

ADHESION OF SALMONELLA SPECIES AND
ESCHERICHIA COLI TO COLLAGEN
FIBRES OF CHICKEN
CONNECTIVE TISSUE

BY

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NOTES

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The terms ligand and adhesin have been used to describe the adhesive structure located on the bacterial cell, and the term receptor to describe the adhesive structure located on the substrate to which bacteria adhere.

The following abbreviations SEM and TEM, are used in this thesis in the following contexts:

SEM: scanning electron microscop(e), (y), or (ic)

TEM: transmission electron microscop(e), (y), or (ic)

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S U M M A R Y

This thesis describes an investigation of some factors affecting attachment of salmonellae and *Escherichia coli* to collagen fibres of poultry breast muscle fasciae. Direct microscopic techniques were used in conjunction with standard microbiological methods as a means of examining the attachment process.

All strains of salmonellae tested, fimbriate *Escherichia coli* and a strain of *Campylobacter coli* adhered to collagen when muscle fasciae was immersed in water containing cells of the appropriate test culture. Adhesion was dependent on water induced changes in fasciae structure and was inhibited or reversed by addition of sodium chloride to the suspending medium. Capsular glycocalyx also prevented attachment of these bacteria to collagen fibres.

TEM studies indicated attached cells were held to the collagen by acidic mucopolysaccharides (or glycosaminoglycans) associated with the intercollagen fibre matrix of fasciae. Subsequent studies showed hyaluronic acid (a predominant glycosaminoglycan associated with collagenous tissue) could inhibit attachment of selected strains of *Salmonella* and *E. coli*, but this inhibition could be reversed by hyaluronidase. Chondroitin-sulphate, a related glycosaminoglycan, only inhibited attachment of *E. coli* strains. This evidence implicated hyaluronate as a key factor in the attachment process.

Since only fimbriate *E. coli* could bind significant amounts of hyaluronic acid, it is suggested these bacteria may bind directly to tissue glycosaminoglycans. Salmonellae, however, apparently require an additional bridging compound (possibly a protein) to mediate adhesion to collagen fibres.

INTRODUCTION

A world-wide increase in the consumption of poultry has led to an increase in the number of cases of enteritis derived from poultry-associated micro-organisms. This trend emphasises the need for an investigation into the attachment of potentially enteropathogenic bacteria to poultry tissues. The attachment of bacteria to meat tissues is a relatively new area of investigation, and one that is gaining interest as the importance of hygiene within the meat processing industry is realised. A number of studies have examined the relationship of bacteria with various meat surfaces: cows' teats (Notermans *et al.*, 1979), chicken and beef meat (Firstenberg-Eden *et al.*, 1978), pork skin, beef and lamb muscle (Butler *et al.*, 1979). However, only one study has unequivocally shown attachment of bacteria to poultry muscle fasciae (Thomas and McMeekin, 1981). This study reported attachment of *Salmonella* to collagen fibres, and suggested tissue mucopolysaccharides were involved in the adhesion process.

Since the mechanism of attachment of bacteria to poultry muscle fasciae is not yet understood, further investigation is necessary to determine the role of mucopolysaccharides in this adhesive system. Such a study should include microscopic evidence of the attachment of these bacteria, investigate the conditions under which they will attach, and attempt to identify the nature of the ligands and receptors involved. This thesis examines some aspects of the attachment of some potentially enteropathogenic bacteria to poultry muscle fasciae. Microscopic techniques have been used in conjunction with standard microbiological methods in attempts to determine the mechanism of adhesion.

LITERATURE REVIEW

INTRODUCTION

There have been a number of studies carried out on the association between bacteria and animal tissues. Although bacterial adhesion to many meat surfaces has been reported, the mechanism of this attachment has yet to be elucidated.

This review will examine various aspects of the relationship between bacteria and meat surfaces, concentrating primarily on poultry tissues. Topics to be discussed include:

- (1) The occurrence of enteric bacteria on poultry
- (2) Cross-contamination of poultry carcasses during processing
- (3) Bacteriological sampling of poultry carcasses
- (4) Retention of bacteria on meat tissues
- (5) Attachment of bacteria to meat tissues
- (6) Histology and physiology of connective tissue
- (7) Adsorption of water by poultry tissues
- (8) Attachment mechanisms of the *Enterobacteriaceae*

2.1 The Occurrence of Enteric Bacteria on Poultry

A world-wide increase in the consumption of poultry has led to an increase in the number of cases of enteritis derived from poultry associated micro-organisms. Data from six countries indicates that 22.9% of the out-breaks of food-borne disease are associated with poultry (Todd, 1978). In the USA from 1966-1974, of the outbreaks of enteritis where a food-vehicle was identified, 15% were found to be caused by contaminated poultry (Horowitz and Gangarosa, 1976). The principal enteric pathogens associated with poultry are *Salmonella* species, *Clostridium perfringens*, *Staphylococcus aureus*, and to a lesser extent *Campylobacter* species, and *Yersinia enterocolitica* (Todd, 1980). The incidence of these organisms may be determined at any stage in processing but to indicate the safety of the carcass for consumption either the post-chill or retail measurements are required.

Reports of post-chill incidence of *Salmonella* on poultry carcasses vary, with levels of 12.9% (Patrick *et al*, 1973), 21.5% (McBride *et al*, 1980), 24% (Hagberg *et al*, 1973), 35% (Roberts, 1972), 37.1% (Bhatia *et al*, 1979), and 47% (Dougherty, 1974) being recorded. The proportions of *Salmonella*-contaminated retail poultry vary even more widely. Some published values are: 0.54%, 1.05% (Sadler and Corstvet, 1965), 1.2% (Norberg, 1981), 27% (Woodburn, 1964), 34% (Hobbs and Gilbert, 1978), 34.8% (Duitschaeffer, 1977), 28.6%, 36.9% (Green *et al*, 1982), 73.6%, 73.9% (van Schothorst *et al*, 1976), and 100% (Dougherty, 1974). Some of these differences may be attributed to the sampling method used, eg. Sadler recorded the intestinal contamination of market poultry whereas the other reports refer to carcass contamination. The value obtained by Dougherty may be misleading as the sample size was small, with only seven carcasses being tested.

Clostridium perfringens is consistently found in high levels on poultry. Post-chill levels have been recorded at 10%, 38.5% (Lillard, 1971), 57%, 100% (Mead and Impey, 1970), and 83% (Hagberg *et al*, 1973). Contamination of retail poultry with *Cl. perfringens* has been reported at 58% (Hall and Angelotti, 1965), and 63% (Roberts, 1972).

Reports from England and Wales attribute 25% of poultry-associated food-poisoning to *Staphylococcus aureus* (Notermans *et al*, 1982). This organism is reported to occur only in low levels prior to processing (10 cells per gram of skin) however, after processing the numbers recorded are significantly higher (over 1 000 cells per gram of skin) (Notermans *et al*, 1982). *Staph. aureus* has been isolated from processed poultry carcasses at levels of 63% (Roberts, 1972), 67% (Hagberg *et al*, 1973), and 80.4% (Gibbs *et al*, 1978).

Campylobacter species have recently been implicated as a cause of gastro-enteritis. These organisms have been isolated from 54% and 62% of fresh poultry (Park *et al.*, 1981), and from 22% of frozen chickens (Norberg, 1981). Little is known about the epidemiology of campylobacteriosis so the relevance of these results to public health cannot be assessed.

One other organism little reported for its involvement in food-borne disease is *Yersinia enterocolitica*. This has been isolated from frozen poultry carcasses at levels of 24.5% (Norberg, 1981), and 29% (Todd, 1980).

2.2 Cross-Contamination of Poultry Carcasses During Processing

During processing the number of contaminated carcasses is rarely reduced and cross-contamination is known to occur. In a typical processing plant there are many sites at which this may happen. Poultry in holding bays may be contaminated externally by micro-organisms excreted from other birds. High levels of bacteria, including *Staph. aureus*, have been found in the air in the holding bay and in the plucking areas (Patterson, 1973). This should not be a problem in contamination of the carcass if factory layout ensures these regions are well separated from the main processing area.

Following slaughter birds are scalded prior to plucking to facilitate the removal of feathers. If a hard scald (58C, 2.5 min.) is used the plucking procedure will remove the entire epidermis with the feathers. A soft scald (52C, 3 min.) does not result in such extensive damage although the epidermal layer is still partially disrupted (Thomas and McMeekin, 1980a). This process exposes dermal tissue to the micro-flora of scald-tank and plucker water. Mulder *et al.* (1978) investigated the cross-contamination occurring

at this stage of processing, simulating external and internal contamination of birds by artificially contaminating them with a strain of *E. coli*. External contamination resulted in the following 650-740 carcasses being contaminated with the test organism. This contamination was shown to occur during both scalding and plucking. Internally inoculated birds only caused cross-contamination during plucking and fewer carcasses (5-55) were affected.

The water of the scald-tank and plucker obviously plays a role in contaminating carcasses. Conditions arising during processing (accumulation of bacteria and organic matter, decrease in pH as a result of urate dissociation) combine to increase the heat resistance of *S. typhimurium* (Humphrey, 1981). Scald-water stabilises at pH 6.0, close to the optimum for heat resistance of this organism. Increasing the pH to pH 9.0 decreases this resistance and reduces total bacterial count by 71.7%, and coli-aerogenes by 87.9% (Humphrey *et al*, 1981).

The plucker itself may also be a source of contamination. Scanning electron microscopy of the rubber fingers used in plucking machines reveals a cracked and pitted surface. Bacteria that become trapped in these areas may multiply and form micro-colonies; these bacteria may not be removed by standard cleaning procedures (Notermans and Kampelmacher, 1983). This mechanism has been postulated as the method by which *Staph. aureus* becomes indigenous to a processing plant (Notermans *et al*, 1982).

Evisceration offers an opportunity for enteric bacteria to contaminate the exterior of the carcass, however this may be kept to a minimum if sufficient and effective spray-cleaning is used. Notermans *et al* (1980) found a significant decrease in the numbers of *Salmonella* on the skin when evisceration was accompanied by spray-cleaning.

Perhaps the most significant area, in terms of carcass contamination, is the chilling stage. Carcasses are most commonly cooled in large spin-chillers where ice and water bring the internal temperature of the carcass down to 6-7°C. During this process the carcasses absorb water causing the skin to swell and deep channels to form (Thomas and McMeekin, 1982). Immersion in water also causes the collagenous tissue to absorb water forming a weak gelatinous layer that is seen as a loose network of swollen collagen fibres when examined by SEM (Thomas and McMeekin, 1981). These crevices and matted fibres may serve to entrap bacteria present in the chill-water. SEM has been used to show bacteria present within a fluid layer on the skin and inside skin channels (McMeekin *et al*, 1979; Thomas and McMeekin, 1980a).

Cross-contamination may be promoted by the high carcass contact that occurs in the chiller. Both increases (Morris and Wells, 1970) and decreases (Hagberg *et al*, 1973; Bhatia *et al*, 1979) in *Salmonella* contamination of poultry carcasses following chilling have been reported. Post-chill decreases in the bacterial load on carcasses have also been reported for total count (86% decrease) (May, 1974) and coli-aerogenes count (87%) (Mead and Thomas, 1973). The contradictory reports may simply reflect the different sampling and enumeration techniques used by each group.

2.3 Bacteriological Sampling of Poultry Carcasses

SEM studies of processed chicken skin have shown that the surface is pitted with feather follicles and creased with deep channels that form after exposure to water (Thomas and McMeekin, 1982). Bacteria may become trapped in these channels (Thomas and McMeekin, 1980a) and in a layer of fluid covering the skin surface. These bacteria may be difficult to remove by conventional cleaning techniques (McMeekin *et al*, 1979) and may also be protected against heat destruction by their location in the skin surface (Notermans and Kampelmacher, 1975a).

It has been noted that the sampling technique used when assessing microbial contamination of material must take into account the structure of the surface being investigated (Patterson, 1971). Poultry skin presents a fatty, irregularly pitted surface in which bacteria may be embedded. These observations suggest swab sampling is unlikely to recover a majority of the organisms present on the carcass area tested. Several studies show that counts obtained by swabbing are consistently lower than those obtained by blending excised tissue (Avens and Miller, 1970; Patterson, 1972; Cox *et al*, 1976). Various rinse procedures have been reported to give good recovery of artificially inoculated micro-organisms from chicken carcasses (Blankenship *et al*, 1975; Blankenship and Cox, 1976; Cox *et al*, 1981), although other workers report low recovery rates (Notermans *et al*, 1975b). Rinse techniques have been shown to be inferior to maceration of excised tissue (Mead and Thomas, 1973; Notermans *et al*, 1975a, 1975b). The effectiveness of maceration as a sampling method was revealed in SEM studies of stomached poultry skin which demonstrated that most of the bacteria were removed by the action of the stomacher (Thomas and McMeekin, 1980b). Recently a scrape-sampling device was developed and this gives only slightly lower counts than maceration methods (not statistically significant) and has the advantage of being non-destructive allowing sampling of carcasses destined for the retail market (Adams *et al*, 1980).

Another factor to be considered when enumerating micro-organisms is the effect of transient stress on the metabolism of the bacteria. During processing poultry-associated micro-organisms are subjected to heat (scalding), cold (chilling), and to anti-microbial agents such as chlorine. These bacteria may become sensitive to selective agents used in media (Busta, 1976). The effect of sub-lethal injury on enumeration of bacteria may be shown by comparing counts on selective and non-selective media. Injured bacteria may account for between 60 and 99% of the numbers recovered after exposure to stress (Scheusner *et al*, 1971; Ray and Speck, 1973b).

Even the heat from agar used in standard pour-plate techniques may be sufficient to cause injury (Ray and Speck, 1973a; Klein and Wu, 1974). In general, non-selective media have been found the media of choice when enumerating micro-organisms (Patterson and Stewart, 1962; Ray and Speck, 1973a).

From these findings it is apparent that the optimum method for bacteriological sampling of poultry carcasses will use a maceration technique (stomaching or blending) and enumerate the organisms on non-selective media.

2.4 Retention of Bacteria on Meat Tissues

In current literature concerned with the mechanism of contamination the terms retention, adsorption, and attachment are sometimes used indiscriminately. For the purpose of this review the following definitions will apply:

- (1) Retention - any process by which a population of bacteria remain in a liquid film on a surface after transient immersion in a bacterial suspension.
- (2) Adsorption - the physico-chemical process by which micro-organisms are sorbed to a solid-liquid interface by short range attraction forces (Marshall, 1976). Adsorbed micro-organisms are readily removed from the surface by shear forces and the process has been referred to as reversible sorption (Marshall *et al*, (1971).
- (3) Adhesion (Attachment) - establishment of an intimate contact between the microbial cell and a surface via polymer bridging (Costerton *et al*, 1981) or organelles such as fimbriae and holdfasts (Corpe, 1980). Most bacteria which become irreversibly attached to a surface apparently do so without involvement of special organelles discernible by either light or scanning

electron microscopy (Corpe, 1980).

Retention of bacteria on surfaces is characterised by a direct correlation between the numbers retained and the suspension concentration. This may be interpreted graphically using the Freundlich equation:

$$\log R = \log A + n \log C$$

where A is a constant, n is the slope of the line, R is the number of bacteria retained by the surface, and C is the bacterial suspension population density. When $n = 1$, there is no active uptake of the bacteria i.e. retention is occurring. When n is greater than one some form of active attachment is occurring (Thomas *et al*, 1977). The Freundlich equation has been used to demonstrate bacterial retention on agar dip-slides (Thomas *et al*, 1977), chicken skin (McMeekin and Thomas, 1978), pork skin and beef and lamb muscle (Butler *et al*, 1979).

The initial work on the association between bacteria and meat involved agitating pieces of chicken skin in a suspension of bacteria for up to 20 minutes before removing the tissue and draining it. Bacteria remaining on the surface were deemed to be attached and were enumerated using plate count techniques (Notermans and Kampelmacher, 1974). Motile bacteria were held in higher numbers than non-motile organisms and the numbers retained depended on the time of immersion and the suspension concentration of the bacteria. Similar results have been obtained for other meat tissues: cows' teats (Notermans *et al*, 1979), chicken and beef meat (Firstenberg-Eden *et al*, 1978), pork skin, beef and lamb muscle (Butler *et al*, 1979). When analysed with the Freundlich equation it becomes apparent that these results depict retention rather than attachment. Other workers report that the motility status of the test organism had a negligible effect on its retention on chicken tissue (McMeekin and Thomas, 1978). SEM of retained bacteria shows no specific sites of accumulation (Firstenberg-Eden *et al*, 1979; Thomas and McMeekin, in press).

Later work suggested that some of the bacteria were held only loosely in a water-film on the skin surface and could be easily removed by washing whereas the more firmly held organisms required tissue maceration to release them. (Notermans and Kampelmacher, 1975b). This is consistent with other information obtained by SEM (Thomas and McMeekin, 1980b) and with the theory that the organisms are not actively attached to the surface. Bacteria requiring maceration for enumeration may be trapped in pits and crevices on the skin surface.

It has been postulated that bacteria retained on meat are adsorbed to the meat surface (Firstenberg-Eden, 1981) and may then consolidate this position by the production of extra-cellular fibrils (Firstenberg-Eden *et al*, 1978; 1979; Notermans *et al*, 1979; Firstenberg-Eden, 1981). SEM has shown fibril production and micro-colony formation on tissue incubated following exposure to bacterial suspensions (Firstenberg-Eden *et al*, 1979; Butler *et al*, 1980; Schwach and Zottola, 1982; Yada and Skura, 1982).

2.5 Attachment of Bacteria to Meat Tissues

The only documented instance of bacteria attaching to tissue appears to be the attachment of *Salmonella* species to chicken muscle fascie. Salmonellae suspended in low ionic strength media attach specifically to the collagen fibres of the muscle fascie. This does not occur if the suspension fluid is saline and saline rinses will remove attached cells. The attachment is apparently not affected by motility and requires the muscle tissue to be immersed in the medium for an extended period of time (30 minutes) (Thomas and McMeekin, 1981). During this time the tissue undergoes changes in micro-topography with the collagen fibres absorbing water and swelling to form a dense network over the surface of the tissue. These changes occur irrespective of the ionic strength of suspending media (Thomas and McMeekin, 1982).

Attachment is inhibited by 0.01M EDTA, however replacement of divalent cations fails to reinstate attachment indicating an ionic bonding between cell and collagen is unlikely. If the bond involved was ionic a wide range of types of bacteria would be expected to attach, however of those tested (*Pseudomonas*, *Alteromonas*, *Micrococcus*, *E. coli*, *Proteus*, *Salmonella*) only the *Salmonella* species attached (Thomas and McMeekin, unpublished data).

Pretreatment of chicken muscle with cetyl pyridinium chloride (CPC), a substance known to precipitate acid mucopolysaccharides, inhibits the attachment of *Salmonella* to the collagen fibres, and tissue treated with this does not take on the typical swollen characteristics of untreated tissue implying that the inter-fibrillar matrix material is altered in some way. TEM of attached bacteria revealed them to be enmeshed in a tissue-derived substance that binds polycationized ferritin indicating a negatively charged tissue component, such as a polysaccharide, may be involved (Thomas and McMeekin, unpublished data).

The alteration of tissue structure caused by absorption of water is an essential prerequisite for the attachment of *Salmonella* to chicken collagen fibres. A brief review of the physiology and histology of connective tissue, and the effect and mechanism of water absorption by these tissues is thus required.

2.6 Histology and Physiology of Connective Tissue

Connective tissues are tissues which support specialized structures and organs of the body. These tissues are intrinsically associated with avian musculature. Individual muscle fibres are surrounded by a delicate endomysium, bundles of muscle fibres are enclosed within a perimysium, and the entire muscle is sheathed in a layer of connective tissue known as the epimysium (Hodges, 1974). It is this sheath that forms the fascia of chicken breast meat that is exposed by the removal of the overlying skin.

The basic structure of connective tissue is a fibrous network of collagen, elastin, and reticulin, enmeshing an acidic proteoglycan-water matrix (Bayliss, 1982). This matrix, or extracellular ground substance, comprises interstitial fluid, glycoproteins, acidic mucopolysaccharides, and non-collagenous proteins. The interstitial fluid is continuous with the lymphatic system and may account for 5-20% of the connective tissue. In some cases the fluid may have the properties of a colloidal suspension. Acidic mucopolysaccharides are composed of an hexosamine plus either uronic acids or galactose units. Common mucopolysaccharides are hyaluronic acid, chondroitin, chondroitin-sulphate, keratin sulphate and heparin sulphate. Hyaluronic acid is a large, coiled, interwoven molecule (Gardner, 1965). It has the important task of binding water and is characteristic of tissues with a high water content. Hyaluronic acid is largely free within the extracellular matrix, whereas the other mucopolysaccharides are bound via proteins to cells and fibres (Chvapil, 1967).

Of the fibres in connective tissue collagen is the most abundant. Collagen fibres are composed of numerous fibrils, in turn composed of overlapping tropocollagen molecules. The tropocollagen molecule is a superhelix consisting of three chains of coiled helices of amino acids. The amino acid composition of collagen is 30% glycine and 20% hydroxyproline, proline, and hydroxylysine. The abundance of glycine permits the super-coiled structure of the molecule, while the high proportion of proline is thought to allow water absorption and swelling of the collagen fibres (Gardner, 1965).

2.7 Absorption of Water by Poultry Tissues

During processing poultry carcasses are exposed to large volumes of water at the scalding, washing, and chilling stages. Some of this process water may be absorbed into

the tissues of the bird. Carcass immersion in water first occurs at scalding where high temperatures may remove the entire cuticle from the skin (Suderman and Cunningham, 1980). This may favour absorption of water during scalding and in subsequent processing stages (Erdsieck, 1975). Though washing may contribute to water absorption, the majority of water uptake occurs during immersion chilling.

