cultures of organisms can be separated from each other on the basis of differences in their adsorption to cation-exchange resin at different pH values (Fig. 4). A further property of organisms which can be used in their separation is the hydrophobicity of their surface proteins. The affinity of different organisms for hydrophobic gel is shown in Fig. 5. Based on these observations, we are developing techniques to separate mixtures of organisms in foods. Thus there are possibilities for the enrichment in numbers of specific groups of organisms over others by physical processes. This may increase the sensitivity of instrumented techniques for the detection of certain organisms, e.g. salmonellae by the Fluorescent Antibody Technique, described later.

Identification

Many kits are currently available for the rapid identification of organisms, for example species of yeasts or Enterobacteriaceae. These are based on the miniaturisation of conventional biochemical techniques and thus save on media and preparation time. Results are obtained in 24 hours or, for some kits, 5 hours. A more recent development for this type of test is to read the results automatically using a scanner linked to a computer, which will automatically tell the operator the identity of the organism from a stored list of biochemical profiles.

Gas-liquid chromatography has also been used for identification of organisms in pure culture, but the technique is at present still in its infancy and used only as a research tool. Organisms can be differentiated by gas-liquid chromatography of either their pyrolysis products¹¹ or their metabolites¹². The technique has not been used for foods to date, but has proved useful for taxonomic studies.

The Fluorescent Antibody Tech-

nique (FAT)^{13,14} is however being used in the food industry. The basis of the technique is to couple a fluorescent dye to the antibody specific for the organism to be detected (e.g. salmonellae¹⁴). Samples of a culture of the food are stained with the fluorescent antibody which binds to the organism which will then fluoresce under an ultra-violet microscope. However, in the testing of foods there is interference from non-specific absorption of fluorescent antibody by food particles, and therefore the method has not gained widespread acceptance. The separation techniques described earlier could be useful in "cleaning up" the sample before staining, thereby reducing interference.

Detection of staphylococcal enterotoxins in foods

Certain strains of *Staphylococcus aureus* produce extracellular proteins known as enterotoxins which, on consumption, produce food poisoning symptoms including nausea, diarrhoea and abdominal cramping. Various methods are available for detection of enterotoxins, but the procedure most frequently used for foods is double-immunodiffusion¹⁵. This involves complex and lengthy extraction and purification of the sample but has been demonstrated to perform satisfactorily in many laboratories. A result may be obtained within a week, but frequently takes longer.

More recently, novel techniques known as enzyme-linked immunoassays have been proposed¹⁶ which greatly simplify both the extraction and detection of enterotoxins, giving a result in approximately 7 hours. Many of these assays require a solid phase to which antibodies or antigens are attached; these are known as Enzyme-

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have described an immobilised enzyme reactor containing lactase, which produces glucose from lactose, and glucose oxidase, which produces hydrogen peroxide by reaction of dissolved oxygen with the glucose. The amounts of hydrogen peroxide required are very low since the thioxyanate reaction products are bacteriocidal at extremely low concentrations. However, the thiocyanate concentrations in milk are low and very variable, depending on the diet of the cows, and it may be necessary to add small amounts of thiocyanate for optimum effect.

The system has been tested on a laboratory scale with whey and it has been demonstrated that after passage through the lactose-glucose oxidase reactor, the whey is bacteriocidal to *Pseudomonas fluorescens* and that the bacteriocidal effect bears an inverse relationship to flow rate through the column. It is believed that this system has not yet been applied commercially.

Various other uses for glucose oxidase or glucose oxidase-catalase products have been proposed (Table 6), most have been proved to work on a technical level but have not been commercialised, probably due to economic factors. Many of these applications were originally investigated when the cost of the enzymes relative to other raw materials was higher.

TABLE 6 Miscellaneous Proposed Uses for Glucose Oxidase Products

Product	Effect	Ref.
Dried meat	Removal of glucose to improve long-term storage	17
Canned foods	Prevention of corrosion	18
Frozen shrimps	Prevention of discolouration	19
Dried milk	Improved storage stability	20

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Linked Immunosorbent Assays (ELISA). ELISA techniques can detect approximately one-thousandth of an emetic dose.

The advantages of speed and simplicity of ELISA over the more conventional methods for detection of enterotoxins make them an attractive prospect for the future. The Food RA is currently investigating the use of ELISA for detecting staphylococcal enterotoxins in foods.

Conclusions

There is a wide variety of rapid methods available for microbiological determinations, ranging from a very rapid technique for enumeration of microorganisms in meat in 20-25 minutes, to procedures to speed up plating and counting. There are many

It is clear that food microbiology is moving away from the traditional colony counting techniques towards more rapid, automated, instrumented measurements. So far, attempts have

been made only to compare these new methods with the colony count, not to

Since we know that the colony count is subject to many errors, it seems more appropriate to assess the new methods in terms of the acceptability of food. It may well be that measurements with a more metabolic basis, e.g. impedance, ATP, provide a more accurate representation of the potential for spoilage of the food than merely counting colonies on an agar plate¹⁷.

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P.D.Patel & J.M.Wood

Applied Microbiology Section, Leatherhead Food Research Association
Randalls Road, Leatherhead, Surrey, KT22 7RY.

The potential of chromatographic separation techniques for
the manipulation of viable micro-organisms.

Most conventional techniques for the detection and estimation of micro-organisms are based upon growth of the organisms to produce a signal e.g. colony or turbidity detectable by eye. Within very broad limits the initial concentration of organisms and the nature of the food material have little effect upon these methods.

The more recently introduced techniques e.g. luminometry, direct epifluorescent filtration technique and impedance measurements have a different basis of measurement and in these techniques the initial concentration of micro-organisms and the nature of the food can have a considerable effect. In these and future instrument-based techniques the ability to manipulate (i.e. enrich, concentrate or separate) micro-organisms by physical techniques may offer advantages in preparing samples for analysis.

Viable cells of food associated organisms were examined for their affinity for several chromatographic materials; cation exchange resin, hydrophilic/hydrophobic gel and an immunosorbent magnetic gel. Differences in the affinity of the organisms for these materials could be exploited to separate or enrich considerably one species from binary mixtures of organisms in broth or artificially contaminated food. A ten-fold enrichment of Salmonella typhimurium was achieved from a mixture containing either E.coli or Citrobacter freundii.

The results of these studies demonstrate the potential of chromatographic materials as a basis for novel methods of preparing samples for analysis by the rapid instrumental techniques now emerging in microbiology.

TECHNIQUES FOR THE MANIPULATION OF VIABLE MICRO-ORGANISMS by P.D.

Patel (*Leatherhead Food Research Association, Randalls Road, Leatherhead, Surrey
KT22 7RY, U.K.*)

Most conventional techniques for estimating micro-organisms are based on growth amplification, e.g. colony count or turbidity. In general, the initial concentration of organisms and nature of the food material have little effect upon these methods. Techniques introduced more recently, e.g. luminometry, direct epifluorescent filtration technique and impedance measurements, have a different basis of measurement and both the initial concentration of micro-organisms and the nature of the food can have a considerable effect. In these and future instrument-based techniques, the ability to manipulate (i.e. enrich, separate or concentrate) micro-organisms by physical techniques may offer advantages in preparing samples for analysis. Several approaches are used to separate micro-organisms from food components. The differential centrifugation method involves a low-speed centrifugation to separate 'large' suspended particles, followed by high-speed centrifugation of the supernatant to concentrate the organisms. The cation exchanger Bio-Rex 70 adsorbs meat proteins but not the indigenous Gram-negative flora, which can then be concentrated by filtration or centrifugation. An anionic flocculant, CA243, can aggregate suspended meat particles (e.g. in Mechanically Recovered Meats) but not the microbial flora, which can subsequently be separated and concentrated. The differences in buoyant densities between micro-organisms and food components (e.g. in yoghurt) have been exploited to separate micro-organisms by density gradient centrifugation. Various micro-organisms associated with foods show differences in affinity for several chromatographic materials, e.g. ion exchange resins, hydrophilic-hydrophobic gels and affinity media. These differences have been exploited to separate or enrich considerably one species from binary mixtures of organisms in broth or artificially contaminated food. Overall, these studies demonstrate the potential of novel separation, enrichment and concentration procedures as a basis of preparing samples for analysis by the rapid instrumental techniques now emerging in microbiology.