Chilling of the poultry carcass is most commonly done by immersion in a mixture of ice and water for 20-60 minutes (Mead, 1976) although 24 hour immersion in slush ice may also be used. The uptake of moisture from the cooling medium is most rapid during the initial 30 minutes of immersion (Sanders, 1969). Indications are that a small amount of water is absorbed through the outer surface of the skin (Veerkamp *et al*, 1973) mainly through the feather follicles and injured surfaces (Sanders, 1969). The primary route of entry for water into tissues is through cut surfaces. This water accumulates in blisters under the skin and then penetrates through the muscle. Absorption of water into muscle is a time dependent process, occurring after 24 hours in slush ice but not after 45 minutes in ice water (Erdsieck, 1975). Factors affecting moisture uptake include time of chill (water absorption is directly proportional to holding time in the chilling medium (Fromm and Monroe, 1958; Erdsieck, 1975)), agitation of the carcass and cuts in the carcass (Heath *et al*, 1968). High pre-chill temperatures were reported to favour water absorption (Erdsieck, 1975). Percentage moisture uptake varies at different sites on the carcass with neck tissue absorbing more water than other areas.

Within tissue, water absorption is largely a function of the connective components. The matrix acidic mucopolysaccharides and the collagen proteins form a highly hydrophilic colloid, capable of binding a considerable quantity of water. Hyaluronic acid is characteristic of tissues with a high water content and tissue hydration is specifically

connected only with this substance. During hydration of hyaluronic acid the molecules of water are placed not only inside its chain but also around it, allowing uptake of 1 000 to 1 800 grams of water per gram of hyaluronic acid (Chvapil, 1967).

2.8 Attachment Mechanisms of the *Enterobacteriaceae*

Like many micro-organisms enterobacteria may adhere to surfaces by either specific or non-specific mechanisms. Non-specific attachment is usually preceded by adsorption of the bacterium onto the substrate. The extent to which adsorption occurs is dependent on the surface properties of both substrate and bacterium, and on the composition of the surrounding medium. Adsorbed micro-organisms are held a small yet finite distance away from the surface by one or more of the following forces: hydrophobic interactions (Marshall and Cruickshank, 1973), ionic repulsive forces, van der Waals attractive forces, electrical double layer repulsion energies, and a variety of other physico-chemical forces (Marshall *et al*, 1971). There are several extensive reviews of the adsorption process available (Marshall, 1976; Rodgers, 1979; Jones and Isaacson, 1983). Adsorption may be facilitated by the presence of fimbriae, pili and other extra-cellular filamentous material (Jones and Isaacson, 1983).

Once adsorbed onto a surface bacteria may attach by producing extra-cellular fibrils which attach to receptor sites either directly or via bridging compounds. In many cases adhesion occurs by lectin bridging between two polysaccharide chains (Costerton *et al*, 1978). The bacterial ligand is often a component of the glycocalyx (Costerton *et al*, 1981). The role of the glycocalyx in adhesion of bacteria to surfaces has been extensively reviewed (Costerton *et al*, 1978; Fletcher, 1979; Rodgers, 1979; Ward and Berkeley, 1980; Costerton *et al*, 1981; Sutherland, 1983).

Specific adhesion of the *Enterobacteriaceae* is usually mediated by a variety of fimbriate and non-fimbriate ligands. The most common of these ligands is that located on type I fimbriae. This adhesin, present in strains of *Salmonella*, *Shigella* and *E. coli* (Jones and Isaacson, 1983) enables bacteria to adhere to a variety of cells including erythrocytes, leukocytes, epithelial cells, plant, animal and fungal cells (Gaastra and de Graaf, 1982). The adhesion of type I fimbriae to these cells is inhibited in the presence of D-mannose, methyl- α -D-mannopyranoside, concanavalin A, or by pretreatment of the cells with periodate (Ofek *et al.*, 1977). Bacteria possessing type I fimbriae are agglutinated by both bound and soluble yeast mannans, an effect which is inhibited in the presence of D-mannose or methyl- α -D-mannopyranoside (Ofek *et al.*, 1977). Addition of mannose or mannose derivatives (but not other sugars) to *E. coli* attached to epithelial cells causes rapid displacement of the organisms from the cells (Ofek and Beachey, 1978). Washing of bacteria and epithelial cells following their exposure to mannose reinstates the adhesive ability of type I fimbriae. The above data indicates that the receptor site for the type I fimbriae adhesin is probably a mannose-like carbohydrate moiety. The inhibitory effect of mannose is reversible and is probably a result of direct competition for the ligand between mannose molecules and the receptor sites.

There are a number of fimbrial non-mannose sensitive haemagglutinins associated with enteropathogenic *E. coli*. The plasmid-coded K88 antigen is present on thin flexible fimbriae, the expression of which is suppressed at 18C. To date, three variants of this adhesin have been described - K88ab, K88ac, and K88ad (Gaastra and de Graaf, 1982). *In vivo* K88⁺ cells adhere to the mucosa of the small intestine whereas K88⁻ cells remain free in the lumen (Jones and Rutter, 1972). Each of the three variants of K88 displays a different adhesion pattern to the five phenotypes of porcine intestinal brush border epithelial cells (Gaastra and de Graaf, 1982).

In vitro *E. coli* isolated from pigs attach to isolated porcine intestinal epithelial cells whereas non-porcine *E. coli* do not attach (Wilson and Hohmann, 1974). Adhesion of K88⁺ strains to porcine brush border epithelial cells occurs at 37C whereas haemagglutination of erythrocytes by these same strains is stable only at 4C or lower, the cells becoming dispersed if warmed to room temperature. This indicates either that attachment to erythrocytes and epithelial cells is mediated by different adhesins or that erythrocytes carry only a few receptor sites for the K88 antigen and kinetic energy at room temperature is sufficient to dislodge bacteria from these. The receptor site to which the K88 adhesin attaches is thought to be a galactose disaccharide (Gaastra and de Graaf, 1982; Jones and Isaacson, 1983).

The K99 antigen is similar to K88 in that it is plasmid-borne, is not expressed when cells are cultured at 18C, is carried on thin, flexible fimbriae and causes mannose-resistant haemagglutination at 4C. The K99 antigen is usually found on strains of *E. coli* isolated from calves and lambs although one instance of it occurring on porcine *E. coli* has been reported (Moon *et al*, 1977). This adhesin is responsible for the attachment of *E. coli* to calf intestinal brush border epithelial cells (Burrows *et al*, 1976). The receptor site for the K99 adhesin is thought to be sialic acid (Jones and Isaacson, 1983). Recently, a new antigen, F41, was found on a mutant K99⁺ *E. coli* strain. This adhesin exhibited mannose-resistant haemagglutination, attached to calf enterocytes and is fimbriae-related. It differs from K99 in its electrophoretic mobility (Morris *et al*, 1982).

A large percentage (86%) of strains of *E. coli* isolated from cases of human diarrhoea possess the colonization factor antigen (CFA). This is carried on thin flexible fimbriae, the presence of which is coded for by a plasmid (Evans *et al*, 1977). The CFA adhesin causes mannose-resistant

haemagglutination of human erythrocytes and adhesion of *E. coli* to the intestinal villous surfaces of infant rabbits (Evans *et al.*, 1978). The receptor site for CFA is thought to be sialic acid (Jones and Isaacson, 1983).

The 987P adhesin of *E. coli* is carried on fimbriae morphologically similar to type I fimbriae. It differs from other fimbriate adhesins in that it has no detectable haemagglutinating activity (Jones and Isaacson, 1983). The receptor site for this adhesin is a galactose disaccharide (Lindberg, 1982).

Mannose-resistant haemagglutinins may be found on species of *Proteus* (MRE/P) and *Klebsiella* (MRE/K). These adhesins are associated with type 4 and type 3 fimbriae respectively and mediate attachment to fungal hyphae, root hairs (MRE/K) and yeast cells (MRE/K, MRE/P) (Duguid and Old, 1980). Several strains of *Salmonella* have also been found to produce both mannose-sensitive and mannose-resistant haemagglutinins. The mannose-resistant adhesin has been implicated in the attachment of *S. typhimurium* to HeLa cells (Jones and Richardson, 1981). A few strains of *Salmonella* also exhibit non-fimbrial mannose-resistant haemagglutination (Duguid *et al.*, 1966).

MATERIALS AND METHODS

3.1 Cultures

Salmonella typhimurium (strain M48) was obtained from University of Tasmania, Hobart, Australia. *S. singapore*, *S. agona*, *S. havana*, *S. liverpool*, and *S. anatum* were provided by the Salmonella Reference Laboratory, Adelaide, Australia. *S. typhimurium* (1138), *S. typhimurium* (450), *S. newport*, and *S. mississippi* were supplied by the Dairy Research Institute, Palmerston North, New Zealand. *S. typhimurium* (11681), *S. agona* (MSS), *S. montevideo*, *S. cholera-suis*, *S. derby*, *S. hindmarsh*, *S. bovis-morbificans*, *Campylobacter coli*, and *C. jejuni* were provided by the Veterinary Faculty, Massey University, Palmerston North. *Staphylococcus aureus*, *Escherichia coli* (C1212-77), (C1214-77), and (04) were obtained from the Massey Culture Collection. *E. coli* (30-19), (20-19), and (20-6) were recent isolates from processed poultry.

All strains of *Salmonella* and *E. coli* were maintained on nutrient agar slopes (Appendix 1) and cultured for experimental purposes in 50ml nutrient broth (Difco) in a 250ml flask overnight at 37C, unless otherwise stated. Strains of *Campylobacter* were maintained on blood agar plates (Appendix 1) and were cultured on this medium in an atmosphere of 5% carbon dioxide. In addition, *S. montevideo* and *S. agona* were grown in a nitrogen deficient broth (NDB) (Appendix 1) and *E. coli* C1212-77, *S. typhimurium* 11681, and *S. cholera-suis* on a minimal medium (Appendix 1) to induce extensive glycocalyx production. Strains cultured with these media were incubated at 37C for 24 hours.

All nutrient broth cultures were examined for production of flagella and fimbriae with the aid of a transmission electron microscope and a negative stain technique. The staining technique used was as follows: formvar coated copper electron microscope grids were floated on a drop of overnight nutrient broth culture for ca. 30 seconds, removed and blotted dry, then floated on a drop of 1% (w/v) uranyl acetate solution for a further 10 seconds. Excess stain was removed by blotting and the grids allowed to air dry prior to

examination in a Philips EM-200 TEM unit operated with an accelerating voltage of 60kV.

3.2 Preparation of Cell Suspensions

Nutrient broth cultures of *Salmonella* and *E. coli* were harvested by centrifugation (6 000 rpm for 20 min.) then resuspended in glass distilled water to 1×10^8 cells per ml. *Campylobacter* cells were harvested by washing plates three times in sterile phosphate buffered saline (PBS) (Appendix 1), centrifuged (6 000 rpm for 20 min.) and resuspended in glass distilled water to 1×10^8 cells per ml. Cultures grown in NDB were harvested by centrifugation (6 000 rpm for 20 min.), while those grown on solid minimal media were harvested by washing plates three times with sterile PBS and centrifuged (6 000 rpm for 20 min.) followed by resuspension in glass distilled water to 1×10^8 cells/ml. The presence of a glycocalyx was confirmed microscopically by the negative staining technique described by Norris and Swain (1971) using 10% Nigrosin with Rose-Bengal as a counter-stain.

3.3 Samples

Fresh chicken pieces, processed 1-2 days previously (soft scald), were purchased from a local retail outlet, and whole chicken carcasses were supplied fresh (on the day of process) from a local poultry processing plant. The skin was carefully removed from the breast areas and thin pieces of breast muscle (ca. 1 cm x cm) with the fascia intact were excised and used for all attachment experiments.

3.4 Vero Cell Culture and Maintenance

Vero cells were maintained at 37C in sterile glass cell culture bottles containing carbon dioxide-saturated Eagles growth medium (Appendix 2). To transfer cells to coverslips the cultures were briefly washed with trypsin-Versene (Appendix 2), then exposed to trypsin-Versene until the cells had lifted from the glass surface. Detached cells were resuspended in 10ml growth medium, an aliquot containing 5×10^5 cells placed in a 45mm x mm petri dish containing 3-4 sterile coverslips, and the volume made up to 5ml with growth medium. Cells were incubated three days at 37C in an atmosphere of 5% carbon dioxide.

3.5 Chick Kidney Cell Culture and Maintenance

Kidneys were removed aseptically from point of hatch chickens, rinsed in Earle's washing solution (EWS) (Appendix 2), transferred to 20ml 0.1% trypsin solution (Appendix 2), chopped finely and transferred to 20ml fresh 0.1% trypsin in a sterile conical flask containing a magnetic stirrer. The tissue was stirred at 37C for approximately 30 minutes to disperse the cells. The resultant suspension was centrifuged (80 x g for 5 min.) in graduated conical glass centrifuge tubes, resuspended in 10ml Hank's growth medium (HTC) (Appendix 2), centrifuged (80 x g for 5 min.), and resuspended in 500x their volume HTC. The cells were dispensed into tubes (1ml) or petri dishes (5ml) containing sterile coverslips. Tubes were incubated at 37C without rolling; plates at 37C in an atmosphere of 5% carbon dioxide. After two days HTC was removed and replaced with the same volume of Earle's maintenance medium and incubation was continued as before.

3.6 Extraction of Glycosaminoglycans from Chicken Comb

Fresh chicken combs were washed to remove blood, sliced into small pieces (2-3mm wide strips), dried in several changes of acetone over a period of 3-4 days, then air-dried. Dried comb (100g) was suspended in 1 000ml phosphate-cysteine buffer (Appendix 1) containing 50mg papain (BDH), then incubated at 60C for 24 hours. The resulting digestion mixture was filtered through Whatman No. 1 paper under vacuum, then under gravity. The filtrate was dialysed for two days in cold, running tap water then filtered by suction through a Celite 545 pad (Hopkins and Williams Ltd).

Sodium chloride (35g) was added to the dialysate and the volume made up to 1 500ml with distilled water. This solution was warmed to 37C and 350ml of 2% cetyl pyridinium chloride (CPC) added slowly with stirring. This solution was left to stand at 37C overnight then centrifuged at 20C (10 000 rpm for 30 min.) to remove any precipitate. The supernatant was dialysed against cold, running tap water for 2-3 days, and the precipitate saved for the isolation of chondroitin-6-sulphate/dermatan sulphate. The dialysed supernatant was used to isolate hyaluronic acid.

1. Chondroitin-6-sulphate/dermatan sulphate.

The precipitate from above was washed with 250ml 0.3M sodium chloride, recovered by centrifugation (10 000 rpm for 30 min.), then dissolved in 200ml 2.0M sodium chloride and 40ml methanol with any insoluble residue being removed by centrifugation. The supernatant was added to 600ml 75% ethanol and left to stand overnight. The bulk of the supernatant was removed by suction and the precipitate formed was recovered by centrifugation, washed in 95% ethanol, 100% ethanol, and ether, then air-dried. The resulting white solid contained both chondroitin-6-sulphate and dermatan sulphate.

2. Hyaluronic acid.

Any gummy aggregate formed in the dialysed supernatant was saved. The remaining fluid was made 0.03M in sodium chloride and 150ml 2% CPC added. After several hours any gummy aggregate formed was recovered by decantation and centrifugation. The combined aggregates were dissolved by prolonged stirring in 250ml methanol and 250ml 2M sodium chloride. The solution was clarified by centrifugation, added to 3 000ml 75% ethanol and allowed to stand overnight. The bulk of the supernatant was removed by suction and the precipitate recovered by centrifugation, washed in 95% ethanol, 100% ethanol, and ether, then air-dried to give a crude extract of hyaluronic acid.

3.7 Chemical Analysis of Glycosaminoglycans

Uronic acids were measured by the carbozole method (Davidson, 1966). This method is sensitive over the range of 5-40ug uronic acid and the pink chromophore formed is stable for 16 hours (Appendix 3). Amino sugars were measured using Erlich's reagent following the method of Gatt and Berman (1966) (Appendix 3). Total sulphate was measured using the method of Dodgson (as described by Sinsberg, 1972). Sulphate is liberated from the glycosaminoglycan by acid hydrolysis. The inorganic sulphate forms a stable barium sulphate precipitate in the presence of gelatin and can be measured spectrophotometrically (Appendix 3).

3.8 Haemagglutination Test

The haemagglutination test used was similar to that described by Reid (1982). Bacterial suspensions of 1×10^{10} organisms/ml PBS (pH7.2) were used; 50ul was added to four wells in a white porcelain tile. Fresh, human, group A and

O erythrocytes (collected in 10% (3.8% solution) sodium citrate) were washed four times in PBS (pH7.2) and resuspended to 3% (v/v) in PBS; 50ul of human A erythrocytes was added to wells 1 and 2, and 50ul of human O erythrocytes was added to wells 3 and 4. To wells 1 and 3 was added 50ul PBS, and to wells 2 and 4 was added 50ul PBS containing 1% α -D-mannose. Each bacterial strain was thus tested for the presence of a mannose-sensitive haemagglutinin (MSHA) and a mannose-resistant haemagglutinin (MRHA). The porcelain tiles were rocked gently then placed at 4C for 60 minutes, after which time the haemagglutination patterns were read. In each batch of tests, two controls were included. *E. coli* C1212-77 is known to possess both a MSHA and a MRHA after growth in broth, and it was used to confirm that the test was working and sensitive. A second control of PBS without bacteria was incorporated to confirm the absence of erythrocyte auto-agglutination.

3.9 Attachment of Bacteria to Cultured Cells

Two ml of a suspension of bacteria in PBS (pH7.2) (1×10^9 cells/ml) were transferred to a coverslip culture of either Vero or chicken kidney cells (previously washed three times in PBS) and incubated at 37C for 30 minutes. Cells were washed five times by immersion in PBS, then rinsed in a gentle stream of PBS, fixed in 10% formal saline, rinsed with PBS, and allowed to air dry.

The number of bacteria attached was determined by indirect immunofluorescence. A dry, fixed preparation was incubated (30 minutes at 37C) with a drop of rabbit-immunoglobulin (Wellcome Reagents Ltd., 1/8 dilution) to the test organisms, washed in three changes of PBS for 3, 5, and 7 minutes, incubated with one drop fluorescein-labelled sheep anti-rabbit immunoglobulin (Wellcome) (30 min. at 37C), washed as before, air-dried, and examined with a Leitz ultra-violet microscope using filtered light from a mercury-vapour lamp.

3.10 Attachment of Bacteria to Chicken Muscle Fascie

The ability of test isolates of bacteria to attach to the surface of muscle fascie connective tissue was assessed using a modification of the procedure outlined by Thomas and McMeekin (1981). Pieces of breast muscle with the fascie intact, were agitated in distilled water for 20 minutes. These pieces were then removed, drained and agitated for 10 minutes in 100ml volumes of distilled water or saline which contained ca. 10^8 cells/ml of the test organism. All test media were vigorously agitated by magnetic stirring and were maintained at ca. 20C. After immersion in the test suspensions, the muscle tissue was rinsed five times in the appropriate sterile suspending medium to remove unattached bacteria and then prepared for SEM or TEM as described below.

The role of chicken tissue extracts in the attachment process was examined by incubation of the test bacteria in spin-chiller water (obtained from a local poultry processing plant), saline or water extracts of poultry tissue (10g of tissue agitated for 2 hours in 100ml 0.87% saline or distilled water, followed by overnight dialysis against cold, running tap water). Test bacteria were incubated in these solutions for 20 minutes prior to the addition of the pre-swollen tissue. Treated tissue was prepared for SEM as described below.

The effect of chicken comb glycosaminoglycan (GAG) fractions (hyaluronic acid or chondroitin-6-sulphate) on attachment of bacteria to muscle fascie was assessed by addition of 1mg or 0.1mg GAG/ml to suspension media. Similarly, the effect of fructose, mannose, glucose, galactose, glucuronic acid and glucosamine was assessed by the addition of the sugars to the suspension medium to give a final concentration of 1% (w/v). Test bacteria were incubated in these solutions for 20 minutes prior to addition of pre-swollen tissue for a further 10 minutes. The treated tissue was then prepared for SEM as described below.

In attempts to identify the nature of the adhesin mediating attachment of bacteria to muscle fasciae, some test suspensions of bacteria were treated as follows. Suspensions of bacteria containing 5×10^9 cells/ml were incubated for 60 minutes at 37C in solutions of trypsin (Sigma type III from bovine pancreas; 1 000 BAEE units/ml in 0.046M Tris-HCl buffer, pH7.5, 0.0115M in CaCl_2), lipase (Sigma type I from wheat germ; 10 units/ml in 0.15M sodium acetate buffer, pH6.5), hyaluronidase (Sigma type I from bovine testes; 640 NF units/ml in 0.1M sodium phosphate buffer, pH6.5, 0.15M in NaCl), and periodate (0.01M sodium periodate in 0.87% NaCl). The treated suspensions were then centrifuged (6 000 rpm for 20 minutes), washed in distilled water, resuspended in water (ca. 10^8 cells/ml) and tested for their ability to attach to chicken muscle fasciae. Control suspensions were treated as above except without the appropriate enzyme or periodate. Treated tissue was then prepared for SEM as described below.