APPLICATION OF ENZYME IMMUNOASSAY TECHNIQUES FOR THE ESTIMATION OF STAPHYLOCOCCAL ENTEROTOXINS IN FOODS

P.D. Patel

*Microbiology Section, Leatherhead Food Research Association,
Randalls Road, Leatherhead, Surrey KT22 7RY, Great Britain*

ABSTRACT

Staphylococcal enterotoxins responsible for staphylococcal food-poisoning outbreaks occur in foods in very small quantities ($\leq 1 \mu\text{g}$ enterotoxin/100 g food). The conventional double-immunodiffusion method for detecting enterotoxins takes ≥ 3 days and has a sensitivity ranging from $0.1 \mu\text{g}$ to $2 \mu\text{g}/100 \text{ g}$ food. Therefore there is a distinct need for developing more rapid and sensitive techniques for estimating the enterotoxins.

This paper reviews the more rapid (< 1 day) and sensitive ($1\text{--}10 \text{ ng}$ enterotoxin/ml) enzyme-linked immunoassay techniques which have been applied for estimating the enterotoxins in foods.

Current research based on a novel technique called Inhibition Magnetic Enzyme ImmunoAssay for staphylococcal enterotoxins is described in some detail.

Finally, the future impetus of enzyme-linked immunoassay for estimating the enterotoxins is discussed.

INTRODUCTION

Normally, foods implicated in staphylococcal food-poisoning outbreaks contain small amounts of enterotoxins (Minor & Marth, 1976). Enterotoxins A and D are most frequently encountered in food-poisoning outbreaks (Holbrook & Baird-Parker, 1975). Since enterotoxin A usually occurred in concentrations of $\leq 1 \mu\text{g}/100 \text{ g}$ of food it was suggested that the sensitivity of any enterotoxin assay technique should be between $0.125\text{--}0.25 \mu\text{g}$ of enterotoxin per 100 g of food sample (Reiser *et al.*, 1974).

The procedures currently used for the detection and estimation of staphylococcal enterotoxins in foods are complex and lengthy but have been demonstrated to perform satisfactorily in many laboratories. The procedures normally involved are the extraction of enterotoxin from the food (2–3 days) followed by detection of the enterotoxin by the microslide double-immunodiffusion technique (Crowle, 1958; a further 2–3 days). Thus a result may be obtained within a week but frequently takes longer (Holbrook & Baird-Parker, 1975). The sensitivity of double-immunodiffusion techniques for detecting enterotoxins in foods varies from 0.1 to $2 \mu\text{g}$ enterotoxin per 100 g of food sample (Niskanen, 1977).

On the other hand, radio immunoassay (RIA) techniques are rapid in regard to the total time taken for estimation of enterotoxins in foods (≤ 1 day) and have been used in several laboratories (Miller *et al.*, 1978; Areson *et al.*, 1980; Bergdoll & Reiser, 1980).

Fig. 1. Elution of *clostridium perfringens* enterotoxin from Sephadex G-100

The column (85 cm $\times$ 2.5 cm diameter) of Sephadex G-100 was equilibrated in 0.02 M-phosphate buffer, pH 6.8, containing 0.001% (w/v) sodium merthiolate. The 0–15% satd. $(\text{NH}_4)_2\text{SO}_4$ fraction was resuspended in equilibration buffer before addition to the column and eluted with the same buffer. Fractions (10 ml) were collected at a flow rate of 20 ml/h.

The need for enterotoxin for structure/function studies and production of poly- and mono-clonal antibodies is such that there is a need for larger-scale production of *Cl. perfringens* enterotoxin. We have attempted to produce enterotoxin from 20–40 litre batch cultures of *Cl. perfringens* type A. Attempts to scale up from 2 to 20 litres highlighted certain problems. The highest degree of sporulation achieved in uncontrolled 20 litre batch incubations was only 20–30%. Since the extent of sporulation is perhaps the single most important step in the production of toxin, we are investigating ways of improving the extent of sporulation in large cultures.

Bacteria from large-volume cultures are conveniently harvested by using continuous-flow type centrifuges, but this process caused disruption of sporulating *Cl. perfringens*, releasing enterotoxin into the spent medium. Ultrafiltration using the Pellicon Cassette System (Millipore) provides a more gentle approach to harvesting sporulating bacteria. At

a pressure of 10 lb/in² (69 kPa), 20 litres of culture may be concentrated to less than 3 litres within 60 min, so the bacteria are concentrated and washed ready for disruption in less time than when harvested by centrifugation, thus decreasing the risk of natural lysis and release of toxin during harvesting.