3.11 Scanning Electron Microscopy

Samples of tissue were fixed at 4C overnight in a glutaraldehyde solution (TAAB Lab., Equip., Ltd., Reading, England, 2% (v/v) in 0.1M Sorensen's phosphate buffer, pH7.2), then washed four times in 0.1M phosphate buffer to remove unbound glutaraldehyde. The specimens were dehydrated in a graded ethanol series (30-50-70-90-95-100% x 3, using distilled water as the diluent) and sliced into smaller pieces (2-3mm x mm). These pieces were infiltrated with acetone (50-100% x 3, using absolute ethanol as the diluent) and critical point dried in carbon dioxide using a Polaron E-3000 critical point drying unit (Polaron Equipment Pty Ltd., Watford, England). Dried samples were mounted on SEM stubs, with double sided tape, and coated with approximately 20nm of gold by DC sputtering (Polaron Sputter Coater, Model E5100). Coated specimens were examined in a Qwikscan Model 100 field emission SEM, operated with an accelerating voltage of 16kV.

3.12 Transmission Electron Microscopy

Conventional Preparative Methods

Tissue samples were placed in glutaraldehyde fixative solution (TAAB Lab., 2% (v/v) in 0.1M cacodylate buffer pH7.2), and kept overnight at 4C, washed four times in cacodylate buffer followed by post-fixation in osmium tetroxide (1% (w/v) in 0.1M cacodylate buffer, pH to 7.6), for 4 hours at room temperature. Specimens were washed in 2% aqueous sodium acetate, stained for one hour in 2% aqueous uranyl acetate then dehydrated through a graded ethanol series (30-50-70-90-95-100 x 3, using distilled water as the diluent). Ethanol was replaced with acetone (50-100 x 3, using absolute ethanol as the diluent).

All dehydrated specimens were embedded in Spurr resin (Spurr, 1969). Ultrathin sections were cut from the embedded material using a LKB Huxley Ultramicrotome and collected on bare 400 mesh copper electron microscope grids. The mounted sections were stained with an alcoholic solution of 2% (w/v) uranyl acetate, washed with distilled water and stained with 0.4% (w/v) lead acetate. The prepared sections were then examined in a Phillips EM-200 TEM unit operated with an accelerating voltage of 60kV.

Cell suspensions to be examined by TEM were first pelleted by centrifugation, embedded in 2% Noble agar, fixed in a glutaraldehyde solution for 2 hours at room temperature, washed in cacodylate buffer then post-fixed with osmium tetroxide. The pellet of cells was washed in 2% aqueous sodium acetate, stained with uranyl acetate, dehydrated and embedded in Spurr resin as described above.

Cytochemical Preparative Methods

Some strains of bacteria known to attach to collagen fibres, were tested for ability to bind hyaluronic acid by incubating the cell suspensions of these bacteria in a 0.1% solution of hyaluronic acid in distilled water.

After 30 minutes incubation the bacteria were recovered by centrifugation (6 000 rpm for 20 min.) and prepared for TEM using the ruthenium red and poly-cationized ferritin labelling techniques described below. The same labelling techniques were used to locate GAG material associated with muscle fasciae with or without attached bacteria.

Specimens to be stained with ruthenium red were fixed in the standard glutaraldehyde fixative (described above) which contained 0.05% (w/v) ruthenium red. Materials treated with this modified fixative were then washed in cacodylate buffer containing 0.05% ruthenium red followed by post-fixation with osmium tetroxide, stained, dehydrated and embedded in Spurr resin as per conventionally prepared material.

Negatively charged groups associated with cell or tissue surfaces were located with the aid of poly-cationized ferritin (Sigma) using a procedure outlined by Weiss *et al* (1979). Samples to be labelled with ferritin were washed in Tris-buffered saline, Tris-buffered saline -NH₄Cl, and Tris-buffered-saline x 2, following primary glutaraldehyde fixation, then incubated 30 minutes at room temperature with poly-cationized ferritin (0.75mg/ml Tris-buffered saline). The reaction was halted by a ten-fold dilution with Tris-buffered saline, then the samples were washed with cacodylate buffer, post-fixed in osmium tetroxide and further prepared for TEM as per conventionally prepared material.

RESULTS

4.1 Attachment of Bacteria to Cultured Cells and Human Erythrocytes

Test strains of bacteria used in this study were examined for their ability to adhere to human erythrocytes (haemagglutination), Vero cells and chicken kidney cells. The results of these adhesion experiments are summarised in Table 1. Only fimbriate strains of *E. coli* were able to agglutinate erythrocytes, and *E. coli* C1212-77 produced both mannose-sensitive and mannose-resistant haemagglutinins. Similarly *E. coli* C1212-77 was the only test culture able to attach in significant numbers to Vero cells. SEM was used to confirm that bacteria attached to the monolayers of cells and not the glass substrate (Figs. 1a, b, c).

4.2 Attachment of Bacteria to Chicken Muscle Fasciae

Results of experiments designed to assess the ability of various test strains of bacteria to adhere to collagen fibres of chicken muscle fasciae are presented in Table 1. Of the cultures employed, only the *Salmonella* strains, *Campylobacter coli*, and fimbriate *E. coli* strains were found to adhere to collagen fibres when cells of these cultures were suspended in media of low ionic strength (Figs. 2a, b, c, d, 3a, c). None of these cultures were however, able to adhere to collagen when suspended in saline solutions. Attachment only occurred when muscle tissue was immersed in water and allowed to swell. Bacteria did not attach to the fasciae surface of fresh muscle that had been only transiently immersed in water and/or test suspensions (Fig. 3b). Bacteria which attached to collagen were usually found as either multilayers of cells surrounding groups of collagen fibres, or as single layers of cells more or less covering groups or individual fibres.

TABLE 1: Adhesion of test cultures of bacteria to chicken muscle fascie, Vero cells, chicken kidney cells and human erythrocytes.

TABLE 1:

Organism	Attachment to			
	Chicken Muscle Fascie +/-	Vero Cells No./cell	Chick Kidney Cells No./cell	Red Blood Cells +/-
<i>S. typhimurium</i> (M48)	+	3.0	0.03	-
<i>S. typhimurium</i> (11681)	+	0.4	nt	-
<i>S. typhimurium</i> (1138)	+	nt	nt	-
<i>S. typhimurium</i> (450)	+	nt	nt	-
<i>S. singapore</i>	+	1.1	0.02	-
<i>S. agona</i>	+	0.8	0.14	-
<i>S. agona</i> (MSS)	+	0.2	nt	-
<i>S. havana</i>	+	0.8	0.003	-
<i>S. montevideo</i>	+	0.5	nt	-
<i>S. liverpool</i>	+	0.8	0.04	-
<i>S. anatum</i>	+	0.2	0.01	-
<i>S. cholera-suis</i>	+	0.7	nt	-
<i>S. derby</i>	+	0.6	nt	-
<i>S. hindmarsh</i>	+	1.2	nt	-
<i>S. bovis-morbificans</i>	+	0.3	nt	-
<i>S. newport</i>	+	nt	nt	-
<i>S. mississippi</i>	+	nt	nt	-
<i>E. coli</i> (C1212-77)	+	14.6	nt	+
<i>E. coli</i> (C1214-77)	+	nt	nt	+
<i>E. coli</i> (04)	+	nt	nt	+
<i>E. coli</i> (M23)	-	nt	nt	-
<i>E. coli</i> (30-19)	-	nt	nt	-
<i>E. coli</i> (20-19)	-	nt	nt	-
<i>E. coli</i> (20-6)	-	nt	nt	-
<i>C. coli</i>	+	nt	nt	-
<i>C. jejuni</i>	-	nt	nt	-
<i>Staph. aureus</i>	-	nt	nt	-

+ attachment; - no attachment; nt not tested

FIGURE 1: SEM of Vero cells attached to glass.

(a) Control Vero cells not exposed to bacteria.
Magnification = x 4000

(b) Vero cells incubated with *S. singapore*. Note
attached bacteria (arrows).
Magnification = x 4000

(c) Vero cells incubated with *E. coli*. Note the
high numbers of attached bacteria (arrows).
Magnification = x 4000

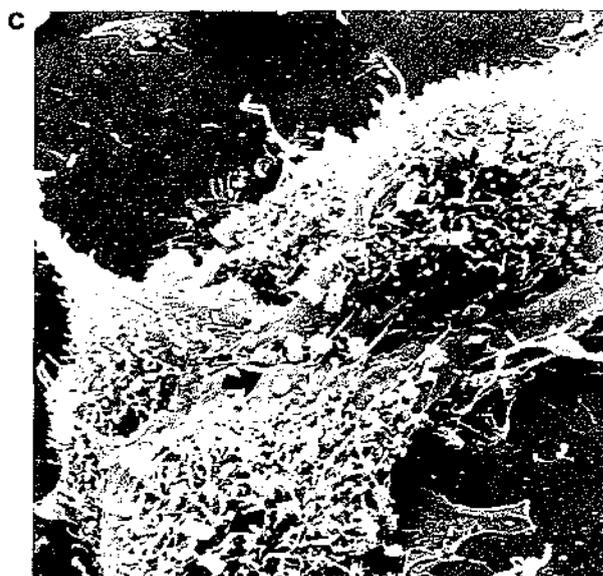
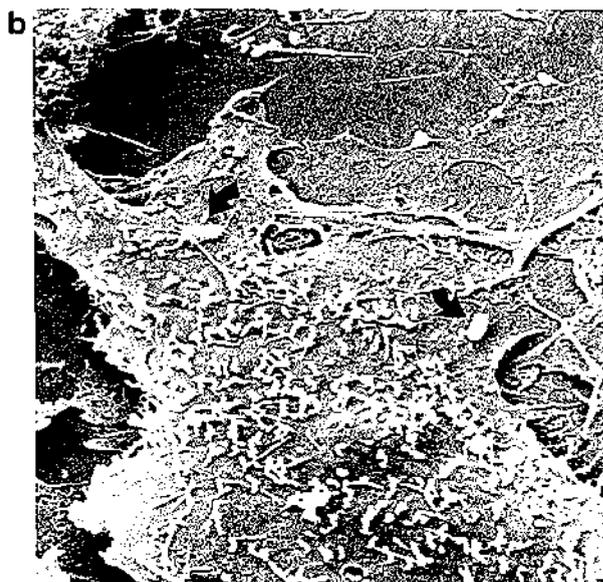
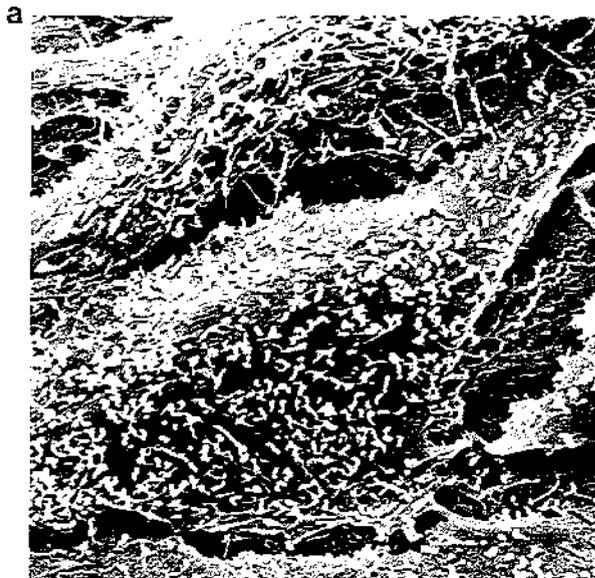


FIGURE 2: SEM of bacteria attached to chicken muscle fascie.

(a) Control muscle fascie immersed in sterile water for 30 minutes. Note the loose network of connective tissue fibres covering the surface of the muscle.

Magnification = x 4000

(b) *S. typhimurium* strain M48 cells attached to groups of collagen fibres.

Magnification = x 8000

(c) *S. typhimurium* strain 11681 cells attached to collagen fibres. Note absence of bacteria from the surrounding muscle tissue.

Magnification = x 4000

(d) *C. coli* cells attached to groups of collagen fibres.

Magnification = x 6000

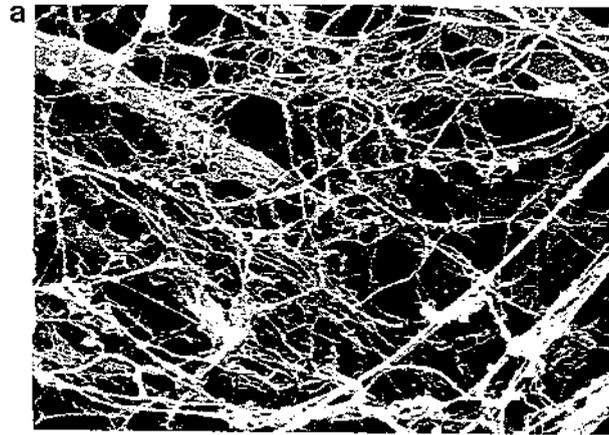
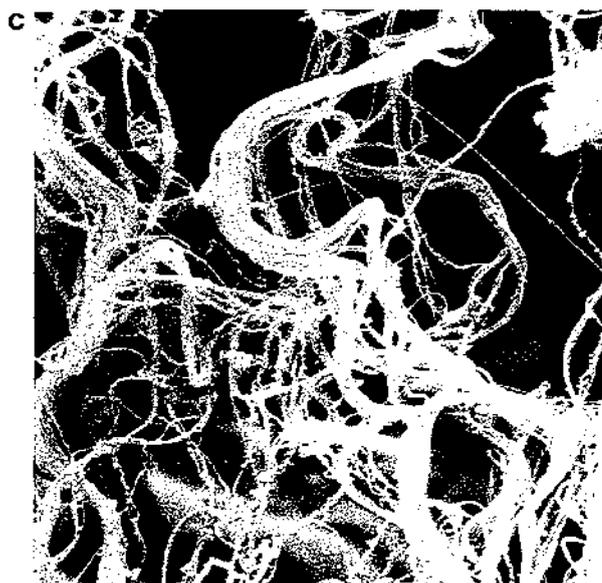
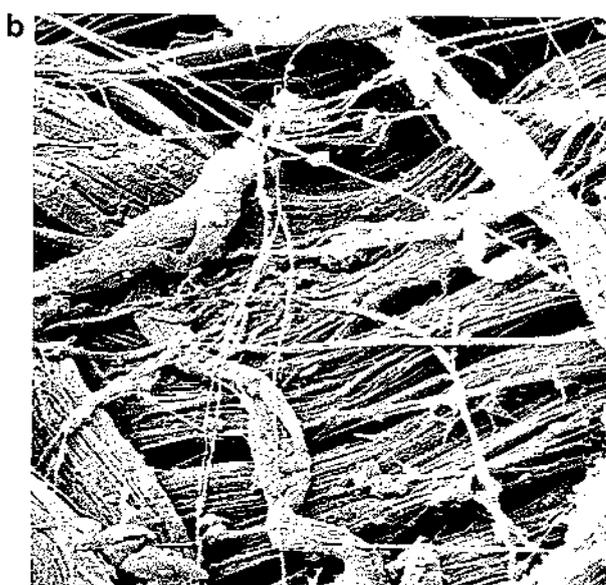
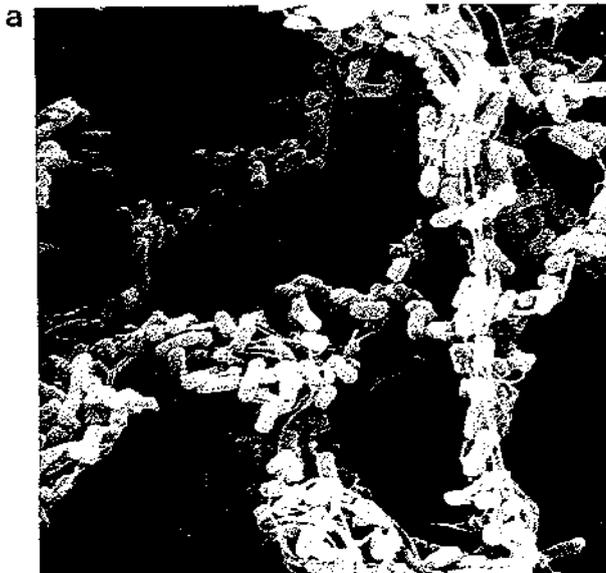


FIGURE 3: SEM of bacteria attached to chicken muscle fascie.

- (a) *E. coli* C1212-77 cells attached to collagen fibres.
Magnification = x 4000

- (b) Fascie exposed only briefly to a suspension of *E. coli* C1212-77 cells. The connective tissue remains closely associated with the muscle surface. Bacteria failed to adhere to the unswollen collagen fibres.
Magnification = x 4000

- (c) Fascie exposed for 30 minutes to a suspension of the non-fimbriate *E. coli* (20-19). These bacteria did not attach to the swollen collagen fibres.
Magnification = x 4000



Cultures of bacteria able to attach to collagen fibres did not produce a significant glycocalyx when cultured in nutrient broth. However, when *S. montevideo*, *S. agona*, *E. coli* C1212-77, *S. typhimurium* 11681, and *S. cholera-suis* were cultured on either nitrogen-deficient or minimal medium, these cultures produced extensive glycocalyces and were noticeably less able to adhere to collagen fibres compared with nutrient broth cultured cells (Figs. 4a, b, c, d, e).

Suspensions of *E. coli* C1212-77, *S. agona*, and *S. singapore* treated with lipase, trypsin or hyaluronidase prior to exposure to muscle fascie, retained their ability to adhere to collagen fibres (Table 2). In fact, SEM examination of tissue specimens suggested treated cells attached as well as control suspensions. Furthermore, periodate treatment of test suspensions did not affect adhesion of *S. singapore* or *S. agona* to collagen, but did inhibit adhesion of *E. coli* C1212-77.

4.3 Effect of Glycosaminoglycans, Component Saccharides and Tissue Extracts on Attachment of Bacteria to Muscle Fascie

Table 3 summarises the results of experiments designed to assess the effects of glycosaminoglycans isolated from comb tissue, and their component saccharides on attachment of *E. coli* C1212-77, *S. singapore* and *S. agona* to collagen fibres of muscle fascie.

Hyaluronic acid added to distilled water suspensions of these bacteria to give a final concentration of 1.0mg glycosaminoglycan/ml, completely inhibited attachment of these bacteria to collagen (Figs. 5a, c; 6a, c). Similarly, chondroitin-sulphate inhibited attachment of *E. coli* C1212-77 but this glycosaminoglycan did not inhibit attachment of *S. singapore* or *S. agona* to collagen fibres.

FIGURE 4: SEM of bacteria possessing a glycocalyx attached to chicken muscle fascie.

- (a) Fascie with attached *S. agona* cells.
Magnification = x 8000

- (b) *S. montevideo* attached to collagen fibres.
These bacteria were typically present only as clumps of cells.
Magnification = x 4000

- (c) Higher magnification of (b) showing extra-cellular material on the bacteria.
Magnification = x 10 000

- (d) *S. typhimurium* strain 11681 cells attached to collagen fibres.
Magnification = x 6000

- (e) *E. coli* C1212-77 cells. Relatively few bacteria have attached. In contrast to cells grown in nutrient broth, bacteria cultured in glycocalyx inducing media did not adhere prolifically to muscle fascie.
Magnification = x 4000

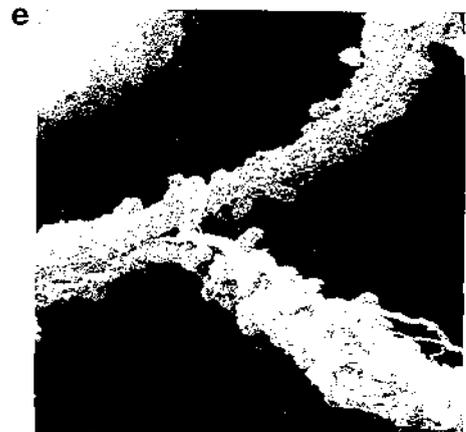
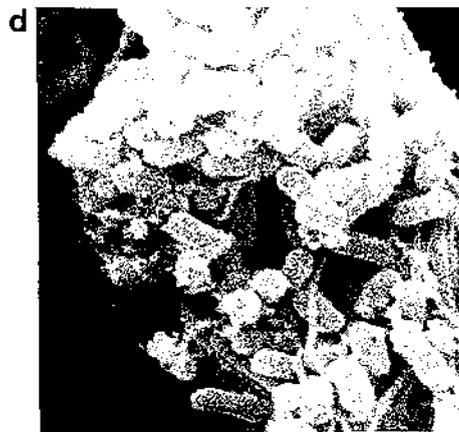
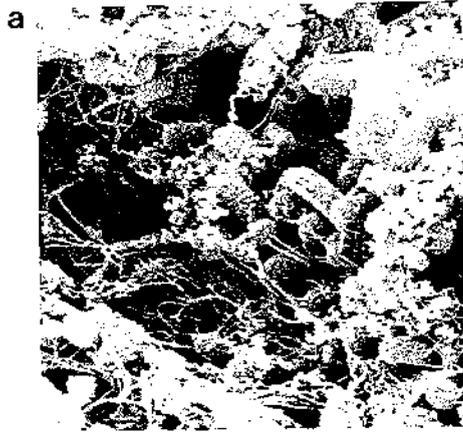


TABLE 2: Adhesion of enzyme treated bacteria to poultry muscle fascie.

TABLE 2:

Treatment	Attachment of		
	<i>E. coli</i> C1212-77	<i>S. singapore</i>	<i>S. agona</i>
Hyaluronidase	+	+	+
Hyaluronidase control	+	+	+
Trypsin	+	+	+
Trypsin control	+	+	+
Lipase	+	+	+
Lipase control	+	+	+

+ attachment

TABLE 3: Effect of chicken comb glycosaminoglycans and component saccharides on attachment of bacteria to collagen fibres of muscle fascie.

TABLE 3:

Component	Attachment of		
	<i>E. coli</i> C1212-77	<i>S. singapore</i>	<i>S. agona</i>
Hyaluronic acid 1mg/ml	-	-	-
Hyaluronic acid 0.1mg/ml	+/-	+/-	nt
Chondroitin-sulphate 1mg/ml	-	+	+
Glucose 1%	+	+	+
Fructose 1%	+	+	+
Mannose 1%	+	+	+
Galactose 1%	+	+	+
Glucosamine 1%	+/-	+/-	+/-
Glucuronic acid 1%	+/-	+/-	+/-
Distilled water control	+	+	+

+ attachment

- no attachment

+/- partial inhibition of attachment;

nt not tested

FIGURE 5: SEM's showing the effect of hyaluronic acid on attachment of *S. singapore* to chicken muscle fascie.

- (a) Cells incubated in a 0.1% (w/v) solution of hyaluronic acid. Attachment was completely inhibited.
Magnification = x 4000

- (b) Cells incubated in a 0.01% (w/v) solution of hyaluronic acid. Attachment was partially inhibited.
Magnification = x 4000

- (c) Cells incubated in the absence of hyaluronic acid.
Magnification = x 4000

- (d) Cells incubated in a 0.1% (w/v) solution of hyaluronic acid, then treated with hyaluronidase. Attachment was partially restored.
Magnification = x 4000

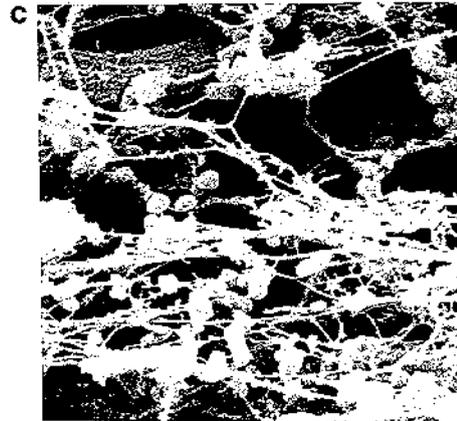
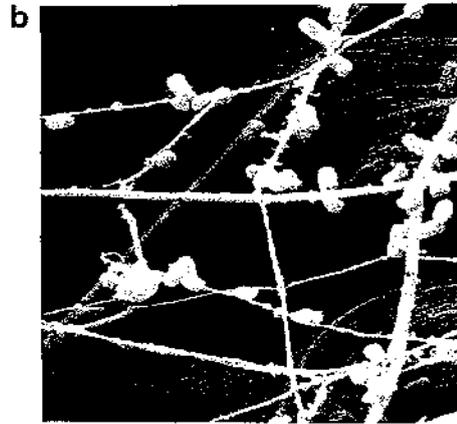
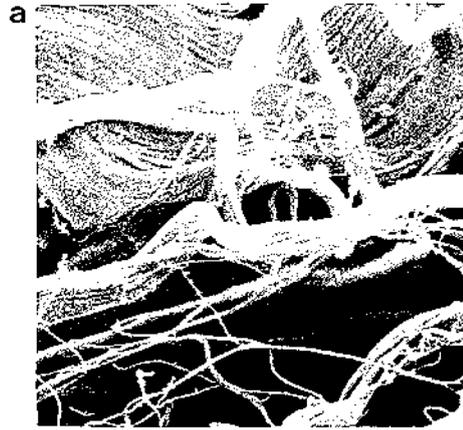


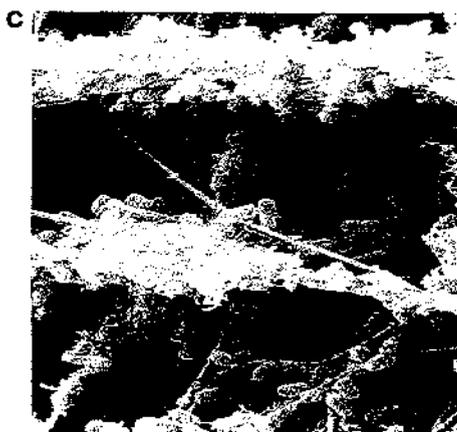
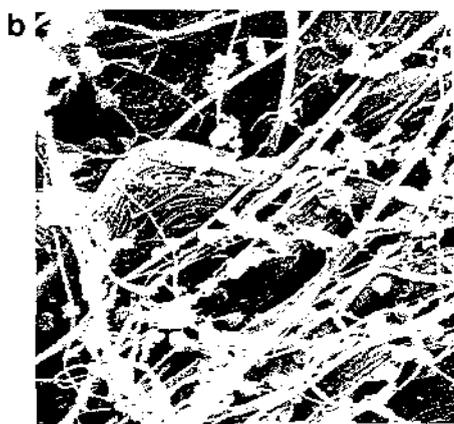
FIGURE 6: SEM's showing the effect of hyaluronic acid on attachment of *E. coli* C1212-77 to chicken muscle fasciae.

- (a) Cells incubated in a 0.1% (w/v) solution of hyaluronic acid. Attachment was completely inhibited.
Magnification = x 4000

- (b) Cells incubated in a 0.01% (w/v) solution of hyaluronic acid. Attachment was partially inhibited.
Magnification = x 4000

- (c) Cells incubated in the absence of hyaluronic acid.
Magnification = x 4000

- (d) Cells incubated in a 0.1% (w/v) solution of hyaluronic acid, then treated with hyaluronidase. Attachment was partially restored.
Magnification = x 4000



Concentrations of 0.1mg hyaluronic acid/ml suspension achieved only partial inhibition of attachment (Figs. 5b, 6b). However, when hyaluronic acid treated suspensions were incubated with hyaluronidase prior to addition of muscle tissue the ability of *E. coli* C1212-77, *S. agona*, and *S. singapore* to adhere to muscle fasciae, was restored (Figs. 5d, 6d).

The component disaccharides of hyaluronic acid and chondroitin-sulphate, glucosamine and glucuronic acid, partially inhibited attachment of all three strains to collagen (Table 3) although none of the other sugars tested inhibited attachment to collagen.

Table 4 summarises the results of experiments which examined the effect of tissue washings on attachment of *E. coli* C1212-77, *S. agona* and *S. singapore* to collagen fibres. When spin-chiller water was used as a suspension fluid, attachment of these cultures was partially inhibited (Fig. 7a). However, when dialysed water extracts of poultry tissue were used as a suspending fluid, all strains attached as well as control treatments (Fig. 7b). Dialysed saline extracts of poultry tissues appeared to enhance adhesion of these organisms compared with a distilled water control (Figs. 7c, d).

4.4 Transmission Electron Microscopy of Bacteria Attached to Chicken Muscle Fasciae

The presence of surface-associated, negatively-charged material in samples of *E. coli* C1212-77, *S. agona*, or *S. singapore* attached to poultry muscle fasciae was detected by treatment of the samples with poly-cationized ferritin. Sectioned tissue from control samples of water-swollen chicken tissue indicated ferritin was bound to the surface of individual collagen fibres (Fig. 8a).

TABLE 4: Effect of dialysed muscle tissue extracts on attachment of bacteria to collagen fibres of muscle fascie.

TABLE 4:

Extract	Attachment of		
	<i>E. coli</i> C1212-77	<i>S. singapore</i>	<i>S. agona</i>
Distilled water control	+	+	+
Spin-chiller water	+/-	+/-	+/-
Water extract	+	+	+
Saline extract	+	+	+

+ attachment

+/- partial attachment

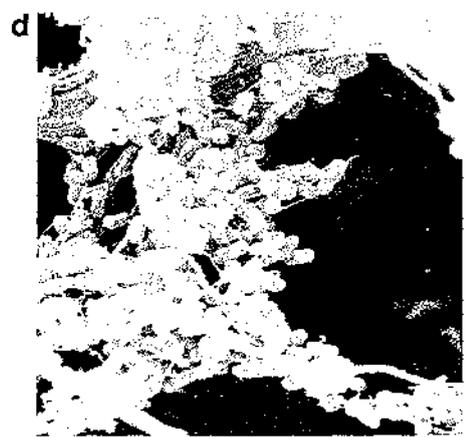
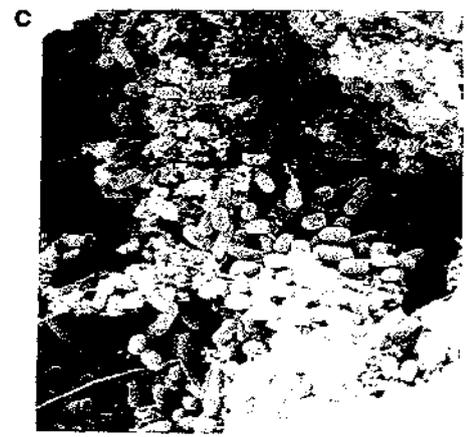
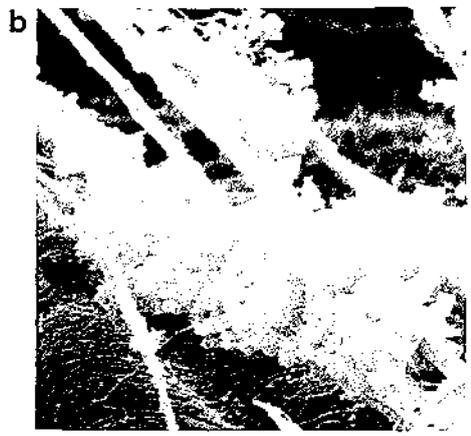
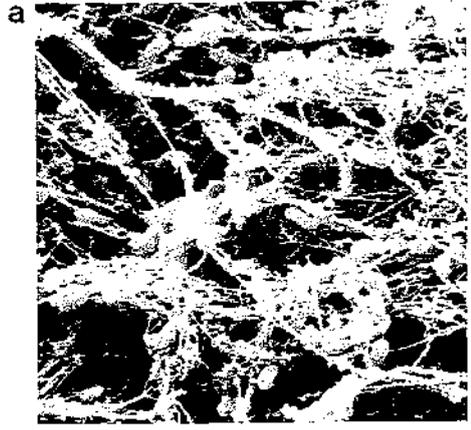
FIGURE 7: SEM of *S. singapore* cells attached to chicken muscle fascie following incubation in tissue washings.

- (a) Cells incubated in spin-chiller water.
Magnification = x 4000

- (b) Cells incubated in dialysed water extract of poultry tissue.
Magnification = x 4000

- (c) Cells incubated in dialysed saline extract of poultry tissue.
Magnification = x 4000

- (d) Cells incubated in distilled water.
Magnification = x 4000



However, suspensions of test bacteria not exposed to poultry tissue did not bind poly-cationized ferritin (Figs. 9a; 12a), although *E. coli* C1212-77 attached to chicken collagen fibres revealed a dense accumulation of ferritin-labelled material around the periphery of the cells, and in places adjoining the cells to adjacent collagen fibres (Figs. 13a, b). Similarly treated samples of *S. singapore* and *S. agona* attached to muscle fascie bound much less ferritin around the exterior of the cells and virtually no labelled material was found between bacterial cells and associated collagen fibres (Fig. 10a; 11a).

Ruthenium red was used to stain negatively-charged, carbohydrate-containing material associated with poultry tissue and attached bacteria (*E. coli* C1212-77, *S. agona*, *S. singapore*) (Luft, 1971). Control suspensions of bacteria did not bind any ruthenium red (Figs. 9b; 12b) however sections of water-swollen chicken muscle fascie showed ruthenium red binding material surrounding individual collagen fibres (Fig. 8b). TEM examination of sections of *E. coli* C1212-77 attached to poultry muscle fascie showed ruthenium red stained material surrounding the bacterial cells and in places linking cells to nearby collagen fibres (Fig. 13c). This layer of ruthenium red binding material was not as extensive as that noted for similar samples treated with poly-cationized ferritin. Sections of *S. agona* and *S. singapore* attached to poultry muscle fascie showed no ruthenium red binding material associated with the bacteria (Figs. 10b; 11b).

4.5 Adsorption of Hyaluronic Acid by Bacteria

Thin sections of *E. coli* C1212-77 and *S. singapore* incubated in the presence of hyaluronic acid were examined by TEM. Samples were treated with either ruthenium red or poly-cationized ferritin to locate extra-cellular, negatively-charged material. Suspensions of *E. coli* C1212-77 exposed to

FIGURE 8: TEM of muscle fasciae immersed in sterile water for 30 minutes.

(a) Tissue treated with poly-cationized ferritin. Note the ferritin-binding charged material surrounding the collagen fibres.
Magnification = x 64 100

(b) Tissue treated with ruthenium red. Note negatively-charged material between the collagen fibres which has bound this stain.
Magnification = x 64 100

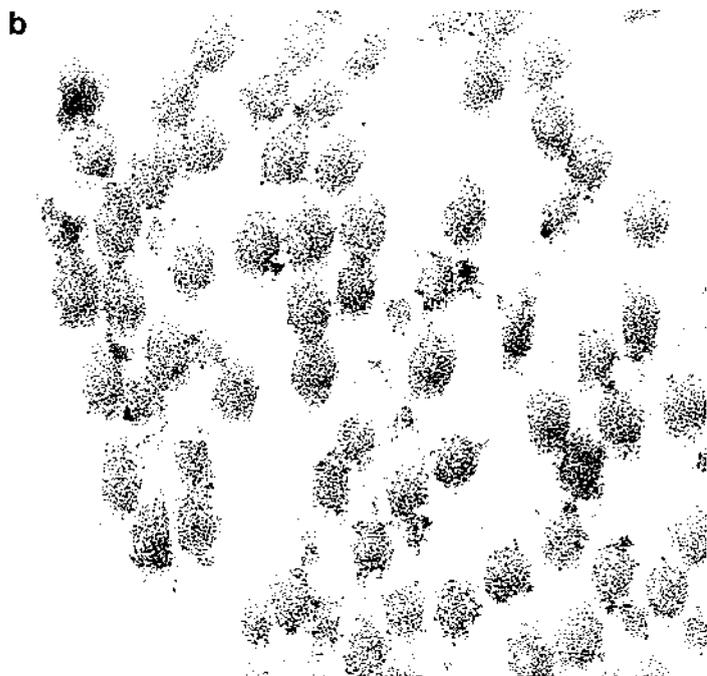
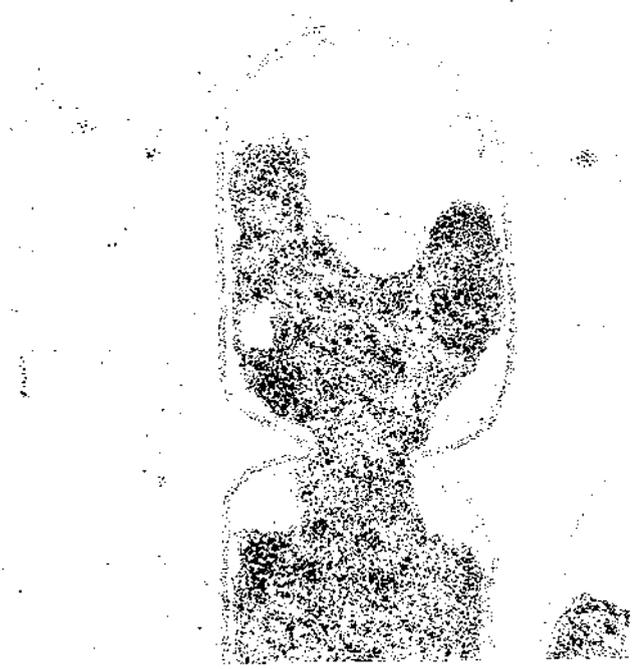


FIGURE 9: TEM of control preparations of *S. singapore* cells.

(a) Bacteria treated with poly-cationized ferritin.
No ferritin label has been bound.
Magnification = x 64 100

(b) Bacteria treated with ruthenium red. No
ruthenium red has been bound by these cells.
Magnification = x 64 100

a



b

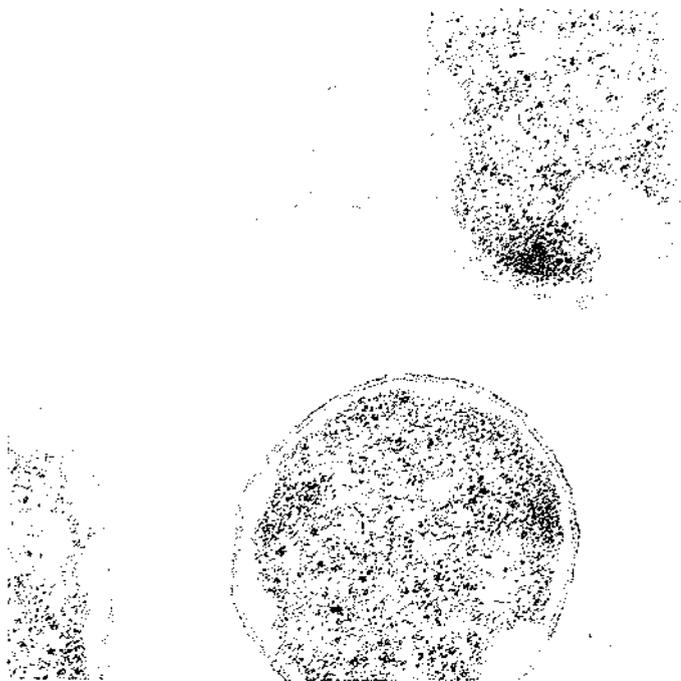


FIGURE 10: TEM of *S. singapore* cells attached to chicken muscle fascie.

(a) Samples treated with poly-cationized ferritin. Note the ferritin has been bound by material surrounding the collagen fibres and by a thin layer of material around the periphery of the bacterial cell (arrows).
Magnification = x 64 100

(b) Samples treated with ruthenium red. There is no significant binding of this stain by attached cells.
Magnification = x 64 100

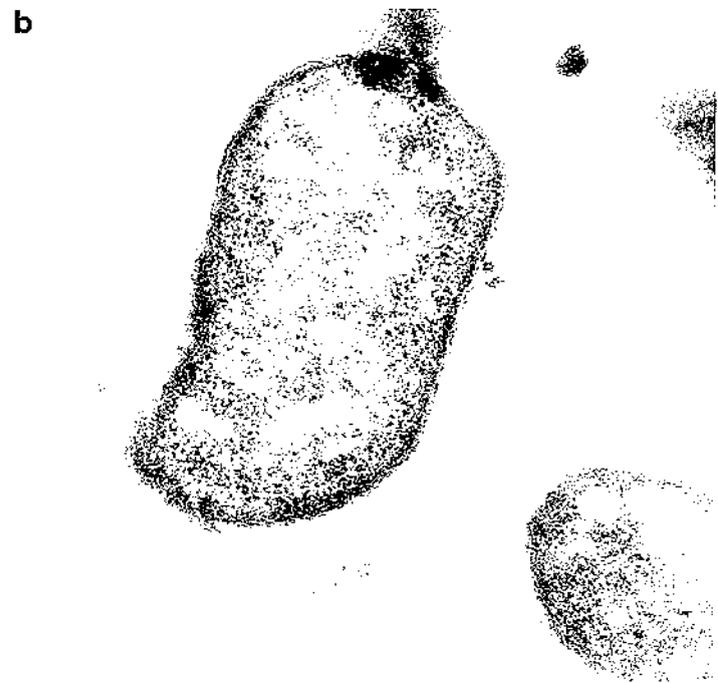
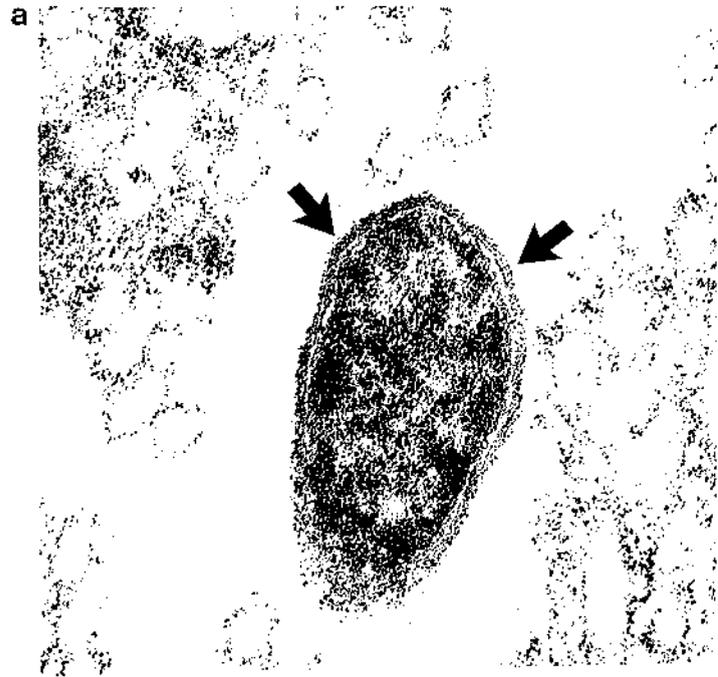


FIGURE 11: TEM of *S. agona* cells attached to chicken muscle fasciae.

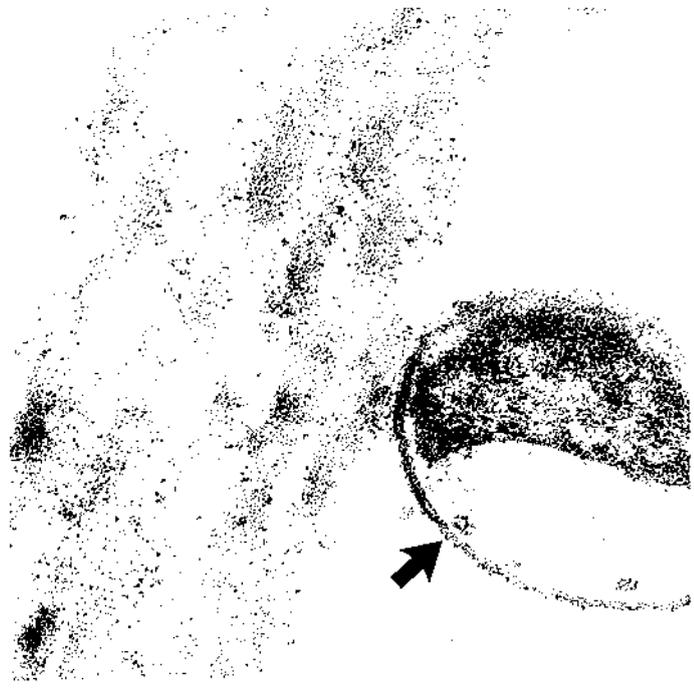
(a) Samples treated with poly-cationized ferritin. Ferritin has been bound by material surrounding the collagen fibres and by a thin layer of material on the periphery of the cell (arrows). Note the close association of bacterial cell and collagen.