Subsequently, harvested bacteria are usually disrupted by sonication, which is very effective, but may lead to denaturation of toxin if improperly controlled (Sakaguchi *et al.*, 1973) and is inefficient for large-scale disruption. Consequently, suspensions of sporulating *Cl. perfringens*, grown in large-volume cultures were disrupted in a French pressure cell at 12 000 lb/in² (82.8 MPa); this was rapid and effective and did not diminish toxin activity.

We have found that the previously described procedures for the production and purification of *Cl. perfringens* type A enterotoxin (Granum & Whitaker, 1980; Sakaguchi *et al.*, 1973) give variable yields of toxin, especially from larger cultures. The use of ultrafiltration as an efficient means of harvesting the sporulated cells and their subsequent disruption by the French pressure cell are significant improvements in the large-scale purification of the enterotoxin. Although the $(\text{NH}_4)_2\text{SO}_4$ precipitations remove many contaminating proteins, there are marked and variable losses of toxin [22% and 20% for the 40%- and 15%-satd. $(\text{NH}_4)_2\text{SO}_4$ steps respectively]. The use of alternative purification procedures, such as ion-exchange or hydrophobic interaction chromatography, may be useful alternatives in the purification process that might replace the use of $(\text{NH}_4)_2\text{SO}_4$ with large-scale preparations and give improved and consistent yields of enterotoxin.

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The effect of *Escherichia coli* enterotoxin and methotrexate on drug absorption

JAMES LYNCH and MICHAEL L. LUCAS

Institute of Physiology, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

In the present study, two procedures were used to induce small-bowel derangement. Primarily, challenge with *Escherichia coli* (heat-stable) toxin was investigated. This was compared with the effect of methotrexate. Methotrexate causes histologically verifiable damage and modifies absorption (Altmann, 1974; Bowring & May, 1976), whereas the effect of toxin challenge on absorption is relatively uninvestigated. Two drugs, aminopyrine (a weak base) and salicylate (a weak acid), were used to test absorption.

Male Wistar rats (250 g) were anaesthetized with urethane (1.5 g/kg body wt., intraperitoneally). Drug solutions (1 mM, in 25 ml of Krebs–Henseleit buffer) were recirculated through jejunal loops (15 cm) over a 3 h period, during which

concentration of ¹⁴C-labelled drug was measured by liquid-scintillation counting of serial samples. Water movement was measured by using [³H]polyethylene glycol 4000 as a non-absorbable marker. These solutions were constantly bubbled with O₂/CO₂ (19:1) and were maintained at 37°C in a small reservoir. The pH was monitored with a glass pH-electrode. In the toxin experiments the toxin (120 mouse units/ml) was dissolved in the buffer with the drug. Methotrexate was injected in oil (40 mg/kg, subcutaneously) 48 h before the experiment. All results were standardized for a 10 cm loop of intestine. Loop length was used rather than dry weight, since methotrexate treatment can markedly decrease intestinal weight. Significance was tested by using Student's *t* test.

In control experiments, 31% of salicylate and 30% of aminopyrine were absorbed (dose, 25 μ mol), with net water absorption of 16.9 μ l/min per 10 cm. In addition, pH fell by

Fig. 1. Effect of *E. coli* toxin and methotrexate on (a) the absorption of aminopyrine and salicylate *in vivo* and (b) fluid absorption and acid-base balance *in vivo*.

Results are expressed as means $\pm$ S.E.M., for the numbers of animals shown at the base of each column. Absorption rates are normalized for a 10cm loop of proximal jejunum. Changes in pH of buffer were over 180 min. Initial pH was 7.4. (a) □, Aminopyrine; ▨, salicylate; (b) □, fluid absorption; ▨, change in pH. Significance of differences from control: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

0.24 unit over 180 min. As expected, the toxin decreased water absorption by 73% ($P < 0.01$). Additionally, total reversal of the acidification occurred, the pH rising by 0.13 unit ($P < 0.001$). Salicylate absorption was decreased by 32% ($P < 0.02$) by toxin, with no change in aminopyrine absorption. Pretreatment with methotrexate caused a 99% ($P < 0.001$) decrease in fluid absorption and a decrease in acidification of the perfusate. The pH fell by 0.09 unit ($P < 0.01$). Aminopyrine absorption was decreased by 53% ($P < 0.01$) after methotrexate.

Both treatment with *E. coli* toxin and methotrexate decreased fluid absorption and inhibited acidification, with toxin having the greater effect on luminal pH. The toxin also caused malabsorption of salicylate, but not of aminopyrine. These molecules are passively absorbed and are absorbed to similar extents in the experimental preparation. It is therefore quite surprising that one is malabsorbed, whereas the other is unaffected, despite decreased fluid absorption and therefore decreased solvent drag through the paracellular pathway. The changes in acid-base balance with toxin should affect weak acids and bases differently (Schanker *et al.*, 1958). Alkalinization favours the absorption of aminopyrine and hinders the absorption of salicylate: mucosal pH will alter the amount of undissociated form available for non-ionic diffusion. Methotrexate caused an even greater decrease in fluid absorption than toxin did, but a much smaller change in acid-base balance; the perfusate still became acidified. This may explain the differences between malabsorption caused by toxin and methotrexate. However, methotrexate, in addition to affecting water movement and acidity, also caused secondary ultra-structural changes, which may additionally affect absorption. In conclusion it seems that both *E. coli* toxin and methotrexate can produce malabsorption of drug and fluid in the experimental animal.

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Development of an inhibition Magnetic Enzyme ImmunoAssay (MEIA) technique for determination of staphylococcal enterotoxin B

PRADIP D. PATEL and PAUL A. GIBBS

Microbiology Section, Leatherhead Food R.A., Leatherhead, Surrey KT22 7RY, U.K.

The conventional double-immunodiffusion technique for detection of staphylococcal enterotoxins (Holbrook & Baird-Parker, 1975) is both tedious and lengthy (2–3 days) and has a sensitivity of approx. 0.5 μg of enterotoxin/ml. Several laboratories use radioimmunoassays, which are more rapid (≤ 1 day) and sensitive, with a detection limit of 1–10 ng of enterotoxin/ml (Johnson *et al.*, 1971; Bergdoll & Reiser, 1980). However, radioimmunoassay techniques have disadvantages such as the need to use hazardous radio-labels and expensive instrumentation. More recently, radio-labels have been replaced by enzyme labels in enzyme-linked immunosorbent assay techniques for determination of staphylococcal enterotoxins (Kauffman, 1980; Freed *et al.*, 1982). The sensitivity and speed of these techniques are comparable with those of radioimmunoassay techniques.

We have reported a Magnetic Enzyme ImmunoAssay (MEIA) technique for determining anti-(staphylococcal enterotoxin B) immunoglobulin G-type antibodies (Patel *et al.*, 1984). In this preliminary study we report a modification of MEIA (i.e. an inhibition MEIA; Scheme 1) for determining staphylococcal enterotoxin B.

The materials and methods described in the accompanying communication (Patel *et al.*, 1984) were used. To appropriate wells in a proteose peptone-treated micro-titre plate (M129A; Dynatech Laboratories), 50 μl of purified anti-(enterotoxin B) immunoglobulin G-type antibodies (1:500 dilution) and 50 μl of different concentrations of enterotoxin B (0.01 to 100 μg/ml) were added. The plate was incubated at 37°C for 1½ h. A 100 μl portion of enterotoxin B-coupled Magnogel (Patel *et al.*, 1984) was added, and the plate was incubated at 37°C for 1½ h with shaking. Magnogel was separated by using a magnet, washed with phosphate-buffered saline/Tween and incubated with 200 μl of anti-rabbit immunoglobulin G-peroxidase conjugate (Sigma Chemical Co.) at 37°C for 1½ h with shaking. The Magnogel complex was separated and washed before the addition of 200 μl of the peroxidase-substrate solution (Voller *et al.*, 1979). The plate was incubated at ambient temperature for 30 min with shaking, followed by addition of 50 μl of 2.5 M-H₂SO₄. Magnogel was separated, and 100 μl

1. Immobilization stage
Act-Magnogel AcA-44 + Enterotoxin → Magnogel-Ent
(Magnogel) (Ent) (a)
2. Assay stages
 - (i) Anti-(enterotoxin B) serum + Ent → Ab-Ent + Ab(excess)
or purified immunoglobulin G (sample) (b)
 - (ii) (a) + (b) → Magnogel-Ent-Ab(excess) + Ab-Ent
(c)
 - (iii) (c) + anti-rabbit immunoglobulin G-peroxidase conjugate
(Anti-Ab-Enz)
→ Magnogel-Ent-Ab-anti-Ab-Enz complex

Scheme 1. Principle of inhibition MEIA for the determination of staphylococcal enterotoxin B

Enzyme activity of the complex is proportional to the antibody excess (i.e. total antibody minus enterotoxin in sample).

of the chromogenic product was mixed with 100 µl of distilled water in another micro-titre plate. The absorbance was measured at 490nm on a MicroELISA Mini-reader MR590; Dynatech Laboratories).