Magnification = x 64 100

(b) Samples treated with ruthenium red. There is no significant binding of this stain by attached cells.

Magnification = x 35 700

a



b

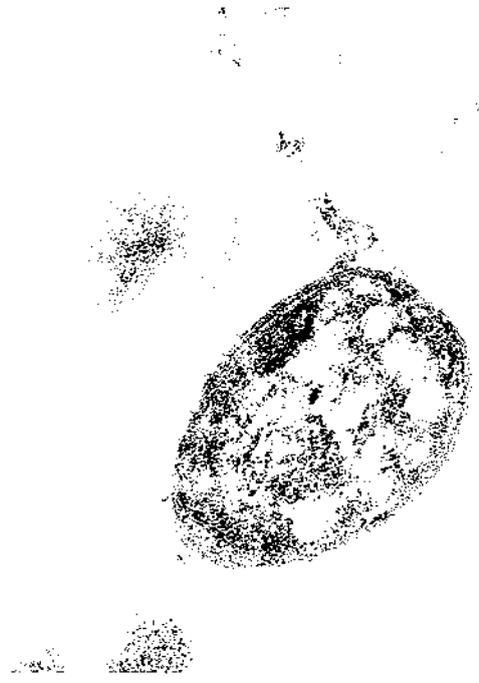


FIGURE 12: TEM of control preparations of *E. coli*
C1212-77 cells.

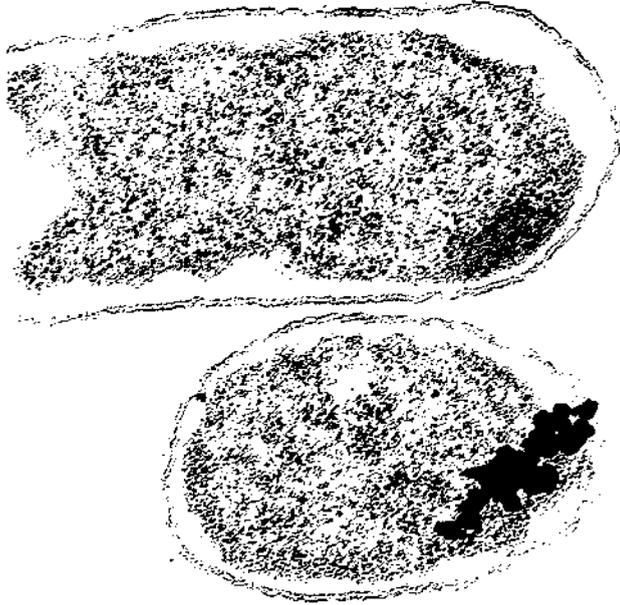
(a) Bacteria treated with poly-cationized ferritin.
Note that no ferritin label is bound by these
bacteria.

Magnification = x 64 100

(b) Bacteria treated with ruthenium red. No
ruthenium red is bound by these cells.

Magnification = x 64 100

a



b

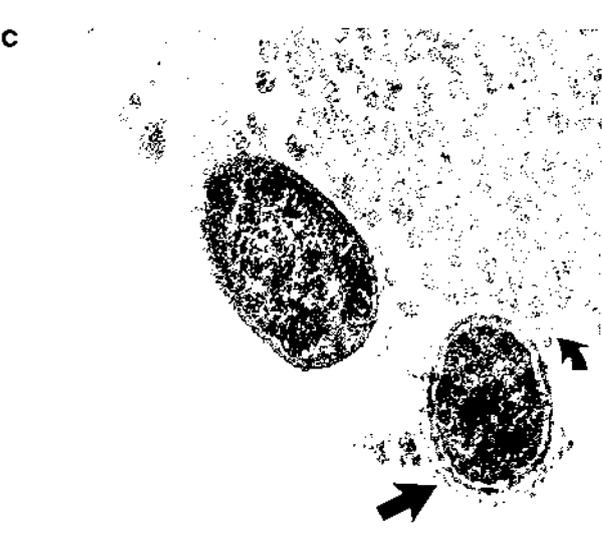
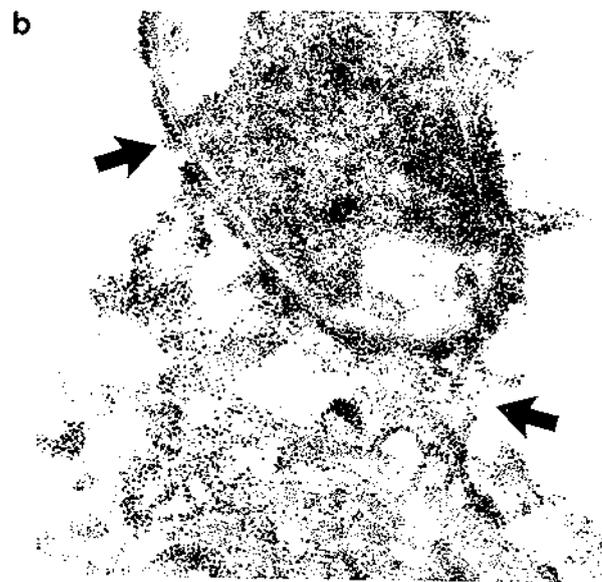
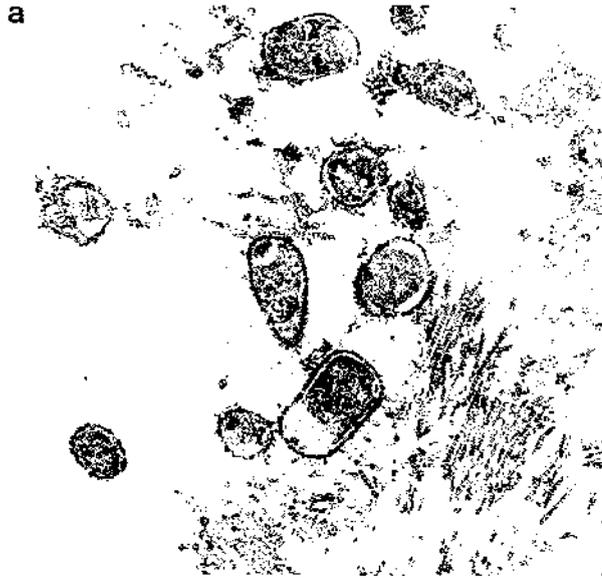


FIGURE 13: TEM of *E. coli* C1212-77 cells attached to chicken muscle fasciae.

(a) Samples treated with poly-cationized ferritin. Note that all attached cells are surrounded by a thick layer of ferritin-binding material. Not all cells are intimately associated with the collagen fibres, however.
Magnification = x 13 600

(b) Higher magnification of cells treated with poly-cationized ferritin. Note the layer of ferritin-binding material surrounding the cell (arrows). Stained material connecting the cell and collagen fibres is also visible.
Magnification = x 64 100

(c) Sample treated with ruthenium red. Note the condensed layer of stained material surrounding the cells and bridging between cells and collagen fibres (arrows).
Magnification = x 35 700



hyaluronic acid showed a dense accumulation of ferritin-binding material around the periphery of the cells (Fig. 14a). Similarly treated preparations of *S. singapore* did not bind any ferritin label (Fig. 15a) neither did control preparations of *E. coli* C1212-77 and *S. singapore* not exposed to hyaluronic acid (Figs. 9a; 12a).

TEM examination of sections of hyaluronic acid treated *E. coli* C1212-77 suspensions stained with ruthenium red, showed substantial aggregates of ruthenium red binding material around the periphery of the cells (Fig. 14b). This material was not as extensive as the ferritin-binding complex mentioned, and appeared to be condensed in localised sites around the cell surface. Ruthenium red stained samples of *S. singapore* exposed to hyaluronic acid did not contain extra-cellular ruthenium red-binding material (Fig. 15b) neither did control suspensions of *E. coli* C1212-77 and *S. singapore* not exposed to hyaluronic acid (Figs. 9b; 12b).

In vitro, addition of ruthenium red to solutions of hyaluronic acid caused aggregates of ruthenium red binding material to form. Addition of poly-cationized ferritin to solutions of hyaluronic acid did not produce aggregates of ferritin-binding material. Auto-agglutination controls of ruthenium red or ferritin mixed with water were negative.

FIGURE 14: TEM of *E. coli* C1212-77 cells incubated in an aqueous solution of hyaluronic acid (0.1% (w/v)).

(a) Sample treated with poly-cationized ferritin. Note that all cells are surrounded by a thick layer of ferritin-binding material (arrows). Magnification = x 64 100

(b) Sample treated with ruthenium red. Note the aggregates of stained material around the periphery of the bacterial cell. (arrows). Magnification = x 64 100

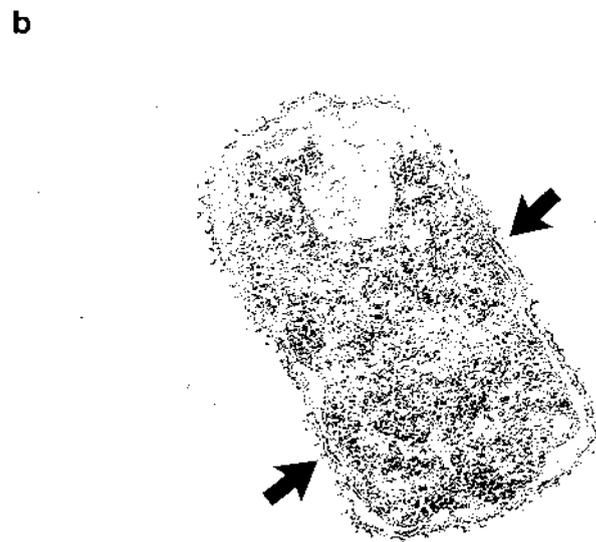
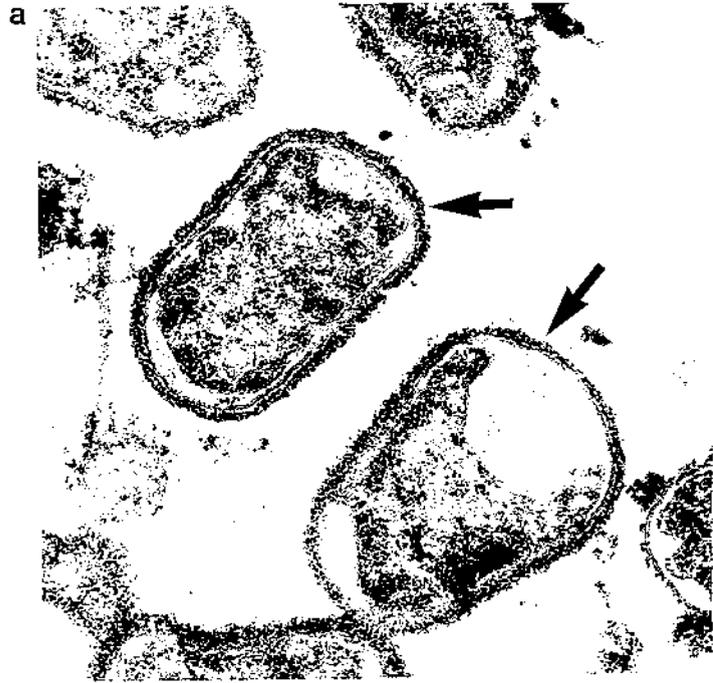
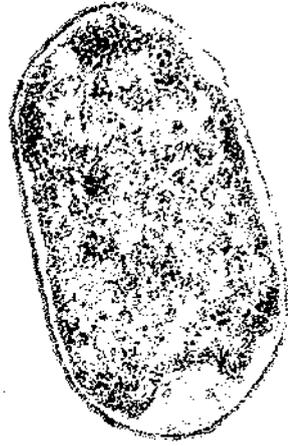


FIGURE 15: TEM of *S. singapore* cells incubated in an aqueous solution of hyaluronic acid (0.1% (w/v)).

(a) Sample treated with poly-cationized ferritin. Note that no ferritin has been bound by the cell surface, indicating lack of adsorption of hyaluronic acid.
Magnification = x 64 100

(b) Sample treated with ruthenium red.
Magnification = x 64 100

a



b



DISCUSSION

During processing poultry carcasses are exposed to large volumes of water, some of which is absorbed into the tissues. Some of this water, especially that in the scald- and chill tanks, may have a high bacterial load and some of these organisms may become trapped, attached or adsorbed onto the poultry tissues (Mulder *et al*, 1978; Thomas and McMeekin, 1980). Of these organisms the major health risk may be presented by those adsorbed onto the tissue and held in the water-film surrounding the carcass. These organisms can easily contaminate working surfaces and utensils which may in turn lead to the contamination of other foodstuffs and result in an outbreak of enteric disease. Although firmly attached and trapped micro-organisms are less likely to cause this type of contamination (Notermans *et al*, 1975b) they may be somewhat protected from the effects of heat and survive if cooking time and temperature are inadequate (Firstenberg-Eden, 1981). Apart from hygiene aspects the significance of attached bacteria lies also in the microbiological assessment of carcass quality. If carcasses are sampled by non-destructive techniques such as swabbing and rinsing of tissue, then a proportion of the bacterial population may remain undetected. Destructive tissue maceration techniques such as blending and stomaching however, will release nearly all trapped and attached micro-organisms from the tissue so a more realistic estimate of the microbiological population may be obtained (Thomas and McMeekin, 1980).

Results presented in this thesis are, at best, qualitative due to the lack of a suitable attachment assay system. During preliminary experiments various methods for assessing the attachment of bacteria to chicken muscle fasciae were investigated. Collagen fibres teased from treated tissue were examined by light microscopy, but attached bacteria were difficult to differentiate from connective tissue due to the thickness of the tissue preparation and lack of a suitable specific stain. Similar problems impaired the use of fluorescent antibody labelling techniques.

Spectrophotometric measurement of changes in the suspension fluid optical density following introduction of tissue was also unsuccessful as only a small proportion of the bacteria present adhered to the fasciae. Enumeration of bacterial suspension density before and after exposure to fasciae was impaired by a similar lack of sensitivity. Consequently SEM procedures used by Thomas and McMeekin (1981) were used for routine assessment of attachment, despite the extensive preparation necessary and the restriction on the surface area that could be examined. Hence, although the presence of attached bacteria on muscle fasciae can be reported with some confidence using this method, the absence of attachment is less certain, due to inherent variability of samples. Extensive repetition of experiments was used to confirm results and overcome this problem.

Previous studies of attachment of bacteria to poultry tissues (Notermans and Kampelmacher, 1974; 1975b; Firstenberg-Eden *et al*, 1978; Notermans *et al*, 1980) have concluded from cultural experiments that a variety of bacterial types are capable of adhering to chicken skin, muscle and muscle fasciae. However, none of these studies presented any direct evidence to substantiate these claims. In fact, the only clear case of attachment of bacteria to meat tissues so far reported is that of Thomas and McMeekin (1981).. This report demonstrated salmonellae could adhere to collagen fibres of meat connective tissue (fasciae) only under specific conditions. The two strains of *Salmonella* used only attached when muscle pieces and bacteria were immersed in media of low ionic strength and substantial water-induced changes in tissue microtopography had occurred. Results presented in this thesis substantially support these findings. All strains of *Salmonella* tested were found to adhere to poultry muscle fasciae, as did three fimbriate strains of *E. coli* and one strain of *Campylobacter coli*. Other types of micro-organisms which did not adhere to collagen included several non-fimbriate strains of *E. coli*, *Staph. aureus*, and *Campylobacter jejuni*. Unpublished studies (personal communication, Dr C.J. Thomas) have also demonstrated

strains of *Alteromonas putrefasciens*, *Pseudomonas* species, *Proteus* species, and *Micrococcus* species will not adhere under conditions necessary for attachment of adherent microorganisms.

Despite the obvious cultural and antigenic differences between *E. coli* and *Salmonella* species, the mechanism of attachment of adherent strains of these bacteria to muscle collagen is remarkably similar in several important aspects. Both types of bacteria:

- (1) adhere to the muscle fascie only when suspended in media of low ionic strength
- (2) do not attach to fascie when suspended in saline
- (3) detach from fascie when rinsed in saline media

Nevertheless, aspects of adhesion of these bacteria to other substrates are quite dissimilar. The *E. coli* strains are for instance, fimbriate, agglutinate red blood cells, and will attach well to cultured cell substrates. Adherent strains of *Salmonella* used possessed none of these features commonly associated with adhesiveness. However, since only fimbriate strains of *E. coli* adhered to collagen fibres of meat tissue it seems likely that these structures must be implicated as adhesive factors for the collagen system. Therefore, at the molecular level, the *E. coli* and the *Salmonella* strains used must attach to collagen fibres in quite different ways. The role of fimbriae in attachment does however, need to be confirmed. This could be achieved by demonstrating inhibition of attachment of bacteria to fascie previously exposed to a cell-free extract of type I fimbriae. Removal of fimbriae from bacteria should also inhibit the attachment of these cells to collagen fibres.

If, as is suggested, *E. coli* and *Salmonella* attach in different ways, then the common effects of salts on attachment could possibly occur as a result of one of two mechanisms:

- (1) salt may physically or chemically alter the structure and function of receptor sites for bacterial ligands
- (2) salt may effect removal of some labile component of the adhesive system from the tissue.

The first possibility does not seem likely since a variety of adhesive systems reported for *E. coli* and *Salmonella* operate well under physiological conditions (Duguid *et al.*, 1966; Ofek *et al.*, 1977). The second concept however, is more attractive and is supported to some extent by experimental data. In addition the concept would not preclude attachment by either *E. coli* or *Salmonella* to some component associated with collagen under either high or low ionic conditions. It is therefore proposed salt acts by loosening the bond between the bacterial cell-receptor complex and the collagen.

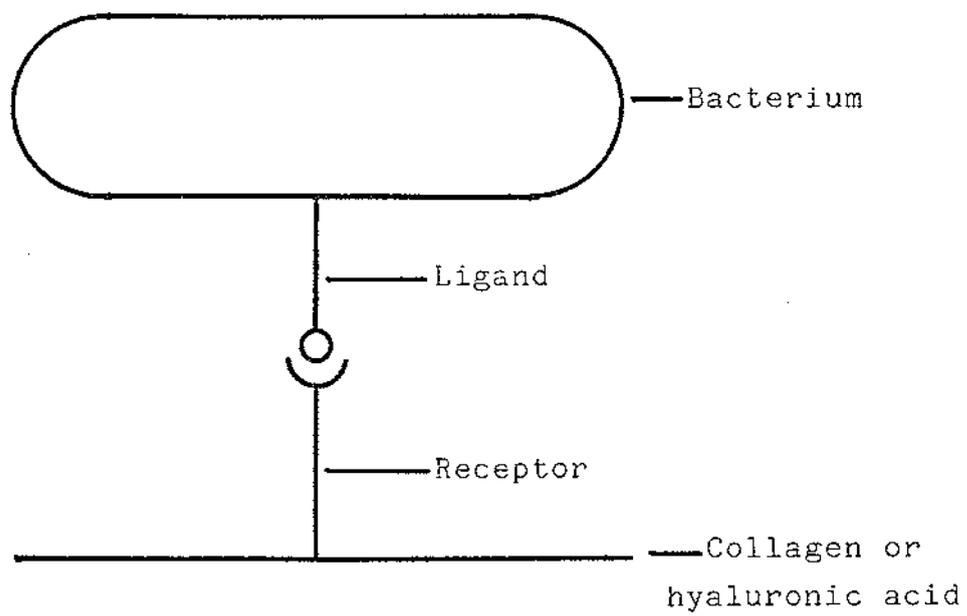
There is a deal of evidence available to support the concept that receptor sites for the adhesion process are located on collagen. The fact that water-induced changes in tissue micro-topography are necessary for attachment implies the receptors are associated with the collagen fibres. In addition, electron microscopic evidence presented suggests material from the inter-collagen fibre matrix is responsible for adhesion of bacteria to the collagen (Figs. 10a; 11a; 13a, b). Since the process of water absorption by connective tissue aids localized dispersion of this highly charged material from the matrix, it also seems plausible to suggest that this material is only loosely associated with the collagen. Since this material binds ruthenium red and poly-cationized ferritin, it may be polysaccharide in nature. This is supported by unpublished data (personal communication, Dr C.J. Thomas) which has shown CPC (a quaternary ammonium compound which selectively precipitates acid mucopolysaccharides (Scott, 1955)) will inhibit attachment of salmonellae to water-treated connective tissue. Since mucopolysaccharides are more readily eluted from tissue by salt solutions than by

water alone (Bayliss, 1982), the nature of the adhesive material and its loose association with collagen, may explain the fact that bacteria detach from collagen when rinsed with physiological saline. Consequently the acidic polysaccharide which is apparently responsible for attachment of bacteria to the collagen, may represent a labile receptor component associated with collagen.