The enzyme activity of the Magnogel complex was inversely proportional over a 10-fold range to the initial toxin concentration (Fig. 1). The inhibition MEIA for determining enterotoxin B is rapid (<1 day) and has a sensitivity of approx. 200ng of toxin/ml, which is 20-fold less than that of radioimmunoassay or enzyme-linked immunosorbent assay but comparable with that of the conventional double-immunodiffusion technique.

The major advantage of magnetic enzyme immunoassays over enzyme-linked immunosorbent assays or radioimmunoassays is the simple and rapid magnetic separation of bound from unbound enzyme label. With further development, enzyme-linked immunosorbent assays (and inhibition MEIA) may become a rapid simple alternative to double-immunodiffusion for measuring staphylococcal enterotoxins in foods.

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PAUL NEAVES, LEONARD F. J. WOODS, PRADIP D. PATEL and PAUL A. GIBBS

Microbiology Section, Leatherhead Food R.A., Leatherhead, Surrey KT22 7RY, U.K.

The detection and identification of *Clostridium botulinum* in foods and pure culture is dependent on detection of its potent protein neurotoxin (Hobbs *et al.*, 1982). The only specific and sensitive assay for toxin is the mouse injection test, presumptive positive reactions being confirmed by immunological protection of further mice injected with

Fig. 1. Titrations of various concentrations of enterotoxin B against a constant concentration (1:500 dilution) of anti-(enterotoxin B) immunoglobulin G-type antibodies

○, 1:100 dilution (a) or 1:50 dilution (b) of enterotoxin-coupled Magnogel used; ●, 1:50 dilution of enterotoxin-coupled Magnogel, 1:500 dilution of Spicer-Edwards serum versus various concentrations of enterotoxin B (negative serum control). The arrows (←) indicate enterotoxin B-coupled Magnogel [1:100 dilution for (a) and 1:50 dilution for (b)] and 1:500 anti-(enterotoxin B) immunoglobulin G-type antibodies, enterotoxin B not used (control).

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Effect of *Clostridium botulinum* toxin on neuroblastoma cells

PAUL NEAVES, LEONARD F. J. WOODS, PRADIP D. PATEL and PAUL A. GIBBS

Microbiology Section, Leatherhead Food R.A., Leatherhead, Surrey KT22 7RY, U.K.

The detection and identification of *Clostridium botulinum* in foods and pure culture is dependent on detection of its potent protein neurotoxin (Hobbs *et al.*, 1982). The only specific and sensitive assay for toxin is the mouse injection test, presumptive positive reactions being confirmed by immunological protection of further mice injected with

mixtures of sample and antiserum. Since there is a strong desire to decrease the numbers of experimental animals, an alternative method is required.

We studied the effects of botulinum toxin on nucleic acid and choline content of cells of a mammalian neuroblastoma line cultured *in vitro*. Cells of strain NB41A3 (obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.) were grown at 37°C in an appropriate medium as described in the Flow Laboratories Tissue Culture catalogue. Cultures were incubated until confluent either as monolayers in culture flasks (75cm² area) containing 50ml of growth medium or

in suspended culture by attachment to micro carrier beads (0.5g dry wt. of Cytodex 1; Pharmacia) in flasks containing 500ml of medium in stirred suspension.

For nucleic acid staining experiments, cells were sub-cultured by preparing a suspension in trypsin/EDTA (Cumming, 1970), inoculating into sterile Leighton tubes containing coverslips and growth medium (1ml) and incubating overnight at 37°C. Cells were then exposed to purified type A neurotoxin, to type A crystalline toxin-haemagglutinin complex or to filtered, toxic, culture supernatant of *Cl. Botulinum* type A (N.C.T.C. 7272). Toxin samples were added to the culture medium at 200 mouse LD₅₀/ml and re-incubated at 37°C for 1 to 6 days. The monolayers on coverslips were then fixed and stained with the nucleic acid-specific fluorescent dye Acridine Orange by the method of Cumming (1970), and the preparations were viewed by u.v. microscopy. Monolayers were also incubated for 7 days in culture flasks containing growth medium and in the presence (200 mouse LD₅₀/ml) or in the absence of toxin, after which the nucleic acid composition was determined quantitatively. Cell suspensions were prepared by trypsinization, and nucleic acids were extracted by the method of Volkin & Cohn (1954). Nucleic acid concentrations were determined by colorimetric assay, by the methods of Burton (1956) for DNA and Dische (1955) for RNA. Choline concentrations in extracellular medium of Cytodex cultures exposed to crystalline toxin (1000 mouse LD₅₀/ml) overnight were determined by a luminometric technique based on that of Israël & Lesbats (1980). Samples for assay were placed into luminometer cuvettes containing 0.2M-Tris/HCl buffer, pH 8.6, luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) and horseradish peroxidase. After stabilization, choline oxidase was added and peak light output was recorded.