One possible simple model of the mechanism of attachment is depicted in Fig. 16. The model is one based on those presented by Ofek and Beachey (1980). In essence, bacterial surface associated ligands enable coupling of the cells to complementary receptor sites associated with the collagen. While a deal of evidence is available to support the existence of a polysaccharide receptor, data concerning the nature of the ligand is confusing. Pre-treatment of both *E. coli* and *Salmonella* strains with lipase, trypsin and hyaluronidase failed to inhibit attachment, suggesting the ligand was not lipid, proteinaceous or polysaccharide. Furthermore, periodate treatments did not affect attachment of *Salmonella* although *E. coli* was completely inhibited indicating that some carbohydrate material may be implicated as a component of the ligand for *E. coli*. However, the involvement of a proteinaceous ligand cannot be discounted since many fimbrial ligands of *E. coli* are trypsin resistant (Jones and Isaacson, 1983) and require treatment with staphylococcal protease V8 before proteinaceous materials can be removed. Furthermore, only a restricted range of enzymes were used. Other experiments which examined the effect of various sugars (mannose, glucose, fructose, galactose) on attachment indicated that neither the ligand nor the receptor were analagous to any of these sugars. Obviously, further work is necessary in order to establish the nature of the ligands of *E. coli* and *Salmonella*. This could include an examination of the effects of a wider range of enzymes and sugars, as well as the role of sugar specific lectins on attachment of bacteria to muscle fascie. Inhibition of attachment by any of these treatments would provide information about the nature of the ligands.

FIGURE 16: Proposed model for the mechanism of adhesion of bacteria to poultry collagen fibres.

FIG 16:



The receptor site for the bacterial ligand may be part of one of a number of tissue polysaccharides associated with collagenous material. These include hyaluronic acid, chondroitin-sulphates, keratin sulphate, and heparin sulphate. Of these, hyaluronic acid is the only component to remain relatively loosely bound within the inter-collagen matrix, where it is linked to a small protein molecule. The remaining polysaccharides are present as complex proteoglycans, covalently bound to cells and collagen fibres via protein linkages. This implies that hyaluronic acid should be more easily dispersed from between collagen fibres when water-induced changes occur. In addition hyaluronic acid is the major component of connective tissue that is involved with the process of water retention and absorption. Water-induced changes in connective tissue are more likely to affect the structure and location of this polysaccharide and hyaluronic acid will be eluted at lower salt concentrations than other tissue polysaccharides thus is more likely to be affected by physiological saline. On the basis of data presented it seems that hyaluronic acid is the most likely candidate of the tissue mucopolysaccharides to possess the receptor site for bacteria adhering to connective tissue.

This suggestion is supported firstly by the observation that crude extracts of hyaluronic acid prepared from chicken comb completely inhibited attachment of bacteria to muscle fasciae, and secondly, that this inhibition could be reversed by hyaluronidase. Presumably hyaluronic acid binds to bacterial cell surface ligands thereby inhibiting attachment. In addition, component disaccharides of hyaluronic acid, glucosamine and glucuronic acid, each exert a degree of inhibition of adhesion when present in the suspension fluid at concentration of 1% (w/v). This perhaps indicates that the receptor site may be partially altered on these commercially prepared disaccharides. It may however, be possible to produce an oligosaccharide receptor which inhibits attachment, by specific enzymatic digestion of hyaluronate.

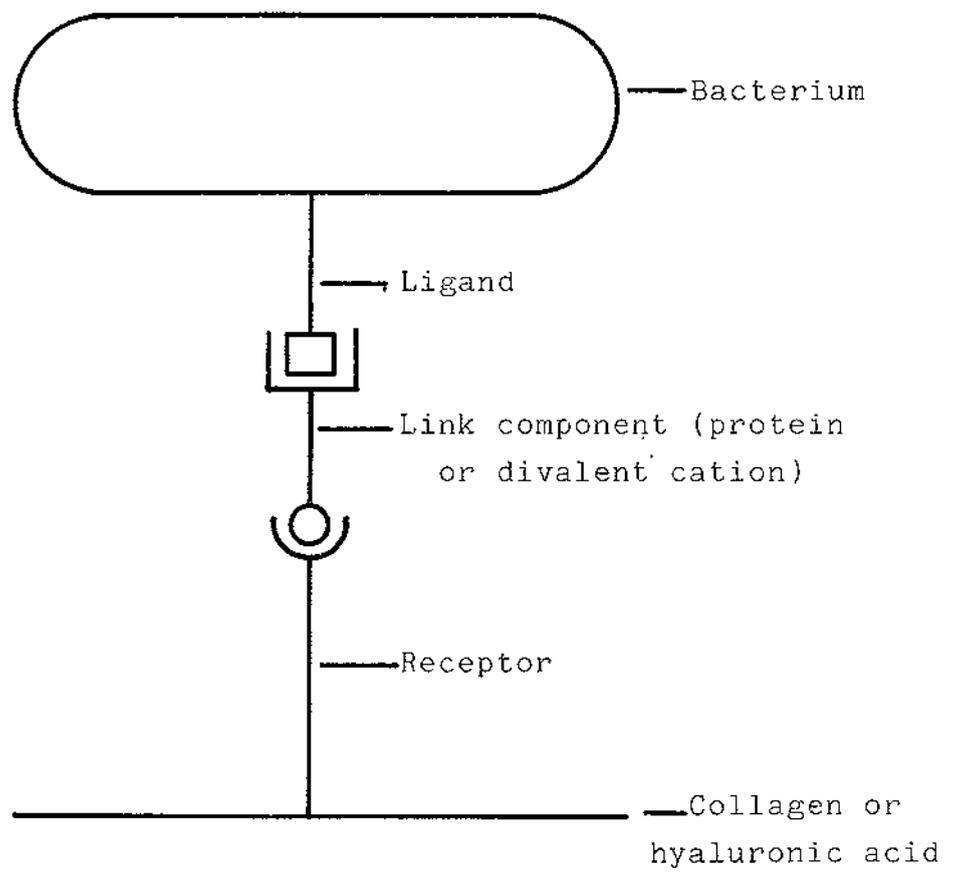
It thus seems that the tissue component responsible for the attachment of bacteria to collagen is the matrix-associated hyaluronic acid. Although this is present in only low concentrations in tissue, high concentrations (0.1% (w/v)) are required to inhibit attachment as at the surface of the fasciae the concentration of hyaluronic acid is high relative to the concentration in the surrounding aqueous medium. This is reflected in the inability of a 0.01% (w/v) suspension of hyaluronate to cause complete inhibition of attachment. However, hyaluronate is not the only tissue polysaccharide to inhibit attachment of bacteria to poultry muscle fasciae. A mixture of chondroitin-sulphate/dermatan sulphate, isolated concurrently with the hyaluronic acid, was found to inhibit attachment of *E. coli* but not *Salmonella*. This further indicates that the attachment of these organisms is mediated by different mechanisms, although chondroitin and dermatan have been shown to inhibit adhesion of other cells that bind hyaluronic acid (Underhill and Dorfman, 1978; Saunders and Miller, 1980).

If hyaluronate is indeed a key tissue associated receptor for complementary ligands associated with *Salmonella* and *E. coli*, it could be expected that these cells should bind hyaluronate *in vitro*. In fact, although *E. coli* C1212-77 bound hyaluronate, none of the salmonellae tested did so. Consequently it seems reasonable to suggest the ligands of *E. coli* will bind directly with hyaluronic acid as depicted in Fig. 16. However, adsorption of *Salmonella* species to isolated hyaluronate or collagen fibres seems somewhat more complicated. Salmonellae may require the presence of an additional factor to link the bacterial ligand to the receptor (see Fig. 17). Three possible factors may be involved and these should be investigated more closely:

- (1) Divalent cations may be necessary to link negatively-charged ligands to similarly charged receptor sites. There is some evidence to suggest this is not the case, however. Pre-treatment of fasciae with 0.01M EDTA inhibited attachment of salmonellae to muscle

FIGURE 17: Proposed model for the mechanism of adhesion of salmonella cells to poultry collagen fibres or hyaluronic acid.

FIG 17:



fasciae and this could not be reversed by addition of millimolar quantities of either Mg^{++} or Ca^{++} (unpublished data, Dr C.J. Thomas). This suggests ionic binding is not involved in linking receptor and ligand. Furthermore, other studies have shown that binding of hyaluronate to cells is independent of divalent cations (Underhill and Toole, 1979).

- (2) Small proteins attached to native hyaluronic acid may be the link compound required. Underhill and Toole (1979) found high molecular weight hyaluronic acid adhered to cells more readily than low molecular weight preparations. Hyaluronic acid used in experiments presented in this thesis was extracted by papain digestion and CPC precipitation, a procedure known to produce a low molecular weight product (Sinsberg, 1972). High molecular weight hyaluronic acid, obtained by gentle salt extraction from tissue, might therefore adsorb to *Salmonella* cells, since much of the associated protein would remain bound to the polysaccharide.
- (3) Tissue-derived proteins may bridge between ligand and receptor. Additional work required to substantiate this theory could include treatment of cell suspensions with an extract of poultry tissue. If extracts contained the necessary link compound, then adsorption of hyaluronic acid to *Salmonella* cells should be enhanced. Judicious use of enzyme treatments could then be used to characterize the nature of the link. Preliminary experiments based on this approach showed dialysed saline extracts of poultry tissue appear to enhance adhesion of bacteria to chicken collagen fibres. This may be caused by tissue proteins bridging between ligand and receptor. Although hyaluronic acid is easily extracted by salt solutions it is likely to be present in extracts in concentrations insufficient to cause inhibition of attachment.

Involvement of hyaluronic acid in the process of adhesion is not unknown. Culp (1976) tentatively identified hyaluronic acid in a material deposited by cells during growth on plastic dishes, and believed to be involved in the adhesion process. Removal of the material was accomplished by treatment with alkali or sodium dodecyl sulphate, as it was impervious to the effects of the calcium-specific chelating agent, EGTA. In addition a variety of cells produce substances, identified as hyaluronic acid, which facilitate aggregation of certain cultured cells (Pessac and Defendi, 1971). Aggregation was inhibited by treatment of cell supernatants with bovine or streptococcal hyaluronidase. Aggregation of cultured SV-3T3 cells can be inhibited by hyaluronate, chondroitin or oligosaccharides (minimum of six sugars) obtained by enzymatic digestion of hyaluronic acid, and to a lesser extent by dermatan. Treatment of cells with hyaluronate-specific enzymes inhibited attachment. SV-3T3 cells aggregated much faster in the presence of divalent cations than in their absence (Underhill and Dorfman, 1978), however the binding of hyaluronate is independent of these ions (Underhill and Toole, 1979). A mucopolysaccharide similar to hyaluronic acid has also been reported as the ligand involved in the attachment of *Actinomyces naeslundii* cells to human epithelial cells suspended in saline media (Saunders and Miller, 1980). Treatment of the bacteria with hyaluronidase inhibited attachment as did pre-treatment of epithelial cells with hyaluronic acid or chondroitin sulphate.

There are several similarities between the role of hyaluronic acid in the aforementioned adhesion systems and in the attachment of bacteria to chicken collagen fibres. These include the inhibitory effects of hyaluronidase, excess hyaluronic acid and, in some instances, chondroitin. In many cases it appears that the binding of hyaluronic acid is independent of divalent cations as is attachment of bacteria to poultry muscle fasciae (unpublished data, Dr C.J. Thomas). However, there are differences between the adhesion of *A. naeslundii* cells to epithelial cells and the attachment

of bacteria to chicken collagen fibres. The former occurs when cells are suspended in saline, presumably because the hyaluronate-based ligand is firmly bound to the cell wall and is resistant to the eluting effects of saline, unlike the loose association of hyaluronic acid with collagen fibres. Furthermore, adhesion of *A. naeslundii* cells is inhibited by galactose, a sugar which has no effect on the attachment of bacteria to muscle fasciae.

From TEM studies it was implied that the negatively-charged material surrounding attached bacteria was not derived from the cells but from the poultry tissue. However, in some attachment systems polymers produced by bacteria may be responsible for adhesion. Two independent studies on the attachment of *Pseudomonas fragi* to beef surfaces have reported the presence of extra-cellular fibrils apparently mediating adhesion of the bacteria to the meat surface. These fibrils were seen after incubation for four hours at 7C (Schwach and Zottola, 1982), and six days at 4C (Yada and Skura, 1982). However, bacteria that attach to chicken muscle fasciae do so after only brief exposure to the meat surface; attachment fibrils are not seen on bacteria prior to this exposure, and it is unlikely that they would be formed during the short time cells are exposed to meat. In other attachment systems the glycocalyx has been found to assist adhesion of bacteria to substrates (Fletcher and Floodgate, 1973; Costerton *et al*, 1978; Fletcher, 1979; Costerton *et al*, 1981) but Pringle *et al* (1983) and Brown *et al* (1977) found that glycocalyx-enclosed cells adhered less well than naked cells. The inhibitory effect of a glycocalyx may indicate that the bacterial ligand is being physically obscured from the receptor.

The attachment of *E. coli* cells to poultry muscle fasciae is almost certainly mediated by a fimbrial ligand as only fimbriate strains adhere to collagen fibres. However, the role of fimbriae in the attachment of *Salmonella* to poultry muscle fasciae is not so apparent since all adhesive strains of *Salmonella* tested were non-fimbriate.

Attempts to induce fimbriae in some of these strains by cultural means (Old and Duguid, 1970) were not successful. Consequently, the role of fimbriae in adhesion of *Salmonella* to poultry muscle fasciae has yet to be determined.

Ruthenium red has been used for some time in electron microscopy as a specific stain for acid polysaccharides (Fletcher and Floodgate, 1973; Fletcher, 1979; Costerton *et al*, 1981). Staining of hyaluronic acid with ruthenium red was established by Luft in 1971. Further reports indicated that acidic polysaccharides of low charge density such as hyaluronic acid, give only an intermediate reaction with ruthenium red (Rambourg, 1971). In a recent review, however, Sutherland (1983) claimed hyaluronic acid did not stain with ruthenium red. Nevertheless, experimental work presented in this thesis shows addition of ruthenium red caused precipitation and aggregation of commercial (Sigma), and crude extracts of comb hyaluronic acid, indicating binding of ruthenium red by this polysaccharide. This *in vitro* reaction is also reflected in the TEM studies presented. Ruthenium red aggregated and condensed the layer of hyaluronic acid adsorbed by *E. coli* cells (Fig. 14b).

As mentioned previously the studies presented in this thesis were hampered by the lack of a suitable, quantitative assay system. However, a relatively simple and effective assay system has been developed to assess the attachment of *Acinetobacter* cells to purified collagen fibres (Monboisse *et al*, 1979). Addition of dried collagen to a suspension of *Acinetobacter* caused rapid clearing of the suspension. The extent of attachment in this case could be detected by changes in optical density following filtration of the suspension to remove collagen fibres. It may be possible to adapt this system to assess attachment of *Salmonella* and *E. coli* cells. However, it must be remembered that purified collagen may bear little resemblance to the collagen fibres of water-treated muscle fasciae. If this is the case it may be possible to substitute homogenized tissue rich in glycosaminoglycans (eg. tendon or chicken comb) for collagen.

The findings presented in this thesis may have relevance not only to the meat processing industry, but also to other industries utilising animal tissues rich in collagen eg. leather, tripe, and sausage casings. Manufacture of leather from animal hides involves prolonged and repeated immersion of the hides in various aqueous solutions to permit swelling of the tissues (Thorstensen, 1969). Initially the hide may be contaminated with a variety of saprophytic and pathogenic micro-organisms. Depending on the hygiene of the slaughterhouse, cross-contaminants from the intestinal contents, including *Salmonella*, may also be present. Curing of the hide in salt may eliminate many of these bacteria although some pathogens have a degree of resistance to salt. Subsequent soaking utilises salts and disinfectants to reduce the bacterial population although *Bacillus anthracis*, *Pseudomonas aeruginosa*, and *Staph. aureus* may survive. The liming process uses a pH of 12-13, ensuring only spore forming organisms survive. Any subsequent bacterial growth usually occurs as a result of contamination by the environmental flora and not those derived from the hide. The tanning process, usually spread over three weeks, uses warm aqueous solutions at pH3 which effectively inhibit the development of most micro-organisms (Carre *et al*, 1983). Opportunity for *Salmonella* and *E. coli* to adhere to the collagen fibres of leather would therefore be limited by the chemical severity of the processing treatments.

Other collagenous animal by-products involving water immersion processing include tripe and sausage casings. The rumen and reticulum of cattle are marketed as tripe. These tissues are washed, soaked in hot lime water, and scraped to remove the inside mucosal wall and then cooked before they leave the packing house (Levie, 1979). Consequently there is a possibility *Salmonella* and *E. coli* may adhere to the cooked tripe if processing hygiene standards are inadequate, particularly at the post-processing stages of packaging. Furthermore, since most micro-organisms will have been eliminated by the cooking process, contaminants like *Salmonella* will be able to grow without competition from other bacteria.

Sausage casings are manufactured from intestines of sheep and pigs, or from reconstituted collagen. The manufacture of reconstituted collagen involves swelling of the fibres, drying, curing and covering with a hydrophilic coating (Karmas, 1974). It is possible that the processing of collagen renders it unsuitable for bacterial adhesion, however if this is not the case a potentially dangerous situation exists as organisms from the sausage meat or the environment may adhere to the casing.

The mechanism by which *Salmonella* invades the epithelium of the intestine is unknown. However, it is known that the mucous coat covering the intestinal epithelia contains low levels of uronic acids (Glass, 1965) and hexosamines (Glass, 1965; Menguy, 1965). These include both the major constituents of hyaluronic acid, glucosamine and glucuronic acid (Allen and Gardner, 1980). Assuming salmonellae do adhere to one or both of these components, if the necessary link compound is present, salmonellae may establish themselves in the intestine by adhering to the mucous coat as a prelude to infection.

B I B L I O G R A P H Y

ADAMS, B.W., MEAD, G.C., and PENNINGTON, D.E. (1980). A scrape-sampling device for the microbiological examination of poultry carcasses. *British Poultry Science* 21: 71-75.

ALLEN, A. and GARNER, A. (1980). Mucus and bicarbonate secretion in the stomach and their possible role in mucosal protection. *Gut* 21: 249-262.

AVENS, J.S., and MILLER, B.F. (1970). Quantifying bacteria on poultry carcass skin. *Poultry Science* 49: 1309-1325.

BAYLISS, M.T. (1982). Isolation and characterization of proteoglycans. In *Physiological and Pathological Processes of Body Systems*, Karger, Basel, Editors Varma, R.S. and Varma, R., pp5-19.

BHATIA, T.R.S., McNABB, G.D., WYMAN, H., and NAYAR, G.P.S. (1979). Salmonella isolation from litter as an indicator of flock infection and carcass contamination. *Avian Diseases* 24: 838-847.

BLANKENSHIP, L.C., COX, N.A., CRAVEN, S.E., and RICHARDSON, R.L. (1975). Total rinse method for microbiological sampling of the internal cavity of eviscerated broiler carcasses. *Applied Microbiology* 30: 290-292.

BLANKENSHIP, L.C., and COX, N.A. (1976). Modified water rinse sampling for sensitive, non-adulterating salmonellae detection on eviscerated broiler carcasses. *Journal of Milk and Food Technology* 39: 680-681.

BROWN, C.M., ELLWOOD, D.C., and HUNTER, J.R. (1977). Growth of bacteria at surfaces: influence of nutrient limitation. *FEMS Microbiology Letters* 1: 163-166

BURROWS, M.R., SELLWOOD, R., and GIBBONS, R.A. (1976) Haemagglutinating and adhesive properties associated with the K99 antigen of bovine strains of *Escherichia coli*. *Journal of General Microbiology* 96: 269-275.

BUSTA, F.F. (1976). Practical implications of injured micro-organisms in food. *Journal of Milk and Food Technology* 39: 138-145.

BUTLER, J.L., STEWART, J.C., VANDERZANT, C., CARPENTER, Z.L., and SMITH, G.C. (1979). Attachment of micro-organisms to pork skin and surfaces of beef and lamb carcasses. *Journal of Food Protection* 42: 401-406.

BUTLER, J.L., VANDERZANT, C., CARPENTER, Z.L., SMITH, G.C., LEWIS, R.E., and DUSTON, T.R. (1980). Influence of certain processing steps on attachment of micro-organisms to pork skin. *Journal of Food Protection* 43: 699-705.

CARRE, M.C., VULLIERMET, A., and VULLIERMET, B. (1983). *Environment and Tannery*. Centre Technique du Cuir.

CHVAPIL, M. (1967). *Physiology of Connective Tissue*. Butterworths, London.

CORPE, W.A. (1980). Microbial surface components involved in adsorption of micro-organisms onto surfaces. In *Absorption of Micro-organisms to Surfaces*, John Wiley and Sons, New York, Editors Bitton, G., and Marshall, K.C.

COSTERTON, J.W., GEESEY G.G., and CHENG, K.J. (1981). The role of bacterial surface structures in pathogenesis. *CRC Critical Reviews in Microbiology* 8: 303-338.

COX, N.A., MERCURI, A.J., THOMSON, J.E., and CHEW, Y. (1976). Swab and excised tissue sampling for total and *Enterobacteriaceae* counts of fresh and surface-frozen broiler skin. *Poultry Science* 55: 2405-2408.

COX, N.A., THOMSON, J.E., and BAILEY, J.S. (1981). Sampling of broiler carcasses for *Salmonella* with low volume water rinse. *Poultry Science* 60: 768-770.

CULP, L.A. (1976). Electrophoretic analysis of substrate-attached proteins from normal and virus-transformed cells. *Biochemistry A.C.S.* 15: 4094-4104.

DAVIDSON, E.H. (1966). Analysis of sugars found in mucopolysaccharides. In *Methods in Enzymology VIII*, Academic Press, Editor Sinsberg, V. pp 52-60.

DOUGHERTY, T.J. (1974). *Salmonella* contamination in a commercial poultry (broiler) processing operation. *Poultry Science* 53: 814-821.

DUGUID, J.P., ANDERSON, E.S., and CAMPBELL, I. (1966). Fimbriae and adhesive properties in salmonellae. *Journal of Pathological Bacteriology* 92: 107-138.

DUGUID, J.P., and OLD, D.C. (1980). Adhesive properties of *Enterobacteriaceae*. In *Bacterial Adherence*, Chapman and Hall, Editor Beachey E.H. pp 185-217.

DUITSCHAEVER, C.L. (1977). Incidence of *Salmonella* in retailed raw cut-up chicken. *Journal of Food Protection* 40: 191-192.

ERDTSIECK, B. (1975). On the water absorption during poultry processing. *Second European Symposium on Poultry Meat*.

EVANS, D.G., EVANS Jr, D.J., and WEILIE, T. (1977). Haemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhoea: correlation with colonization factor. *Infection and Immunity* 18: 330-337.

EVANS, D.G., EVANS Jr., D.J., WEILIE, S.T., and DUPONT, H.L. (1978). Detection and characterization of colonization factor of enteropathogenic *Escherichia coli* isolated from adults with diarrhoea. *Infection and Immunity* 19: 727-736.

FIRSTENBERG-EDEN, R. (1981). Attachment of bacteria to meat surfaces: a review. *Journal of Food Protection* 44: 602-607.

FIRSTENBERG-EDEN, R., NOTERMANS, S., and VAN SCHOTHORST, M. (1978). Attachment of certain bacterial strains to chicken and beef meat. *Journal of Food Safety* 1: 217-228.

FIRSTENBERG-EDEN, R., NOTERMANS, S., THIEL, F., HENSTRA, S., and KAMPELMACHER, E.H. (1979). Scanning electron microscopic investigations into attachment of bacteria to teats of cows. *Journal of Food Protection* 42: 305-309.

FLETCHER, M. (1979). The attachment of bacteria to surfaces in aquatic environments. In *Adhesion of Micro-organisms to Surfaces*, Academic Press, London, Editors Ellwood, D.C., Melling, J., and Rutter, P. pp 87-108.

FLETCHER, M., and FLOODGATE, G.D. (1973). An electron-microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces. *Journal of General Microbiology* 74: 325-334.

FROMM, D., and MONROE, R.J. (1958). Moisture absorption and retention of freshly eviscerated broilers as influenced by holding time in slush ice. *Poultry Science* 37: 328-331.

GAASTRA, W., and DE GRAAF, F.K. (1982). Host-specific fimbrial adhesins of non-invasive enterotoxigenic *E. coli* strains. *Microbiological Reviews* 46: 129-161.

GARDNER, D.L. (1965). *Pathology of the Connective Tissue Diseases*. Edward Arnold (Publishers) Ltd., London.

GATT, R., and BERMAN, E.R. (1966). A rapid method for the estimation of amino sugars on a micro scale. *Analytical Biochemistry* 15: 167-171.

GIBBS, P.A.A, PATTERSON, J.T., and THOMPSON, J.K. (1978). The distribution of *Staphylococcus aureus* in a poultry processing plant. *Journal of Applied Bacteriology* 44: 401-410.

GLASS, G.B.J. (1965). Fractionation of gastric macromolecular materials by electrophoresis, column chromatography and gel filtration; correlative study. In *Gastric Secretion Mechanisms and Control*. Pergamon Press, Editors Shnitka, T.K., Gilbert, J.A.L., Harrison, R.C. pp 187-209.

GREEN, S.S., MORAN, A.B., JOHNSTON, R.W., UHLER, P., and CHIU, J. (1982). The incidence of *Salmonella* species and serotypes in young whole chicken carcasses in 1979 as compared with 1967. *Poultry Science* 61: 288-293.

HAGBERG, M.M., BUSTA, F.F., ZOTTOLA, E.A., and ARNOLD, E.A. (1973). Incidence of potentially pathogenic micro-organisms in further processed turkey products. *Journal of Milk and Food Technology* 36: 625-634.

HALL, H.E., and ANGELOTTI, R. (1965). *Clostridium perfringens* in meat and meat products. *Applied Microbiology* 13: 352-357.

HEATH III, J.L., DAVIS, B.H., TEEKELL, R.A., and WATTS, A.B. (1968). Water penetration of broiler carcasses. *Poultry Science* 47: 1933-1937.

HOBBS, B.C., and GILBERT, R.J. (1978). *Food Poisoning and Food Hygiene*. Edward Arnold (Publishers) Ltd.

HODGES, R.D. (1974). *The Histology of the Fowl*. Academic Press.

HOROWITZ, M.A., and GANGAROSA, E.J. (1976). Foodborne disease outbreaks traced to poultry, United States, 1966-1974. *Journal of Milk and Food Technology* 39: 859-863.

HUMPHREY, T.J. (1981). The effects of pH and levels of organic matter on the death rates of salmonellas in chicken scald-tank water. *Journal of Applied Bacteriology* 51: 27-39.

HUMPHREY, T.J., LANNING, D.G., and BERESFORD, D. (1981). The effect of pH adjustment on the microbiology of chicken scald-tank water with particular reference to the death rate of salmonellas. *Journal of Applied Bacteriology* 51: 517-527.

JONES, G.W., and RUTTER, J.M. (1972). Role of the K88 antigen in the pathogenesis of neonatal diarrhoea caused by *Escherichia coli* in piglets. *Infection and Immunity* 6: 918-927.

JONES, G.W., and RICHARDSON, L.A. (1981). The attachment to, and invasion of HeLa cells by *Salmonella typhimurium*: the contribution of mannose-sensitive and mannose-resistant haemagglutinating activities. *Journal of General Microbiology* 127: 361-370.

JONES, G.W., and ISAACSON, R.E. (1983). Proteinaceous bacterial adhesins and their receptors. *CRC Critical Reviews in Microbiology* 10: 229-260.

KARMAS, E. (1974). *Sausage Casing Technology*. Noyes Data Corporation.

KLEIN, D.A., and WU, S. (1974). Stress: a factor to be considered in heterotrophic micro-organism enumeration from aquatic environments. *Applied Microbiology* 27: 429-431.

LEVIE, A. (1979). *Meat Handbook*, Fourth edition. A.V.J. Publishing Co. Inc., Westport, Connecticut, USA.

LILLARD, H.S. (1971). Occurrence of *Clostridium perfringens* in broiler processing and further processing operations. *Journal of Food Science* 36: 1008-1010.

LINDBERG, A.A. (1982). Specificity of fimbriae and fimbrial receptors. In Microbiology 1982, American Society of Microbiology, Editor Schlessinger, D. pp 317-323.

LUFT, J.H. (1971). Ruthenium red and ruthenium violet. II Fine structural localisation in animal tissues. Anatomical Records 171: 369-416.

MARKOVITZ, A., SYDISKIS, R.J., and LIEBERMAN, M.M. (1967). Genetic and biochemical studies on mannose-negative mutants that are deficient in phosphomannose isomerase in *Escherichia coli* K12. Journal of Bacteriology 94: 1492-1496.

MARSHALL, K.C. (1972). Mechanism of adhesion of marine bacteria to surfaces. Proceedings Third International Congress on Marine Corrosion and Fouling, Gaithersburg, Maryland, USA. pp 625-632.

MARSHALL, K.C. (1976). Interfaces in Microbial Ecology. Harvard University Press.

MARSHALL, K.C., STOUT, R., and MITCHELL, R. (1971). Mechanism of the initial events in the sorption of marine bacteria to surfaces. Journal of General Microbiology 68: 337-348.

MARSHALL, K.C., and CRUICKSHANK, R.W. (1973). Cell surface hydrophobicity and the orientation of certain bacteria at interfaces. Archives of Microbiology 91: 29-40

MAY, K.N. (1974). Changes in microbial numbers during final washing and chilling of commercially slaughtered broilers. Poultry Science 53: 1282-1285.

McBRIDE, G.A., SKURA, B.J., YADA, R.J., and BOWMER, E.J. (1980). Relationship between incidence of *Salmonella* contamination among pre-scalded, eviscerated and post-chilled chickens in a poultry processing plant. Journal of Food Protection 43: 538-542.

McMEEKIN, T.A., and THOMAS, C.J. (1978). Retention of bacteria on chicken skin after immersion in bacterial suspensions. *Journal of Applied Bacteriology* 45: 383-387.

McMEEKIN, T.A., THOMAS, C.J., and McCALL, D. (1979). Scanning electron microscopy of micro-organisms on chicken skin. *Journal of Applied Bacteriology* 46: 195-200.

MEAD, G.C. (1974). Microbiology of the poultry carcass and processing plant. *Royal Society of Health Journal* 96: 164-168.

MEAD, G.C., and IMPEY, C.S. (1970). The distribution of *Clostridia* in poultry processing plants. *British Poultry Science* 11: 407-414.

MEAD, G.C., and THOMAS, N.L. (1973). The bacteriological condition of eviscerated chickens processed under controlled conditions in a spin-chilling system and sampled by two different methods. *British Poultry Science* 14: 413-419.

MENGUY, R. (1965). Regulation of gastric mucus secretion. In *Gastric Secretion - Mechanisms and Control*, Pergamon Press, Editors Shnitka, T.K., Gilbert, J.A.L., and Harrison, R.C. pp 177-185.

MONBOISSE, J.C., LABADIE, J., and GOUET, P. (1979). Attachement d'une bacterie collagenolytique a son substrat. *Annales Microbiology (Institute Pasteur)* 130A: 435-440.

MOON, H.W., NAGY, B., ISAACSON, R.E., and ØRSKOV, I. (1977). Occurrence of K99 antigen on *Escherichia coli* isolated from pigs and colonization of pig ileum by K99⁺ enterotoxigenic *E. coli* from calves and pigs. *Infection and Immunity* 15: 614-620.

MORRIS, G.K., and WELLS, J.G. (1970). *Salmonella* contamination in a poultry-processing plant. *Applied Microbiology* 19: 795-799.

- MORRIS, J.A., THORNS, C., SCOTT, A.C., SOJKA, W.J., and WELLS, G.A. (1982). Adhesion *in vitro* and *in vivo* associated with an adhesive antigen (F41) produced by a K99 mutant of the reference strain *Escherichia coli* B41. *Infection and Immunity* 36: 1146-1153.
- MULDER, R.W.A.W., DORRESTEIJN, L.W.J., and VAN DER BROEK, J. (1978). Cross-contamination during the scalding and plucking of broilers. *British Poultry Science* 19: 61-70.
- NORBERG, P. (1981). Enteropathogenic bacteria in frozen chicken. *Applied and Environmental Microbiology* 42: 32-34.
- NORRIS, J.R., and SWAIN, H. (1971). Staining bacteria. In *Methods in Microbiology* 5A, Academic Press, Editors Norris, J.R., and Ribbons, D.W. pp 105-134.
- NOTERMANS, S., and KAMPELMACHER, E.H. (1974). Attachment of some bacterial strains to the skin of broiler chickens. *British Poultry Science* 15: 573-585.
- NOTERMANS, S., and KAMPELMACHER, E.H. (1975a). Heat destruction of some bacterial strains attached to broiler skin. *British Poultry Science* 16: 351-361.
- NOTERMANS, S., and KAMPELMACHER, E.H. (1975b). Further studies on the attachment of bacteria to skin. *British Poultry Science* 16: 487-496.
- NOTERMANS, S., KAMPELMACHER, E.H., and VAN SCHOTHORST, M. (1975a). Studies on sampling methods used in the control of hygiene in poultry processing. *Journal of Applied Bacteriology* 39: 55-61.
- NOTERMANS, S., KAMPELMACHER, E.H., and VAN SCHOTHORST, M. (1975b). Studies of different sampling methods for the determination of bacterial counts from frozen broilers. *Journal of Applied Bacteriology* 39: 125-131.

- NOTERMANS, S., FIRSTENBERG-EDEN, R., and VAN SCHOTHORST, M. (1979). Attachment of bacteria to teats of cows. *Journal of Food Protection* 42: 228-232.
- NOTERMANS, S., DUFRENNE, J., and VAN SCHOTHORST, M. (1980). The effect of cultural procedures on the attachment of bacteria to chicken breast meat. *Journal of Applied Microbiology* 49: 273-279.
- NOTERMANS, S., DUFRENNE, J., and VAN LEEUWEN, W.J. (1982). Contamination of broiler chickens by *Staphylococcus aureus* during processing; incidence and origin. *Journal of Applied Bacteriology* 52: 275-280.
- NOTERMANS, S., and KAMPELMACHER, E.H. (1983). Attachment of bacteria in meat processing. *Fleischwirtschaft* 63: 72-78.
- OFEK, I., MIRELMAN, D., and SHARON, N. (1977). Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptors. *Nature* 265: 623-625.
- OFEK, I., and BEACHEY, E.H. (1978). Mannose binding and epithelial cell adherence of *Escherichia coli*. *Infection and Immunity* 22: 247-254.
- OFEK, I., and BEACHEY, E.H. (1980). General concepts and principles of bacterial adherence in animals and man. In *Bacterial Adherence*, Chapman and Hall, Editor Beachey, E.H. pp 1-29.
- OLD, D.C., and DUGUID, J.P. (1970). Selective outgrowth of fimbriate bacteria in static liquid medium. *Journal of Bacteriology* 103: 447-456.
- PARK, C.E., STANKIEWICZ, Z.K., LOVETT, J., and HUNT, J. (1981). Incidence of *Campylobacter jejuni* in fresh eviscerated whole market chickens. *Canadian Journal of Microbiology* 27: 841-842.

- PATRICK, T.E., COLLINS, J.A., and GOODWIN, T.L. (1973). Isolation of *Salmonella* from carcasses of steam and water-scalded poultry. *Journal of Milk and Food Technology* 36: 34-36.
- PATTERSON, J.T. (1971). Microbiological assessment of surfaces. *Journal of Food Technology* 6: 63-72.
- PATTERSON, J.T. (1972). Microbiological sampling of poultry carcasses. *Journal of Applied Bacteriology* 35: 569-575.
- PATTERSON, J.T. (1973). Air-borne micro-organisms in poultry processing plants. *British Poultry Science* 14: 161-165.
- PATTERSON, J.T., and STEWART, D.J. (1962). Bacteriology of processed broilers. 1. Methods of examination. The Research and Experimental Record of the Ministry of Agriculture, Northern Ireland 11: 57-63.
- PESSAC, B., and DEFENDI, V. (1971). Cell aggregation: role of acid mucopolysaccharides. *Science* 175: 898-900.
- PRINGLE, J.H., FLETCHER, M., and ELLWOOD, D.C. (1983). Selection of attachment mutants during the continuous culture of *Pseudomonas fluorescens* and relationship between attachment ability and surface composition. *Journal of General Microbiology* 129: 2557-2569.
- RAMBOURG, A. (1971). Morphological and histological aspects of glycoproteins at the surface of animal cells. *International Review of Cytology* 31: 57-114.
- RAY, B., and SPECK, M.L. (1973a). Discrepancies in the enumeration of *Escherichia coli*. *Applied Microbiology* 25: 494-498.
- RAY, B. and SPECK, M.L. (1973b). Enumeration of *E. coli* in frozen samples after recovery from injury. *Applied Microbiology* 25: 499-503.

- REID, G. (1982). The *in vitro* attachment of *Escherichia coli* to human uroepithelial cells: investigation of host and bacterial cell factors. Ph.D Thesis, Massey University, Palmerston North, New Zealand.
- ROBERTS, D. (1972). Observations on procedures for thawing and spit roasting frozen dressed chickens, and post-cooking care and storage with particular reference to food poisoning bacteria. *Journal of Hygiene, (Cambridge)* 79: 565-588.
- ROGERS, H.J. (1979): Adhesion of micro-organisms to surfaces: some general considerations of the role of the envelope. In *Adhesion of Micro-organisms to Surfaces*, Academic Press, Editors Ellwood, D.C., Melling, J., and Rutter, P. pp 29-55.
- SADLER, W.W., and CORSTVET, R.E. (1965). Second survey of market poultry for *Salmonella* infection. *Applied Microbiology* 13: 348-351.
- SANDERS, D.H. (1969). Fluorescent dye tracing of water entry and retention in chilling of broiler chicken carcasses. *Poultry Science* 48: 2032-2037.
- SAUNDERS, J.M., and MILLER, C.H. (1980). Attachment of *Actinomyces naeslundii* to human buccal epithelial cells. *Infection and Immunity* 29: 981-989.
- SCHEUSNER, D.L., BUSTA, F.F., and SPECK, M.L. (1971). Injury of bacteria by sanitizers. *Applied Microbiology* 21: 41-45.
- SCHWACH, T.S., and ZOTTOLA, E.A. (1982). Use of scanning electron microscopy to demonstrate microbial attachment to beef and beef contact surfaces. *Journal of Food Science* 47: 1401-1405.
- SCOTT, J.E. (1955). The solubility of cetylpyridinium complexes of biological polyanions in solutions of salts. *Biochemica et Biophysica Acta* 18: 428-429.

SINSBERG, V. (1972). Methods in Enzymology XXVIII, Complex Carbohydrates Part 13, Academic Press.

SPURR, A.R. (1969). A low viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructural Research 26: 31-43.

SUDERMAN, D.R., and CUNNINGHAM, F.C. (1980). Factors affecting adhesion of coating to poultry skin. Effect of age, method of chilling, and scald temperature on poultry skin ultrastructure. Journal of Food Science 45: 444-449.

SUTHERLAND, I.W. (1983). Microbial exopolysaccharides - their role in microbial adhesion in aqueous systems. CRC Critical Reviews in Microbiology 10: 173-201.

SUTHERLAND, I.W., and WILKINSON, J.F. (1965). Depolymerases for bacterial exopolysaccharides obtained from phage infected bacteria. Journal of General Microbiology 39: 373-383.

THOMAS, C.J., McMEEKIN, T.A., and BALIS, C. (1977). Retention of bacteria in liquid films at agar surfaces. Applied and Environmental Microbiology 34: 456-457.

THOMAS, C.J., and McMEEKIN, T.A. (1980). Contamination of broiler carcass skin during commercial processing procedures: an electron microscopic study. Applied and Environmental Microbiology 40: 133-144.

THOMAS, C.J., and McMEEKIN, T.A. (1980b). A note on scanning electron microscopic assessment of stomacher action on chicken skin. Journal of Applied Bacteriology 49: 339-344.

THOMAS, C.J., and McMEEKIN T.A. (1981). Attachment of *Salmonella* spp. to chicken muscle surfaces. Applied and Environmental Microbiology 42: 130-134.

THOMAS, C.J., and McMEEKIN, T.A. (1982). Effect of water immersion on the microtopography of the skin of chicken carcasses. *Journal of the Science of Food and Agriculture* 33: 549-554.

THOMAS, C.J., and McMEEKIN, T.A. (1984). Effect of water uptake by poultry tissues on contamination by bacteria during immersion in bacterial suspensions. *Journal of Food Protection* (in press).

THORSTENSEN, T. (1969). *Practical Leather Technology*. Van Nostrand Reinhold Company.

TODD, E. (1978). Food-borne disease in six countries - a comparison. *Journal of Food Protection* 41: 559-565

TODD, E.C.D. (1980). Poultry-associated food-borne disease - its occurrence, cost, sources and prevention. *Journal of Food Protection* 43: 129-139.

UNDERHILL, C., and DORFMAN, A. (1978). The role of hyaluronic acid in intercellular adhesion of cultured mouse cells. *Experimental Cell Research* 117: 155-164.

UNDERHILL, C.B., and TOOLE, B.P. (1979). Binding of hyaluronate to the surface of cultured cells. *Journal of Cell Biology* 82: 475-484.

VAN SCHOTHORST, M., NORTHOLT, M.D., KAMPERMACHER, E.H., and NOTERMANS, S. (1976). Studies on the estimation of the hygienic condition of frozen broiler chickens. *Journal of Hygiene (Cambridge)* 76: 57-63.

VEERKAMP, C.H., GERRITS, A.R., and MULDER, R.W.A.W. (1973). Water uptake as a consequence of water-chilling poultry. *Proceedings Fourth European Poultry Conference, London*: 277-287.

WARD, J.B., and BERKELEY, R.C.W. (1980). The microbial cell surface and adhesion. In *Microbial Adhesion to Surfaces*, Ellis Horwood Ltd., London, Editors Berkeley, R.C.W., Lynch, J.M., Melling, J., Rutter, P.R. and Vincent, B. pp 47-66.

WEISS, R., SCHIEFER, H.G., and KRAUSS, H. (1979). Ultra-structural visualization of *Klebsiella* capsules by polycationic ferritin. *FEMS Microbiology Letters* 6: 435-437.

WILSON, M.R., and HOHMANN, A.W. (1974). Immunity to *Escherichia coli* in pigs: adhesion of enteropathogenic *Escherichia coli* to isolated intestinal epithelial cells. *Infection and Immunity* 10: 776-782.

WOODBURN, M. (1964). Incidence of salmonellae in dressed broiler-fryer chickens. *Applied Microbiology* 12: 492-495.

YADA, R.Y., and SKURA, B.J. (1982). Scanning electron microscope study of *Pseudomonas fragi* on intact and sarcoplasm depleted bovine *Longissimus dorsi* muscle. *Applied and Environmental Microbiology* 43: 905-915.

APPENDICES

APPENDIX 1MEDIA AND BUFFERSMEDIANitrogen-Deficient Broth (Sutherland and Wilkinson, 1965)

Yeast extract	1.00	g
Casamino acids	1.00	g
Na ₂ HPO ₄ ·12H ₂ O	25.23	g
KH ₂ PO ₄	3.00	g
MgSO ₄ ·7H ₂ O	0.20	g
K ₂ SO ₄	1.00	g
NaCl	1.00	g
CaCl ₂ ·6H ₂ O	0.02	g
FeSO ₄ ·7H ₂ O	1.80	mg
Distilled water	1 000	ml

Glucose solution (10g/40ml water) autoclaved separately and added to give a final concentration of 2% (w/v) (4ml glucose solution/50ml media).