Monolayers incubated for up to 6 days in the absence of botulin toxin and stained with Acridine Orange appeared as small discrete cells comprising bright yellow-to-orange cytoplasm and small distinct green-to-yellow nuclei. This indicated the presence of RNA in the cytoplasm (orange) and DNA in the nucleus (green). Monolayers incubated for 1-24 h in the presence of native neurotoxin, crystalline toxin or a toxic culture supernatant were markedly different and seen as enlarged cells with pale non-fluorescent staining. Cytoplasm appeared dark green and nuclei were enlarged. After 48-72 h incubation, the effect was less marked, monolayers often containing a mixture of normal and enlarged cells. Monolayers incubated for up to 72 h in the presence of diluent alone, boiled (i.e. inactivated) toxin or *Clostridium sporogenes* culture supernatant stained similarly to the medium control monolayers.

Quantitative changes in nucleic acid concentrations were observed when cells were incubated in the presence of neurotoxin (200 mouse LD₅₀/ml). In the absence of toxin, cells contained 34.4pg of RNA/cell and 140pg of DNA/cell, whereas those incubated in the presence of toxin possessed

22.7pg of RNA/cell and 180pg of DNA/cell. The results match those from the staining experiments, suggesting that exposure of cells to toxin resulted in a decrease in RNA content whereas the DNA concentration was slightly increased.

In an investigation of the effect of crystalline toxin on the extracellular choline concentration of neuroblastoma cultures, cells (2.6×10^7 cells/test) were incubated overnight at 37°C in the presence of toxin (1000 mouse LD₅₀/ml). Control cultures showed a small increase in choline from 79ng/ml to 111ng/ml (41%), whereas the choline concentration of toxin-treated cultures increased by 109%. In a second experiment at a lower toxin concentration (500 mouse LD₅₀/ml), the extracellular choline concentration of control cultures decreased by 38% but that of toxin-treated cultures increased by 12%. These results suggest that toxin affects the choline balance of the neuroblastoma cells either by increasing the rate of choline release or inhibiting the rate of choline transport into the cells.

Our studies indicate that methods of detection based on a physiological effect of *Cl. botulinum* toxin appear to offer potential for the development of an alternative to the mouse bioassay. Whereas toxin can be detected immunologically, the cross-reactions and variable sensitivity observed with most immunological methods (e.g. Evancho *et al.*, 1973; Kozaki *et al.*, 1979; Guilfoyle & Mestrandrea, 1980) are difficult problems to overcome. The development of tissue-culture techniques may therefore provide a sensitive and specific method of detection as well as providing a means of studying the mode of action of botulin toxin at the molecular level. However, at this stage, tissue-culture techniques do not have the same degree of sensitivity as the mouse bioassay, and further work is required to make the technique of practical value.

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Development of a Magnetic Enzyme ImmunoAssay (MEIA) technique for determination of anti-(staphylococcal enterotoxin) immunoglobulin G-type antibodies

PRADIP D. PATEL, JOHN M. WOOD and PAUL A. GIBBS

Microbiology Section, Leatherhead Food R.A., Leatherhead, Surrey KT22 7RY, U.K.

Conventional single- and double-immunodiffusion techniques for determining immunoglobulin G-type antibodies

(Hudson & Hay, 1980) are tedious and lengthy (≥ 16 h). Enzyme-linked immunosorbent assays (Engvall & Perlmann, 1971; Kan *et al.*, 1983) and radioimmunoassays (Cripps *et al.*, 1983) have shortened the analysis time to < 7 h. In addition, novel techniques known as Magnetic Enzyme ImmunoAssay (MEIA) techniques for determining immunoglobulins (Guesdon & Avrameas, 1977; Guesdon *et*