Minimal Medium (Markovitz *et al.*, 1967)

NH ₄ Cl	1.000	g
MgSO ₄	0.130	g*
KH ₂ PO ₄	3.000	g
Na ₂ HPO ₄	6.000	g
Glucose	6.000	g*
Mannose	6.000	g*
CaCl ₂	0.278	g*
Thiamine-HCl	10	mg ⁺
L-leucine	50	mg ⁺
L-proline	50	mg ⁺
Adenine	50	mg ⁺
L-histidine-HCl	50	mg ⁺
DL-tryptophan	100	mg ⁺
Agar	1.500	g
Distilled water	1 000	ml

* sterilise separately by autoclaving; + filter sterilise

APPENDIX 1 (Continued)Blood Agar

Tryptone Soya Agar (Oxoid)	40 g
Distilled water	1 000 ml

Heat to dissolve agar then sterilise by autoclaving. Cool to 45C and add 50ml sterile defibrinated horse blood. Mix thoroughly and pour plates.

Nutrient Agar

Nutrient broth (Difco)	8 g
NaCl	5 g
Agar	15 g
Distilled water	1 000 ml

Heat to dissolve agar then sterilise by autoclaving.

BUFFERSSorensen's Phosphate Buffer (pH7.2)

Na_2HPO_4	20.44 g
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	8.74 g
Distilled water to	1 000 ml

Autoclave in 180ml aliquots and store in cold until required. To use, dilute to 0.1M by adding 120ml distilled water to each 180ml aliquot of buffer.

Tris-Buffered Saline (pH7.0)

Tris base	0.121 g
NaCl	4.237 g
Distilled water to	500 ml

Adjust to pH7.0 with 0.1M HCl

APPENDIX 1 (Continued)Tris-Buffered Saline-NH₄Cl

Tris base	0.121	g
NaCl	4.237	g
NH ₄ Cl	2.674	g
Distilled water to	500	ml

Adjust to pH7.0 with 0.1M HCl

Phosphate-Buffered Saline (PBS)

NaCl	8.50	g
Na ₂ HPO ₄ ·12H ₂ O	2.70	g
NaH ₂ PO ₄ ·2H ₂ O	0.39	g
Distilled water	1 000	ml

Adjust to pH required with 0.1M HCl

Phosphate-Cysteine Buffer (pH6.5)

Na ₂ HPO ₄ (0.3M)	190.0	ml
NaH ₂ PO ₄ (4.0M)	6.4	ml
Cysteine-HCl	1.8	g
EDTA	7.4	g
Distilled water to	1 000	ml

APPENDIX 2CELL CULTURE MEDIAPhosphate-Buffered Saline and Mineral Salts (PBS) (pH7.5)

Solution A:	NaCl	8.0 g
	KCl	0.2 g
	Na ₂ HPO ₄ .12H ₂ O	2.9 g
	KH ₂ PO ₄	0.2 g
	Distilled water	800 ml
Solution B:	CaCl ₂ .6H ₂ O	0.19 g
	Distilled water	100 ml
Solution C:	MgCl ₂ .6H ₂ O	0.1 g
	Distilled water	100 ml

Autoclave each solution separately and combine 800ml solution A, 100ml solution B, and 100ml solution C when cool.

Trypsin Solution (0.1% (w/v))

Trypsin (Difco)	0.1 g
PBS (pH7.5)	100 ml

Sterilise by filtration through 0.22um filters (Millipore) after preparation.

APPENDIX 2 (Continued)EARLE'S BASED SOLUTIONSEarle's Stock Solution

NaCl	68.00	g
KCl	4.00	g
MgSO ₄ .7H ₂ O	2.00	g
NaH ₂ PO ₄ .2H ₂ O	1.40	g
Glucose	10.00	g
CaCl ₂ .6H ₂ O	3.94	g*
Phenol red (0.4% (w/v))	25	ml
Distilled water to	1 000	ml

* dissolved separately in a small volume of distilled water

Earle's Lactalbumin Solution (LaE)

Stock solution	500 ml	
Distilled water	4 500 ml	Earle's working solution

Lactalbumin hydrolysate (25g) was added to 500ml working solution; the remainder of the working solution dispensed in 360ml aliquots and all media sterilised by autoclaving. Each 360ml aliquot of working solution was then supplemented with 40ml lactalbumin hydrolysate solution. This final solution is LaE.

Bicarbonate Buffer (4.4% (w/v))

NaHCO ₃	11.00	g
Phenol red (0.4% (w/v))	6.25	ml
Distilled water to	250	ml

Sterilise by autoclaving

APPENDIX 2 (Continued)Earle's Maintenance Medium (EaM)

LaE	400 ml
Bicarbonate buffer (4.4%)	20 ml
Sterile foetal calf serum	10 ml
Benzyl penicillin	43 mg*
Streptomycin sulphate	43 mg*

* dissolved in a small quantity of water and sterilised by filtration.

All components are already sterile and are added aseptically.

Earle's Based Washing Solution (EWS)

LaE	400 ml
Benzyl penicillin	40 mg*
Streptomycin sulphate	40 mg*

* dissolved in a small quantity of water and sterilised by filtration.

All components are already sterile and are added aseptically.

APPENDIX 2 (Continued)HANK'S BASED SOLUTIONSHank's Balanced Salt Solutions

Solution A:	NaCl	160.0 g
	KCl	8.0 g
	MgSO ₄ .7H ₂ O	2.0 g
	MgCl ₂ .6H ₂ O	2.0 g*
	CaCl ₂ .6H ₂ O	5.5 g
	Distilled water to	1 000 ml

* dissolved separately in a small quantity
of distilled water.

Dispensed in 250ml aliquots and autoclaved.

Solution B:	Na ₂ HPO ₄ .2H ₂ O	1.2 g
	KH ₂ PO ₄	1.2 g
	Glucose	20.0 g
	Phenol red (4.4%)	50 ml
	Distilled water to	1 000 ml

Dispensed in 250ml amounts and autoclaved.

Hank's Lactalbumin Solution (LaH)

Solution A	250 ml	
Solution B	250 ml	Hank's working solution
Distilled water	4 500 ml	

Lactalbumin hydrolysate (25g) was added to 500ml working solution, the remainder of the solution dispensed in 360ml amounts, and all media sterilised by autoclaving. Each 360ml aliquot of working solution was then supplemented with 40ml lactalbumin hydrolysate solution. This final solution is LaH.

APPENDIX 2 (Continued)Bicarbonate Buffer (1.4% (w/v))

NaHCO ₃	3.50 g
Phenol red (0.4%)	6.25 ml
Distilled water to	250 ml

Sterilise by autoclaving.

Tryptose Phosphate Broth

Tryptose	20.0 g
Glucose	2.0 g
NaCl	5.0 g
Na ₂ HPO ₄	2.5 g
Distilled water	1 000 ml

Sterilise by autoclaving.

Hank's Growth Medium (HTC)

LaH	400 ml
Bicarbonate buffer (1.4%)	20 ml
Sterile calf serum	50 ml
Tryptose phosphate broth	50 ml
Benzyl penicillin	52 mg*
Streptomycin sulphate	52 mg*

* dissolved in a small quantity of water and sterilised by filtration.

All components are already sterile and are added aseptically

APPENDIX 2 (Continued)VERO CELL MEDIAEagles Growth Medium

BHK (Wellcome Labs.)	41.6 g
Deionized water	3 200 ml

Sterilise by filtration (0.22um filter) and store in 350ml volumes. To each 350ml of base solution add:

PSK	4.0 ml
Bicarbonate buffer (4.4%)	20.0 ml
Sterile bovine serum	40.0 ml
Tryptose phosphate broth	40.0 ml
Fungizone (5mg/ml)	0.2 ml

Aseptically add all components and saturate media with carbon dioxide.

PSK Solution

Benzyl penicillin	10^6 units
Streptomycin sulphate	1 g
Kanamycin	1 g
PBS (pH7.5)	700 ml

Filter sterilise (0.22um filter) and store in aliquots at -20C.

Trypsin-Versene (10 x concentrated)

NaCl	40.0 g
KCl	2.0 g
Glucose	5.0 g
NaHCO ₃	2.9 g
Phenol red (1% (w/v))	1.0 ml
Trypsin	2.5 g
Versene (EDTA)	1.0 g
Distilled water	500 ml

APPENDIX 2 (Continued)

Filter sterilise through a 0.22 μ m filter. Store in 2ml aliquots at -20C. Dilute to working strength with 18ml sterile distilled water just before use.

APPENDIX 3ANALYSIS OF GLYCOSAMINOGLYCANSDetermination of Uronic Acids

Samples (0.5ml containing 0-500ug of GAG) were added to 3.0ml concentrated sulphuric acid-0.025M sodium tetraborate in a glass stoppered tube, cooled to 0C in an ice bath and mixed. The solutions were then heated in a water-bath at 100C for 10 minutes, cooled at room temperature for 5 minutes, and 0.1ml of carbazole reagent added (0.125% carbazole in 95% ethanol). After mixing the samples were heated at 100C for a further 15 minutes, cooled to room temperature and the absorbance read at 530nm. A range of standards (25-100ug glucuronic acid/ml) were prepared and treated as above. For the blank, distilled water replaced the sample and ethanol was used in place of carbazole.

Results

The results of the analysis of the uronic acid content of two isolates of hyaluronic acid are given in Table 5. From Fig. 18 it can be seen that 1mg batch 1 hyaluronic acid contains 240ug uronic acid while 1mg batch 2 hyaluronic acid contains 320ug uronic acid. These values are slightly lower than those obtained for purified hyaluronic acid (47.2% by weight (Sinsberg, 1972)) as would be expected when crude extracts are being analysed.

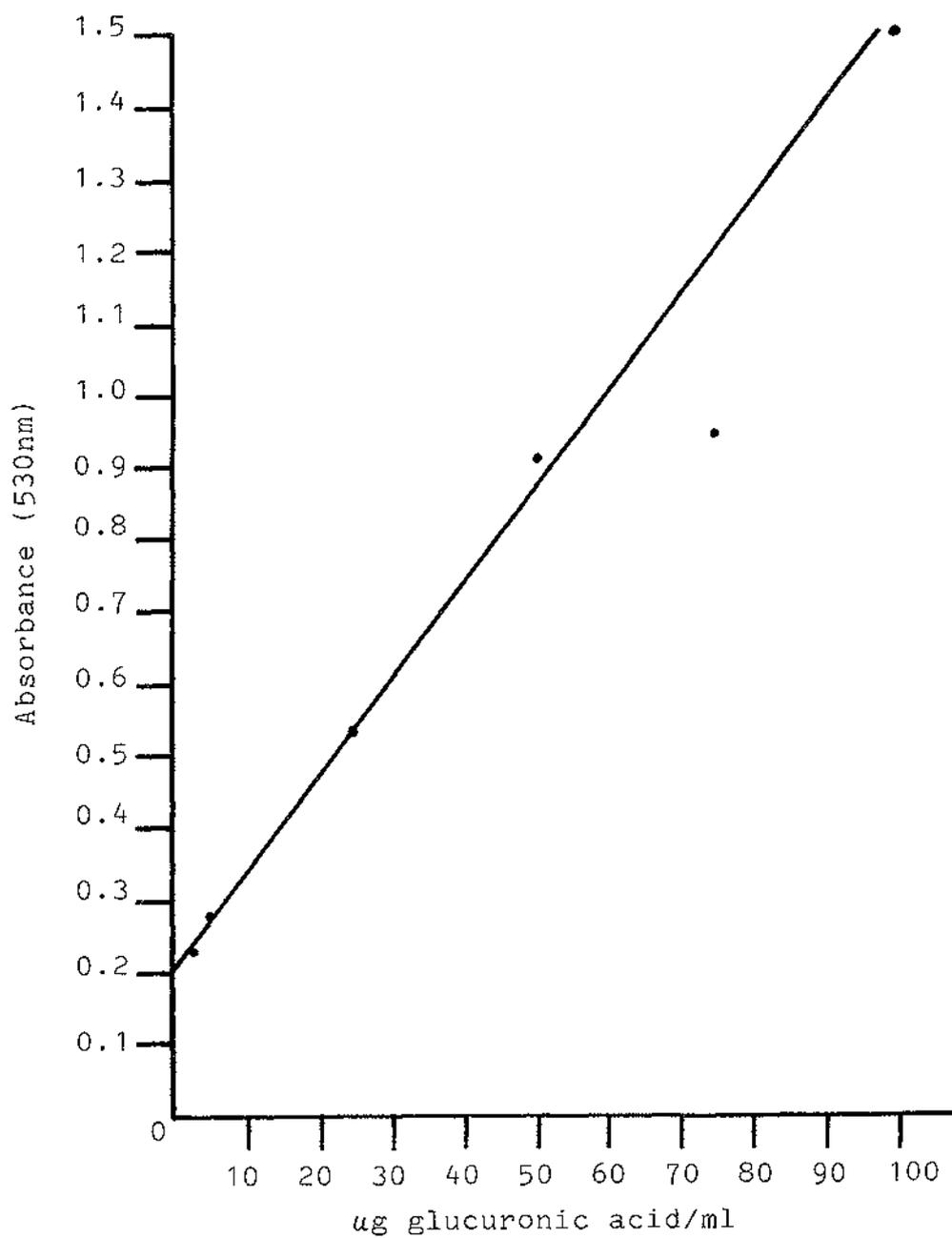
TABLE 5: Analysis of the uronic acid content of
hyaluronic acid.

TABLE 5:

Hyaluronic acid	<i>ug/ml</i>	Absorbance 530nm
Batch 1	50	0.41
	100	0.52
	500	-
	1 000	-
Batch 2	50	0.38
	100	0.62
	500	-
	1 000	-

FIGURE 18: Standard curve for the determination of uronic acid content of polysaccharides.

FIG 18:



APPENDIX 3 (Continued)Determination of Amino Sugars

Samples of polysaccharide (1-20 μ g) were placed into screw-cap Kimax tubes and HCl added to give a final concentration of 2N and a volume of 0.6ml. Tubes were stoppered tightly and hydrolysed at 100C for 10-12 hours. A range of standards (1-20 μ g glucosamine) and a blank were prepared in the same manner. Once hydrolysed, samples were neutralised with 0.4ml 2M sodium carbonate, shaken gently, 0.5ml of 2% acetylacetone in 1.5M sodium carbonate added, and the tightly stoppered tubes were heated in a boiling water-bath for 20 minutes. Once cooled 1ml of ethanol and 0.5ml of Erlich's reagent were added and the tubes shaken vigorously. The colour, which develops in 5 minutes, was read at 530nm.

Results

The results of the analysis of the amino sugar content of the two isolates of hyaluronic acid are given in Table 6. From Fig. 19 it can be seen that both batches of hyaluronic acid contain approximately 30% (w/w) amino sugars. This value is slightly lower than that obtained for purified hyaluronic acid (38.3%) (Sinsberg, 1972) as is to be expected for a crude extract.

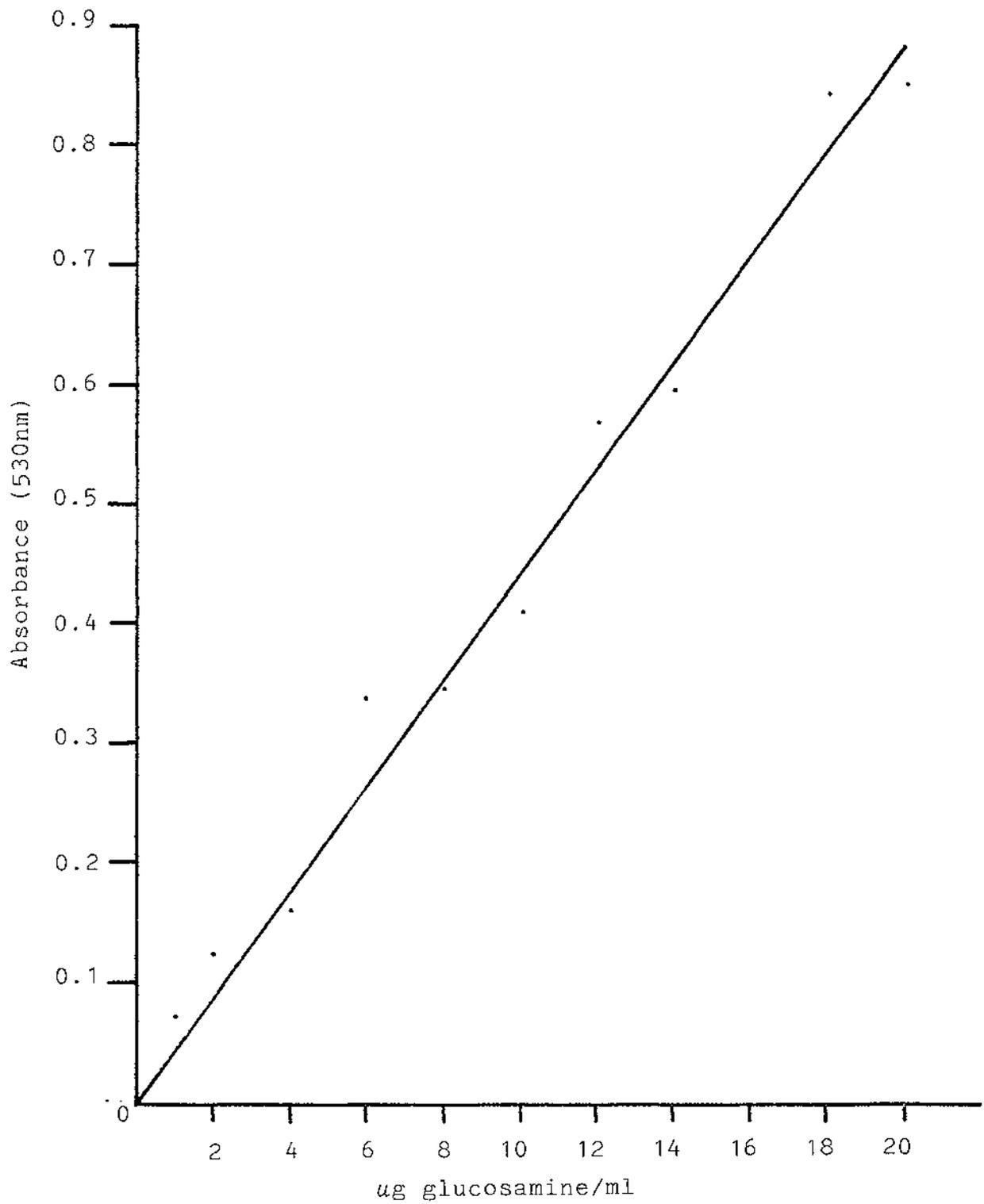
TABLE 6: Analysis of the amino sugar content of
hyaluronic acid.

TABLE 6:

Hyaluronic acid	ug/ml	Absorbance 530nm
Batch 1	10	0.13
	12	0.15
	14	0.19
	16	0.20
	18	0.23
	20	0.27
Batch 2	10	0.14
	12	0.17
	14	0.18
	16	0.20
	18	0.23
	20	0.27

FIGURE 19: Standard curve for the determination of amino sugar content of polysaccharides.

FIG 19:



APPENDIX 3 (Continued)Determination of Total Sulphate

Samples (1-3mg) of polysaccharide were hydrolysed in 1ml of 1M HCl in sealed tubes at 100C for a minimum of 6 hours. After cooling the tubes were opened and the contents left to dry at 40C. The residues were dissolved in 1ml of water, 0.1ml aliquots taken and added to 1.4ml 4% trichloroacetic acid and 0.5ml barium chloride-gelatin reagent (0.5% barium chloride in 0.5% gelatin). Sulphate standards (10-40mg inorganic sulphate/ml water) and a water blank were similarly treated. After mixing the samples were allowed to stand for 15-20 minutes and turbidities measured at 360nm.

Another 0.1ml aliquot of each solution was mixed with 1.4ml 4% trichloroacetic acid and 0.5ml gelatin reagent (0.5% gelatin). These mixtures were also read at 360nm and served to estimate any UV-absorbing substances present in the hydrolysed samples.

Results

The results of the analysis of the total sulphate content of two isolates each of chondroitin-sulphate and hyaluronic acid are given in Table 7. From Fig. 20 it can be seen that batch 1 of chondroitin-sulphate contains ca. 54ug sulphate/mg chondroitin-sulphate, while batch 2 contains 80ug sulphate/mg chondroitin-sulphate. Batches 1 and 2 of hyaluronic acid contain 20 and 0ug sulphate/mg hyaluronic acid respectively. Hyaluronic acid should be sulphate-free however batch 1 of this does contain some sulphate, presumably linked to associated impurities. The experiments described in this thesis were performed using batch 2 hyaluronic acid.

TABLE 7: Analysis of the total sulphate content of
hyaluronic acid and chondroitin-sulphate.

TABLE 7:

Polysaccharide	mg	Absorbance (360nm)		
		Test	Background	Value
Chondroitin-sulphate 1	1	0.76	0.46	0.30
	2	0.88	0.48	0.40
	3	0.91	0.40	0.51
Chondroitin-sulphate 2	1	0.80	0.38	0.42
	2	0.91	0.43	0.48
	3	1.02	0.49	0.53
Hyaluronic acid 1	1	-	-	-
	2	0.74	0.60	0.14
	3	0.88	0.49	0.39
Hyaluronic acid 2	1	0.75	0.50	0.25
	2	0.78	0.52	0.26
	3	0.81	0.50	0.31

FIGURE 20: Standard curve for the analysis of total sulphate content of polysaccharides.

FIG 20:

