Influence of sub-inhibitory concentrations of penicillin G on surface properties of iron-deprived *Staphylococcus aureus* NCTC 6571

by

Pierre Alain Gilbert Domingue

Submitted for the degree of Doctor of Philosophy

University of Aston in Birmingham

September 1986

The influence of sub-inhibitory concentrations of antibiotics on the surface properties of iron-deprived Staphylococcus aureus.

by Pierre Alain Gilbert Domingue

Submitted for the degree of Doctor of Philosophy, University of Aston in Birmingham, Sep 1986

The surface properties of batch cultures of Staphylococcus aureus NCTC 6571 (OXFORD) grown with 48 and 24 minute doubling times in iron-depleted Tryptone Soy Broth were studied after treatment with 1/16 MIC of penicillin G.

Growth rate influenced surface hydrophobicity. Slow-grown phenotypes, particularly iron-poor ones, had low contact angles generally, but these were increased by iron excess and/or penicillin. Opsonisation also significantly increased contact angles. All fast-grown phenotypes had much higher, uniform contact angles regardless of iron or penicillin status.

Low numbers (10-20 cocci:1 neutrophil) of the slow-grown iron-poor phenotype provoked the least chemiluminescence, had the lowest neutrophil association values and were least susceptible to whole blood killing. The other phenotypes behaved in a manner that generally reflected their relative hydrophobicities.

Alterations in growth rate significantly affected Protein A production and adherence to polyvinyl chloride catheters whilst iron deprivation and/or penicillin induced minor changes. Little effect was noted upon the production of staphylocoagulase and alpha-haemolysin.

Immunoblotting profiles were greatly simplified by iron deprivation and/or penicillin treatment. Growth rate alterations had no effect. Non-immune sera recognised the iron-poor preparations better than the iron-rich. Analysis of iron-poor and iron-rich preparations of S.aureus Hopewell, an endocarditis isolate, using the patient's immune serum also confirmed this trend both in immunoblotting and ELISA.

Keywords: Staphylococcus aureus, iron, sub-MIC, growth rate, phagocytosis, immunogenicity

ACKNOWLEDGEMENTS

Thanks are due to the following people. Professor M.R.W. Brown for the opportunity to do the Ph.D. and for its supervision; Dr P. Lambert for the infusion of ideas; Dr R. Klemperer for discussions; Mrs D. Townley and Mr R. Tilling for support especially in finding things; D. Townley, R. Tilling and Ms K. Ward for blood donations; Dr E. Harpur for the use of the multi-barreled pipette; Dr I. Farrell for the clinical material; the staff of the Haematology Laboratory, General Hospital, Birmingham for great assistance especially with techniques; Dr Trevor Baglin, Research Fellow, Haematology Department, Medical School, Birmingham University, for useful chats and expertise; Ms Jane Eminson, Research Fellow, Department of Social Medicine, Birmingham University, for the statistical education.

CONTENTS

List of Figures

List of Tables

Origin and scope of the study

Staphylococcus aureus is a pathogen that after some years of being overshadowed by clinical isolates such as Pseudomonas aeruginosa, is again becoming recognised as a predominant pathogen in the community and in hospitals (Cooke and Marples, 1985a and b; Sheagren, 1984). Despite natural immunity to S. aureus due to sub-clinical exposure people cannot prevent re-infections and eradication in vivo can be delayed with persistence of small numbers of cocci. Attempts to isolate or identify immunogenic protective components of S. aureus have failed, to date. The interaction of S. aureus with host defences is thus partly an immunological puzzle and a better understanding of the organism's characteristics in vivo is needed. However, such characteristics including those of the surface, the first part to come into contact with host defences, are poorly researched. In vitro experiments as usually understood, are unlike conditions in vivo and this affects bacterial properties (Brown and Williams, 1985a and b; Dalhoff, 1985; Lewis, Reeves, Wiedemann and Zinner, 1985). Bacteria in vivo are very often relatively slow-growing. Evidence is now accumulating that they are also nutritionally restricted especially for iron. They may also encounter sub-optimal drug concentrations. In this study, S. aureus NCTC 6571 was grown in batch under conditions that approximated to in vivo. Doubling times were changed by following a procedure for studying surface hydrophobicity (M.R.W. Brown; pers. comm.). Iron deprivation was achieved by treating Tryptone Soya Broth with a chelating resin. Penicillin G was used at a sub-MIC that induced morphological changes but did not alter the growth rate. Phenotypes produced were interacted with components of the host defence system. Attempts were made to characterise the antigens recognised by normal and hyperimmune sera.

INTRODUCTION

1. Introduction

1.1 Characteristics and clinical significance

Staphylococcus aureus belongs to the genus Staphylococcus in the Family Micrococcaceae. They are Gram-positive, non-chaining, non-motile, non-spore forming cocci approximately 0.8 to 1.0 μ in diameter. The other genus in the family, Micrococcus, differs from the genus Staphylococcus in that amongst other things, they have higher GC ratios of DNA and they lack teichoic acids, and glycine in the peptidoglycan (Schleifer, 1983).

The Staphylococci are taxonomically and clinically, conveniently split into staphylocoagulase (Section 1.3.3.1) positive and negative strains. The latter are usually regarded as less pathogenic under normal circumstances, and are represented by, e.g. S. epidermidis.

There is a gradation from the biochemically relatively inactive Micrococci through staphylocoagulase-negative Staphylococci to the active S. aureus strains. The latter group whose characteristics overlap to form a spectrum, originally consisted of six biotypes (Oeding, 1983). These biotypes were summarised as follows. Type A is mainly isolated from man and recognised by production of fibrinolysin. Types B (poultry and swine), C (cattle and sheep), D (hares), E (dogs, horses and minks) and F (foxes and pigeons) are all usually animal associated. Types E and F were so distinctive that they have become S. intermedius (Schleifer, 1983). However, whilst a S. aureus biotype may predominate in a particular host's flora other biotypes may be present. Also, S. aureus biotypes are not strictly species-specific with regard to pathogenicity (Anderson, 1983; Parker, 1983 and 1983/1984).

S. intermedius and some staphylocoagulase-positive strains of

S. hyicus can be differentiated from S. aureus by their inability to ferment mannitol anaerobically and a negative clumping factor reaction (Section 1.2.2.3).

S. aureus produces catalase and a pigment typically described as golden yellow but this characteristic is not so constant between strains. Strong pigmentation confers ecological advantages as resistance to ultra violet, dessication and linolenic acid is increased (Grinsted and Lacey, 1973). S. aureus is a facultative anaerobe and a semi-exacting heterotroph (Koser, 1968). It is one of the more resistant of the non-spore formers. It is susceptible to 60°C for thirty min but can resist drying or 4°C for 3 to 6 months. It is halotolerant.

S. aureus is found in varying numbers in the natural environment and in sewage and foods. However, it is not yet clear whether its presence in soil or water is a primary one or whether it is found there after host shedding because its accepted habitat is the animal body. It is found on the skin of both humans (Noble, 1976; Noble and White, 1983; Parker, 1983/1984; Shanson, 1982; Sheagren, 1984) and animals (Anderson, 1983; Blobel and Brückler, 1984). Humans are the predominant natural reservoir. S. aureus is rare on normal, healthy skin and resident carriage occurs at specific sites especially the anterior nares, the perineum, the axillae and the toewebs. Dispersion from these sites to other parts of the body then occurs (Noble and White, 1983).

Gram-positive bacteria such as Micrococcaceae flourish on the normal skin usually keeping the Gram-negative flora in check (Noble, 1976) and quite why S. aureus is uncommon there is still unknown. Certainly, coagulase-negative staphylococci can inhibit or interfere with S. aureus growth in vitro. Bacteriocins such as Pep 5 from

S. epidermidis and nicin from Streptococcus lactis induce unregulated autolytic activity (Section 1.3.1) in other Staphylococci (Bierbaum and Sahl, 1985). However, there is still no evidence of this in vivo and besides, a proper appraisal of the status of antibiotic production e.g. cyclic peptides, by skin flora is needed. Certainly, S. aureus is more resistant to free fatty acids than S. epidermidis and has less fastidious growth requirements than coagulase-negative Staphylococci (Koser, 1968).

The depth of colonisation of the surface of a particular site by S. aureus is not known. It seems possible, from electron microscopy, that it is mainly the actual skin surface but colonisation of the upper part of the hair follicle lumen is also possible. Application of S. aureus to intact skin can result in infection but usually the skin integrity has to be disrupted.

Nasal carriage in the general British population runs at about 30 to 35% and the factors deciding this carrier state are mostly unknown (Noble and White, 1983; Parker, 1983). The incidence can rise to 60% amongst hospital staff (Shanson, 1982) and also amongst patients on haemodialysis or receiving regular parenteral therapy, e.g. some diabetics. The incidence decreases with age (Noble, 1976).

S. aureus is also found in similar carriage sites in animals. In dairy cattle, it is mostly found on the teats and udders from where it can spread.

The first association of S. aureus with infection was made by Ogston (1882). Since then it has become apparent that few clinically important organisms can produce as wide a range of toxins and enzymes as S. aureus or invade and produce serious infections in any tissues or organs. Also, very few organisms can produce such varied illnesses

ranging from deep-seated systemic infections that are life-threatening or that become chronically debilitating and persistent, to superficial localised lesions. This is despite the natural resistance that hosts have to S. aureus, due in part to repeated exposure to these organisms or related ones. So whereas there is very little ability to prevent superficial colonisation, i.e. the host-parasite relationship approaches symbiosis, the typical host resists progressive disease quite well. Therefore, most S. aureus infections occur in already immunised hosts.

S. aureus is the most common cause of serious skin, soft-tissue and post-traumatic infection in the community. Soft-tissue infections include cellulitis and abscesses. The latter are known as boils or furuncles which are the most common septic lesion, or carbuncles if the lesion is larger and involves invasion of the hair follicles or sebaceous glands. Once established, it can spread directly to deep support structures such as the bones and joints. In Britain, S. aureus is the most common cause of acute and chronic osteomyelitis and septic arthritis (Hickling and Golding, 1984; Shanson, 1982). It is also the most common endocarditis isolate after the Streptococci, causing an acute illness with accompanying bacteraemia, metastatic abscesses and sometimes disseminated intravascular coagulation. The distinguishing feature of S. aureus is bacteraemia, either primary (no defined peripheral focus of infection) or secondary (peripheral focus of infection). Organs that are coincidentally inflamed can then become infected. 10% of bacteraemias can result in endocarditis (Sheagren, 1984).

In British hospitals, it is third behind Escherichia coli and Streptococcus pneumoniae in causing septicaemias and in the period 1975-1980 was responsible for 1:6 of notified cases in England and

Wales (Young, 1982). It is also a significant cause of catheter-related sepsis (Shanson, 1972; Wheat, Kohler and White, 1983).

S. aureus sepsis becomes a communicable disease in situations such as hospitals or in dairy herds. This coupled with advances in serological and genetic analysis has meant an improved understanding of its epidemiology.

S. aureus has persisted and is now re-emerging, especially in its methicillin resistant form, as an important community and hospital pathogen. It is secondary to E. coli in the incidence of hospital-acquired infections (Sheagren, 1984).

It has enormous adaptive capabilities. It is genetically well able to evolve (Lacey, 1975) not only by transduction but also by intra and inter-species conjugation (McDonnell, Sweeney and Cohen, 1983). It can rapidly develop resistance to a wide variety of agents.

Post-operative sepsis in orthopaedic or clean wound surgery is usually caused by S. aureus. It often involves the patient's own flora and not epidemic hospital strains. However, if the infection is acquired during the operation it is usually derived from the staff. Outbreaks of S. aureus sepsis usually originate in the ward and the source is an infected patient with subsequent transmission by the hands of staff (Shanson, 1982).

There are five international phage typing groups I to V, with an unamed group containing type 81. Many strains show lytic patterns found in both I and II, whereas III remains distinct and IV contains bovine strains. Phage typing patterns of individual strains are not characteristic enough to allow identification of the animal host unless serological and biochemical tests are included (Parker, 1983).

However, phage groups I, II and III are most commonly associated

with boils, they also produce lipases. The broadest antibiotic resistance is found in groups I and III.

The hospital epidemics from the 1950s to the mid-1960s were dominated by phage group I S. aureus strains commonly known as 80/81 types and their derivatives the 52/52A/80/81 strains which were exceptionally capable of infecting through healthy skin, exhibited a high clinical attack rate in colonised patients and showed great spreading ability in hospitals. All of these were derived from a single parent strain and were eclipsed by the 1970s by the phage group III or 83/83A/84/85 strains. Their particular feature was that they had appeared almost simultaneously in many countries and not always from the same parent strains, i.e. different geographical strains with convergent phage patterns had emerged. These new S. aureus strains had a lower ability to spread despite their broader spectrum of antibiotic resistance, and so only sporadic or small endemics prevailed. Today however, individual highly resistant strains such as the epidemic methicillin-resistant S. aureus (EMRSA) as opposed to sporadically occurring MRSA, are becoming widely disseminated either locally or regionally, especially in South East England and Merseyside (Cooke and Marples, 1985a and b).

Phage typing patterns are not related to antibiotic resistance patterns although the one is used to study the other. Also, differences in biological characters show only partial association with phage groups. Invasive ability of S. aureus strains is not associated with phage groups but with particular strains and therefore phage typing patterns (Parker, 1983; 1983/1984) and strains with shorter phage patterns cause more serious illnesses (Zierdt, MacLowry and Robertson, 1982).

Animal diseases like those in humans can be superficial e.g.

boils, impetigo, or deep and severe such as furunculosis, arthritis or osteomyelitis and can also be haematogenous (Anderson, 1983; Blobel and Brückler, 1984). Bovine mastitis can be economically very damaging and S. aureus and Streptococci cause 95% of British cases (Anderson, 1983).

1.2 Capsular and envelope components and their biological activities

1.2.1 Capsule

The capsule is an exopolysaccharide entity and has been defined as a covering layer outside the cell wall which has a definite external surface, is demonstrable by the light microscope and therefore has a thickness of 200 nm or greater (Wilkinson, 1958). This contrasts with a microcapsule which is a sub-light microscopic layer outside the cell wall, and which is chemically and immunologically distinct from the cell wall. Slime is another exopolysaccharide that is distinct from capsule and microcapsule and is not attached to the bacterial surface (Wilkinson, 1983; Sutherland, 1977). Confusingly, slime has also been called pseudocapsule.

The capsule can be visualised by ferritin-labelled antibodies or ruthenium red but the latter does cause dehydration and shrinkage. This emphasises that the high water content of capsules leaves them susceptible to shrinkage during staining and fixing. Methods of choice therefore employ an aqueous suspension of S. aureus, e.g. as in the indian ink method, or the Neufeld Quellung reaction where specific anticapsular antibody microprecipitates with, and therefore outlines the capsule. Both techniques are applicable to electron microscopy and the antibody can be made fluorescent. Cultural techniques to demonstrate encapsulation use serum-soft agar; S. aureus Smith-non-

capsulated grows as a compact colony whilst S. aureus Smith-capsulated produces a diffuse colony.

Encapsulation is relatively common in vivo but subculture in vitro rapidly results in loss. When fresh clinical isolates were tested in serum-soft agar, 18.4% were found to be encapsulated, the highest proportion in studies to date. Interestingly, the majority of the strains were capable of producing capsular antigens but full encapsulation was not frequently achieved, at least in vitro (Yoshida, Takahashi, Ohtoma, Minegishi, Ichiman, Kono, Haga and Sanclemente, 1979). Unencapsulated clinical isolates frequently gave rise to encapsulated variants when grown in the presence of neutrophils and anti-peptidoglycan serum (Karawaka and Young, 1979 a and b).

Capsular chemistry analyses are not always comparable because of differences in growth media, extraction and purification methods. It is known that capsules are heteropolymers and that N-acetylated aminouronic acids and N-acetylfucosamine are capsular components in S. aureus Smith-diffuse and S. aureus T respectively. These negatively charged polymers are firmly associated with the cell wall, possibly by covalent bonding. Capsular polysaccharides are provisionally classified into eight antigenic types with no cross-reactivity displayed (Karakawa and Vann, 1982). Serological analysis of world-wide clinical isolates showed that types 5 (26.4%) and 8 (40.7%) predominated and caused 70% of S. aureus bacteraemias and that types 1 and 2, represented by the classical control strains S. aureus M and Smith respectively, were rare! Approximately 24% were non-typable. This could have been due to one or several factors such as the misidentification of micro-capsules, a lack of freshness of referred isolates, and the existence of new antigenic groups (Arbeit, Karakawa, Vann and Robbins, 1984; Nelles, Niswander, Karakawa, Vann

and Arbeit, 1985).

Encapsulated S. aureus are more resistant to phagocytosis than non-capsulated controls whether in vitro or in vivo, and this greater resistance manifested as greater virulence in mice (Koenig, 1972). However, encapsulation per se is not a lethal determinant in that it did not contribute to the survival of S. aureus M within bovine neutrophils (Anderson and Williams, 1985) whilst an encapsulated coagulase-negative Staphylococcus simulans resisted phagocytosis but was not virulent in a murine peritonitis model (Anderson and Wilson, 1981). Thus encapsulation and the choice of host are only part of the factors in pathogenesis of staphylococcal infection.

The capsule does not trigger the alternate complement (C´) pathway and it interferes with opsonisation by both classical and alternate C´ pathways and by antibodies (Quie, Giebink and Peterson, 1981; Peterson, Wilkinson, Kim, Schmeling and Quie, 1978). The capsule does not however interfere with complement activation by peptidoglycan or association of C3b to the wall (Peterson, Kim, Wilkinson, Schmeling, Michael and Quie, 1978; Verbrugh, Peterson, Nguyen, Sisson and Kim, 1982). It also allows immunoglobulin G (IgG) to penetrate and react with, for example, Protein A (Pr A; Wilkinson, 1983). The capsule does though interfere with the phagocytic cell´s recognition of cocci opsonised with either C3b, IgG or both in that it acts as a physical barrier between the opsonic ligands on the bacterial wall and the receptors on the phagocyte membrane (Quie, et al., 1981; Wilkinson, 1983). Encapsulated S. aureus are most efficiently phagocytosed if complement and specific anticapsular antibody are present (Rogers and Melly, 1962; Melly, Duke, Liau and Hash, 1974; Peterson, Kim, Wilkinson et al., 1978b). The antibody

opsonises the capsular surface and so provides ligands for the phagocyte. Various surveys have shown that anticapsular antibodies, usually of relatively low titre and to specific types, are often found in normal human sera (Melly et al., 1974; Rogers and Melly, 1962) and in bovine serum and lacteal secretions (Opdebeeck and Norcross, 1985). This presumably confirms encapsulation of Staphylococci in vivo and the frequency of sub-clinical exposure to them.

1.2.2 The cell envelope

There are numerous reviews of envelope components in Staphylococci and unless otherwise stated, the information cited is drawn from these (Glaser, 1973; Forsgren, Ghetie, Lindmark and Sjöquist, 1983; Hammond, Lambert and Rycroft, 1984; Langone, 1982; Rogers, 1983; Schleifer, 1983, Shockman and Barrett, 1983; Wilkinson, 1983).

The basic difference between Gram-positive and Gram-negative bacteria lies in the chemical composition and therefore the ultrastructure of the cell wall. Electron micrographs of this section show that the Gram-positive wall is more or less homogenous and thick (up to 80 nm) whereas the Gram-negative wall is thinner (20-30 nm) and has a characteristic outer membrane (Glauert and Thornley, 1969). With regard to the major wall polymers, Gram-positives contain

peptidoglycan, PG (50-60% of dry weight of wall preparations)
polysaccharides
teichoic acids and/or teichuronic acids
proteins

the Gram-negative wall contains

relatively little PG (< 10% of dry weight of wall preparations)
lipopolysaccharide
lipoprotein
phospholipids
proteins
ie. Gram-negative walls are rich in lipophilic material

S. aureus has a typical Gram-positive wall (Fig 1a) and its cell surface is a mosaic in which cell wall components and perhaps the cytoplasmic membrane can interact with the environment (Wilkinson, 1983).

1.2.2.1 PEPTIDOGLYCAN (PG. A.K.A. murein, mucopeptide)

PG is the main structural and shape-maintaining polymer and represents the target of β-lactams. The mechanism of growth of this giant macromolecule is such as to maintain the osmostability of the bacterium. Accordingly, growth must not allow gaps to occur causing lysis. Also, septum formation must occur at a position such that each daughter receives at least one copy of the genome. Thus, cell envelope synthesis and DNA replication are closely coupled. The PG, despite its structural role, is flexible and allows moderate swelling or distortion because it has the properties of polyelectrolyte gels (Ou and Marquis, 1970).

PG is a heteropolymer of glycan chains which are relatively uniform and composed of alternating β-1,4 linked N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) (Strominger, Willoughby, Kamiryo, Blumberg and Yocum, 1974). The glycan strands are of 100-150 disaccharide units, and they are cross-linked through short peptide bridges (Fig 1b). These oligopeptide bridges are predominantly tetrapeptides in S. aureus, and attach to the PG at the carboxyl group of the NAM and are not linked directly at their other end as in the Gram-negatives but instead are cross-linked by an interpeptide bridge. This is a pentaglycine or more infrequently, a hexaglycine moiety. The D-alanyl-D-alanine of the pentapeptide is the main Staphylococcal antigenic determinant in PG, but the pentaglycine bridge is the

Figure 1

a) Diagrammatic representation of the Gram-positive envelope.

CAP	capsule
WTA/TU	wall teichoic acid/teichuronic acid
MTA	membrane teichoic acid
PG	peptidoglycan
CM	cytoplasmic membrane

(From Lambert, 1983)

b) Peptidoglycan polymer fragment of the primary structure.

Ac = acetyl

(From Schleifer, 1983)

Fig 1a

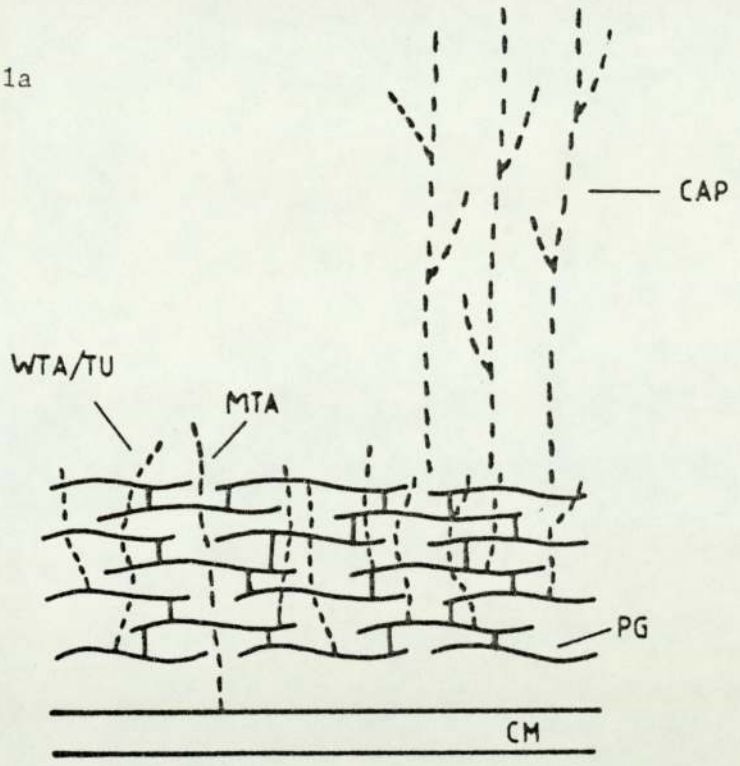
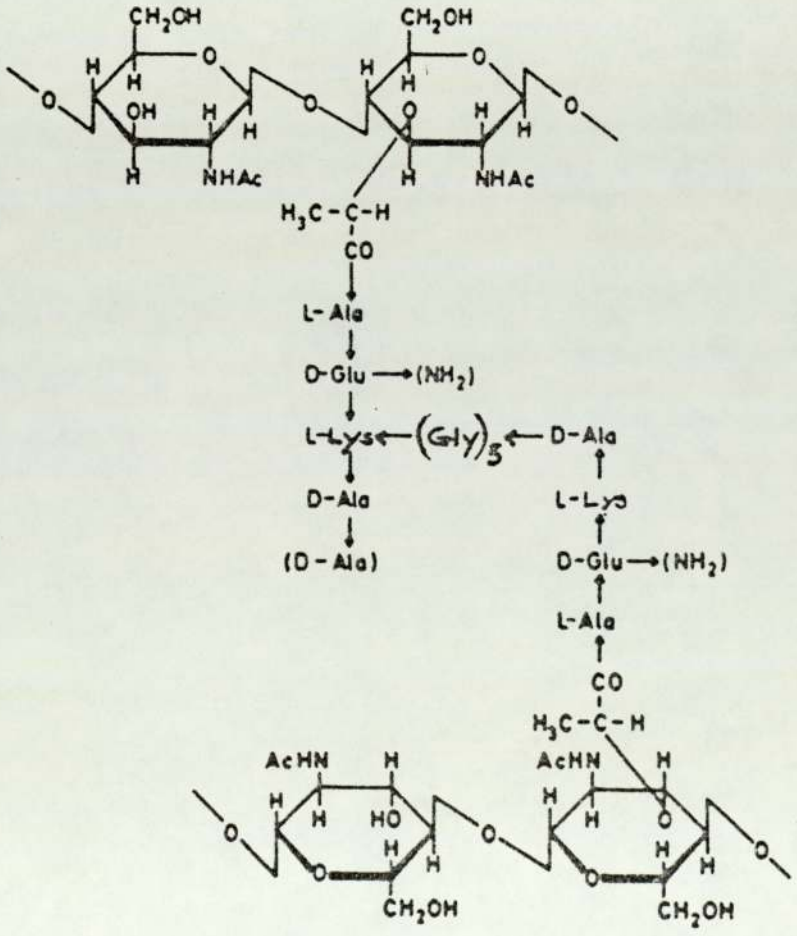

CAP
WTA/TU
MTA
PG
CM

Fig 1b


CH2OH
NHAc
H3C-C-H
CO
L-Ala
D-Glu
(NH2)
L-Lys
(Gly)5
D-Ala
(D-Ala)
AcHN
HO

specific antigenic determinant for S. aureus (Helgeland, Grov and Schleifer, 1973). The cross linkage has a maximum value of approximately 90% (Tipper and Berman, 1969) and is especially decreased by carbon, nitrogen and potassium limitation (Dobson and Archibald, 1978). The total amount of PG can be reduced most effectively by phosphate limitation which also reduces cross-linking and renders envelopes more sensitive to lytic peptidases (Archibald and Heckels, 1975). Muramic acid-6-phosphate has been detected in S. aureus and coagulase-negative Staphylococci. It has been suggested that this represents the attachment point of teichoic acids to PG (Coley, Tarelli, Archibald and Baddiley, 1978).

Oligopeptide-containing PG fragments are actively secreted during cell wall growth and cell division. This secretion shows only minor variations not only between S. aureus strains but between Staphylococci. Apparently, it was just as strong in a capsulated strain as in a non-capsulated strain of S. aureus. Also, sub-MICs of penicillin specifically increased the secretion five-fold and within a short time of addition (Seidl and Schleifer, 1985). The secretion of such fragments has implications for the potential host's response to infection. They are potent B-lymphocyte mitogens and occur in vivo where fragments were found to be uncross-linked and to contain NAG and the D-alanyl-D-alanyl-L-lysine sequence (Babu and Zeiger, 1983; Zeiger, Tuazon and Sheagren, 1981).

PG has a series of striking biological activities. It has a major role in early onset complement and non-complement-associated leukopaenia and thrombocytopaenia (Spika, Peterson, Wilkinson, Hammerschmidt, Verbrugh, Verhoef and Quie, 1982). PG can elicit an endotoxin-like pyrogenic response but its method of preparation is important because lysozyme-derived extracts were least pyrogenic

whilst ultrasonication-derived extracts were the most pyrogenic (Schleifer, 1983). The smallest dose of PG that caused pyrexia in rabbits was 50 μg kg^{-1} of bodyweight which compared to a dose of 0.008 μg kg^{-1} for lipid A. No cross tolerance was conferred by PG to the Escherichia coli endotoxin or vice versa.

Purified PG provoked a weak antibody response in rabbits whilst whole cell usage produced an antibody with good PG specificity (Schleifer, 1983). PG is the key cell wall component involved in opsonisation of S. aureus (Peterson, Wilkinson, Kim, Schmeling, Douglas and Quie, 1978; Verbrugh, van Dijk, Peters, van der Tol and Verhoef, 1979; Wilkinson, Peterson and Quie, 1979). It activates complement mainly by the classical route and because activation of both classical and alternate routes is faster in normal human serum than in Ig-deficient serum, immunoglobulins play a role in maximum rates of complement activation (Peterson, Wilkinson, Kim et al., 1978a). Purified Gram-positive PG is much more immunogenic than Gram-negative PG (Stewart-Tull, 1980) and normal animal (Heymer and Reitschel, 1977) and human sera very often contain anti-PG antibodies (Verbrugh, Peters, Rozenberg-Arska, Peterson and Verhoef, 1981). Also, PG shares several structural features among different bacterial species so host responses to PG may be part of natural immunity to a variety of Gram-positive pathogens. This indicates that colonisation and indeed, sub-clinical Staphylococcal infections are common throughout life and that the presence of anti-staphylococcal antibodies in normal human sera represents the host's natural immunity against what is essentially an endogenous saprophyte/parasite.

Intact S. aureus, crude cell walls, purified cell walls and PG itself are potent chemotaxins for leucocytes because they interact

with human serum to produce C5a (Schmeling, Peterson, Hammerschmidt, Youngki, Verhoef, Wilkinson and Quie, 1979). Macrophages, T and B lymphocytes are the targets for PG and its derivatives (Stewart-Tull, 1980). Regulation can be to potentiate or supress, but the mechanisms are not fully elucidated. It is known though, that cell envelopes and PG (and teichoic acids) stimulate T and B lymphocytes to produce leucocyte inhibitory factor (Rasanen and Arvilommi, 1982).

Inflammation (Section 1.5.2) is a general term for a second line of host defences triggered by breaching of the skin. S. aureus has evolved an ability to offset some of the components of the inflammatory response. A PG-protein complex from virulent strains and known as DOCR (deoxycholate residue) was shown to potentiate skin infections in mice by inhibiting both fluid accumulation and neutrophil chemotaxis (Hill, 1968). DOCR could also inhibit inflammation caused by other agents and it seems to act by inhibiting the kinin release system (Easmon, Hamilton and Glynn, 1973).

PG itself modulates inflammatory reactions as it induces histamine release from human basophil leucocytes by a non-immunological method. Cell envelopes and whole cells were less effective at this but did so by both immune and non-immune mechanisms (Espersen, Jarløv, Jensen, Skov and Norn, 1984). PG and wall teichoic acid but not Pr A, activated prekallikrein amidolytic activity, in an in vitro system using a mixture of proteins involved in the inflammatory response, namely Hageman factor (factor VII), prekallikrein and kininogen (Kalter, van Dijk, Timmerman, Verhoef and Bouma, 1983). PG can induce arthritis and, in rats, its effectiveness increases with increasing glycan chain length (Stewart-Tull, 1980).

Particulate but not soluble PG nor teichoic acids, induces delayed hypersensitivity (Section 1.5.9). There was species but not

strain specificity and this indicates the involvement of the pentaglycine bridge of the PG (Easmon and Glynn, 1978). Hypersensitivity was elicited when TA was covalently bound to soluble PG and it is possible that the covalent link between TA and PG slows degradation of the latter *in vivo* allowing prolonged antigenic stimulation (Phillips, R.D.J.; pers. comm. in Adlam and Easmon, 1985, page 312). Recent experimentation with synthetic immunoadjuvants based upon PG structure such as N-acetyl-muramyl-L-alanyl-D-isoglutamine, i.e. muramyl dipeptide (MDP) have shown promise (Ausobsky, Scuitto, Trachtenberg and Polk, 1984).

1.2.2.2 PROTEIN A (Pr A)

It is a single polypeptide that contains no carbohydrate residues and has a reported molecular weight of 42,000 daltons, i.e. 42 Kd. The protein is bound by its carboxy terminus to an unknown site in peptidoglycan. Pr A has an elongated conformation with one section embedded in the cell envelope and the remaining four sections, all similar, protruding into the environment (Forsgren *et al.*, 1983; Langone, 1982). Pr A is synthesised in some strains, mainly in exponential phase so that levels are constant by stationary phase. It is not vital for coccal viability as during amino acid starvation it can be broken down for endogenous needs (Szewczyk and Mikucki, 1983). In exponential phase cocci, most Pr A is secreted into the medium without passing through a cell wall bound state. Elsewhere, peak Pr A production and alpha-haemolysin production was post-exponential and co-ordinately controlled at the transcriptional level (Janzon, Löfdahl and Arvidson, 1986). It is only rarely detected in coagulase-negative staphylococci whilst between 93% and 99% of *S. aureus* strains contain it (Forsgren, 1970; Lachica, Genigeorgis and Hoeprich, 1979). In

S. aureus Cowan I, a Pr A-positive control strain, it comprises 0.9-1.1% of whole cells and 6.7% of the cell envelope fraction of that organism. It is mainly cell envelope located as L-forms of S. aureus contained only trace amounts (Forsgen et al., 1983; Langone, 1982). Ferritin-labelled immunoglobulin/ electronmicrographic studies showed that it was distributed all over the surface of the coccus (Lind, Reyn and Birch-Anderson, 1964) in discrete patches as determined by pH-electrophoretic values (James and Brewer, 1968a).

Extracellular protein A could be demonstrated in supernatants of cultures of 99% of methicillin-sensitive, penicillin-resistant S. aureus strains but in only 50% of methicillin-resistant S. aureus, these latter strains are also unusual in that they produce reduced amounts of cell-wall bound Pr A (Forsgen et al., 1983).

The exposed region of Pr A contains a site of 58 amino acids (Uhlén, Guss, Nilsson, Gatenbeck, Philipson and Lindberg, 1984) that has the unusual but well exploited (Godding, 1978; McDougall, Kennedy, Hubbard, Browning, Tsang, Winton and McDuffie, 1986) property of binding IgG at its constant or F_c portion which is not responsible for specific antigen-antibody interactions. This non-immune binding is not impeded by capsulation (King and Wilkinson, 1981). One molecule of Pr A can bind two molecules of human IgG at equivalence and this is regarded as a relative affinity by which Pr A's reactions with other immunoglobulins are compared (Forsgen et al., 1983) and at equivalence, Pr A can precipitate approximately 50% of the IgG in normal human serum. In S. aureus Cowan I radio-labelled IgG probes showed there were 80,000 binding sites per coccus (Kronvall, Quie and Williams, 1970).

Pr A does combine with immunological specifity with

immunoglobulins and antibodies to Pr A are found very frequently in human sera and in human colostrum (Oeding, 1984).

Protein B has been described but is now realised to be an extraction degradation product of Pr A; it does not combine with the F_c portion of IgG (Oeding, 1983).

Pr A triggers inflammatory reactions in mice (Kinsman, White and Noble, 1981) and stimulates delayed hypersensitivity in guinea pigs and rabbits (Noble and White, 1983) but not mice (Easmon and Glynn, 1978).

In a mouse mastitis evaluation of the three potential virulence factors, Pr A, staphylocoaglulase and alpha-haemolysin, it was found that both extra high-producing and extra-low Pr A producing mutants were just as virulent as their parents. However, mutants that could not produce either staphylocoagulase or alpha-haemolysin or especially both, were poorly virulent compared to their parents (Jonsson, Lindberg, Haraldsson and Wadström, 1985).

The majority of infections in Systemic Lupus Erythematosus (SLE) patients in one study were caused by S. aureus (Nivid, Linder, Odeberg and Svennsson, 1985) and it was also found that phagocytosis was reduced in SLE sera due to immune complex binding through F_c of IgG, to Pr A of the S. aureus. This appeared to block receptor contact on phagocytes. Tyrosine residues in both Pr A and the immunoglobulins of rheumatoid factors bind to the same histidine residues in F_c of IgG. This "molecular mimicry" by Pr A has been hypothesised to be a possible role for S. aureus in induction of rheumatoid arthritis (Nardella, Teller, Barber and Mannik, 1985).

Pr A-negative phenotypes were more sensitive to phagocytosis than were control phenotypes (Dossett, Kronvall, Williams and Quie, 1969). Pr A does not inhibit phagocytes per se as the rate of phagocytosis of

Pr A-rich strains is enhanced by opsonic sources such as IgG-deficient serum rather than normal human serum or purified IgG. The reduction in phagocytosis could be due to its reaction with the F_c portion of IgG which would affect opsonisation, and its effect upon complement. Perhaps one aspect of Pr A's role in vivo is that it effects this when extracellular, thereby intercepting host defenses (Forsgren et al., 1983). There is though no significant decrease in the clearance of Pr A-rich and Pr A-poor strains from the murine lung by alveolar macrophages. To contrast this, Pr A-rich strains were found to be associated with acute cases of bovine mastitis whilst Pr A-poor strains were associated with chronic cases (Forsgren et al., 1983).

Pr A can elicit Arthus and anaphylactic reactions in laboratory animals, particularly the guinea pig. It activates the C' cascade and prompts histamine release from granulocytes. It is well known for its reaction with the F_c part of IgG 1, 2 and 4 but it also reacts with IgM, IgA and IgE (Harboe and Fölling, 1974; Johansson and Inganas, 1978). The activation of C' is by the classical pathway, the alternate pathway is involved indirectly (Peterson, Verhoef, Sabath and Quie, 1977; Verbrugh, van Dijk, Peters et al., 1979). The sensitivity-promoting action of Pr A may be explained by its combining with F_c of IgG to trigger consumption of C' (Sjöquist and Stålenheim, 1969). It can also impair antibody-complement dependent phagocytosis and subsequent killing by human neutrophils due to the blocking of Cl_q binding to IgG (Schalén, Truedsson, Christensen and Christensen, 1985).

1.2.2.3 Clumping factor (A.K.A. bound coagulase; slide coagulase)

This is a true cell wall component and is absent in L-forms; it is a protein of molecular weight 21 Kd (Switalski, 1976). Peak

production occurs in the exponential phase of batch culture. The majority of S. aureus strains are usually both staphylocoagulase and clumping factor positive but about 3% of staphylocoagulase-positive strains are clumping factor negative and approximately the same proportion of staphylocoagulase-negative strains are clumping factor positive (Jeljaszewicz, Switalski and Adlam, 1983). Clumping occurs in two stages with adsorption of fibrinogen onto the staphylococcal wall followed by clumping.

Fibrinogen binds to clumping factor with a dissociation constant of approximately 10^{-8} and only 20 molecules of fibrinogen can clump one coccus (Hawiger, Hammond, Timman and Budzynski, 1978; Hawiger, Timmons, Strong, Cottrell, Riley and Doolittle, 1982). Clumping factor may not be present in the envelope as single entities but as carbohydrate-containing complexes of 380 Kd, incorporating one or several fibrinogen-binding proteins linked to PG by bands that are not broken by sodium dodecyl sulphate denaturation (Espersen, Clemmensen and Barkholt, 1985). As expected because of its weight and high polysaccharide content clumping factor was highly immunogenic and produced significant protection in mice, when used as a vaccine (Espersen and Clemmensen, 1985). Clumping factor is a more potent activator of both C´ pathways than Pr A (Espersen, 1985) but its overall contribution to activation by whole cocci is still unresolved.

Traditionally clumping factor was a virulence factor in its own right, as is staphylocoagulase. However, clumping factor-positive strains are not more resistant to phagocytosis than are clumping factor-negative strains and examples of the latter are fully virulent. Perhaps in vivo clumping confers a group protective effect (Jeljaszewicz et al., 1983; Switalski, 1976). Certainly S. aureus,

unlike other bacterial pathogens, can interact with fibrinogen *in vivo* and *in vitro*.

1.2.2.4 Agglutinogens

The *S. aureus* envelope contains small amounts of carbohydrate and protein antigens in addition to the major antigens. These are the type specific antigens of which approximately 30 are known (Oeding, 1983 and 1984) and allow reliable serotyping. Because they occur in small amounts, isolation and characterisation has proved difficult, to date. They are though good immunogens. The majority are protein whilst the rest are carbohydrates. Antigen a_5 is like WTA (Section 1.2.2.4) in that it contains ribitol phosphate, NAG and alanine. However, it is serologically distinct from WTA and is present in the envelope in a ten-fold less amount (Ndulue and Flandrois, 1983). Antigen n is composed of two portions, a major polypeptide sequence that is responsible for agglutination and a minor carbohydrate that participates in immunoprecipitation. Antigen h_1, is known to be a 90 Kd protein firmly bound to the PG and it lacks tyrosine (Oeding, 1984).

1.2.2.5 TEICHOIC ACIDS (TA. Greek TEICHOS "city walls")

TAs are located exclusively in the outer layers of Gram-positive bacteria, i.e. in the capsules and walls (wall teichoic acid; WTA) and also in the cytoplasmic membrane (membrane teichoic acid; M.T.A.; A.K.A. lipoteichoic acid, LTA).

There is a great structural diversity within TAs of the Staphylococci but essentially they are water soluble, strongly acidic, negatively charged, linear polymers of glycerol or ribitol phosphate, sugars and/or N-acetylamino sugars (Fig 2a and b). Occasionally, D-alanine is present and it contributes to the regulation of overall

Figure 2

a) Wall teichoic acid (ribitol) of Staphylococcus aureus.

Ala = alanyl

R = α or β N-acetylglucosaminyl

b) Membrane teichoic acid (glycerol) of S. aureus.

Ala = alanyl

R = long chain fatty acid

both ester linked

(Both diagrams from Hammond, Lambert and Rycroft, 1984)

Fig 2a

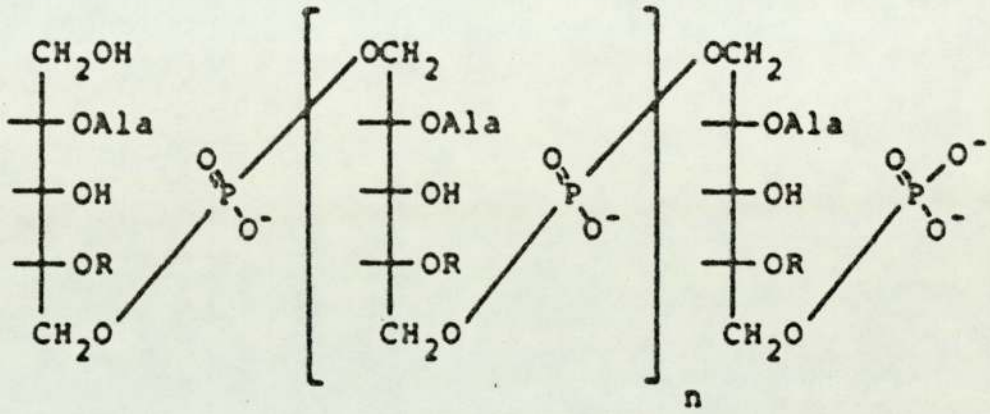
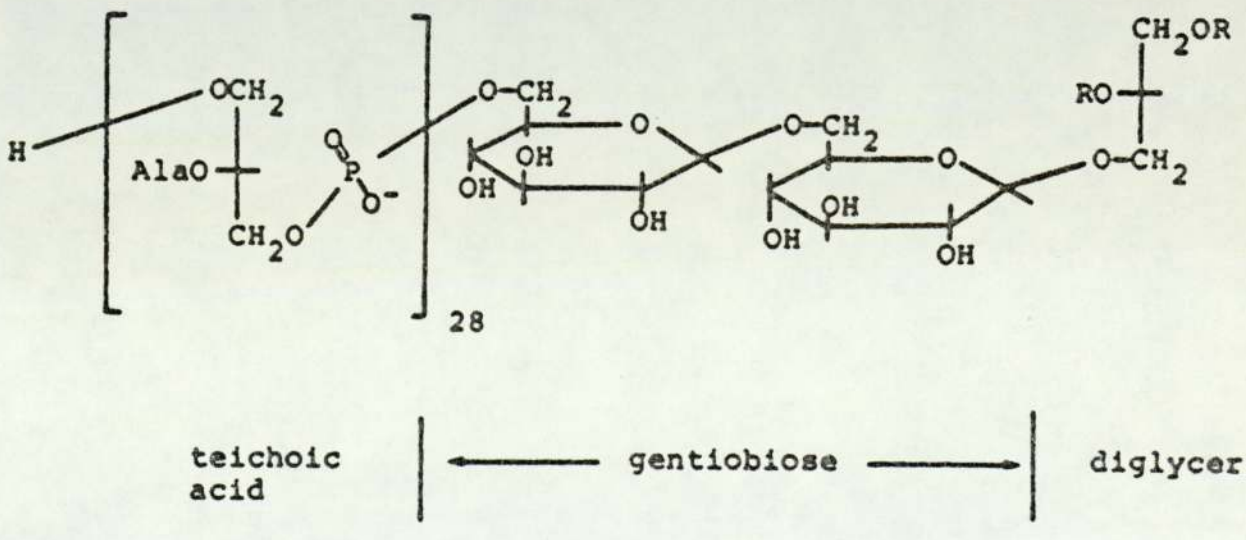

CH2OH
OAla
OH
OR
CH2O
OCH2
O=P
O-
n

Fig 2b


H
OCH2
AlaO
CH2O
O=P
O-
28
O-CH2
OH
O
CH2OR
RO
teichoic acid
gentiobiose
diglyceri

ionic charge of the polymer at physiological pH because its free amino group is protonated and neutralises adjacent phosphate groups (Baddiley, 1972). D-alanine can distort analyses because it is very labile at pH > 7.0 and is lost if reaction conditions become even slightly alkaline (MacArthur and Archibald, 1984).

It would be expected that as TA is strongly negatively charged, it is a major contributor to the overall surface charge of S. aureus. This seems not to be the case due to results with TA-deficient strains (Wood, 1980) and it has been concluded that the contribution is minor (Miörner, Albertsson and Kronvall, 1982) as the TA only contributes at a pH below 5 or 6 (James and Brewer, 1968b).

Ribitol-containing WTA has been found in three Staphylococci to date. Most strains of S. aureus contain a ribitol WTA which, in intact cells, is approximately 30-40 units long. This carries an N-acetylglucosamine at the 4-hydroxyl group of ribitol and D-alanine at the 2-hydroxyl group. S. aureus phage group 187 contains both ribitol and glycerol WTA substituted by N-acetylglucosamine and D-alanine. S. xylosus and S. saprophyticus also contain both WTAs (Endl, Seidl, Fiedler and Schleifer, 1983).

WTA is covalently linked supposedly by the NAG portion of a triglycerol phosphate N-acetylglucosamine residue in ribitol WTA to the 6-hydroxyl group of NAM in PG (Coley, Tarelli, Archibald and Baddilley, 1978). However, the linkage unit is now recognised to be a triglycerol phosphate N-acetylmannosaminyl-N-acetylglucosamine (Kojima, Araki and Ito, 1983). This linkage affects 5-10% of NAM and is much more acid and alkali labile than the endogenous phosphate diester link, and separation methods exploit this.

WTA can account for 30-50% of purified wall and more than 10% of

cellular dry weight. It is responsible for much of the phage binding and serological specificity. The latter is determined by sugar residues, but purified WTA is of too low a molecular weight to precipitate with antibody (Knox and Wicken, 1973).

S. aureus grown under limiting inorganic phosphate conditions tends to replace WTA with a phosphate-free anionic polymer of NAG and glucoronic acid called teichuronic acid (Archibald, 1974; Ellwood and Tempest, 1972).

Membrane teichoic acids, MTA, are ubiquitous in Gram-positive bacteria, except in some Micrococci where they are replaced by a succinylated mannan (Powell, Duckworth and Baddiley, 1975).

MTA isolation and purification is difficult but all so far examined from Staphylococci have glycerol as the polyol (Coley, Duckworth and Baddiley, 1972; Lambert, Hancock and Baddiley, 1977). MTA consists of 28-30 units of 1,3-phosphodiester linked glycerol phosphate which is substituted with gentiobiose (diglucose) and D-alanine. It is located in the cytoplasmic membrane and protrudes through the cell envelope, with the hydrocarbon chains of the acyl substituents interchelated with lipid bilayers of membrane. Anchorage is by a covalent phosphodiester link to the hydroxyl group on a glycolipid (Duckworth, Archibald and Baddiley, 1975). This glycolipid consists of gentiobiose which couples a diglyceride unit to the remainder of the MTA chain. MTA thus has a hydrophilic main body and a hydrophobic glycolipid anchor on the outer surface of the cytoplasmic membrane and is therefore an amphiphile.

Synthesis of MTA was reported to be unaffected by phosphate depletion (Ellwood and Tempest, 1972) which means it could be irreplaceable. Its biosynthesis certainly dominates S. aureus membrane lipid metabolism (Koch, Haas and Fischer, 1984) and turnover

of alanine in MTA acts as the source of alanine incorporated into WTA (Haas, Koch and Fischer, 1984). D-alanine levels in MTA are maintained by ATP-dependent esterification of fresh D-alanine (Koch, Doker and Fischer, 1985).

MTAs have direct contact with the cell envelope and it is suggested that a WTA-MTA cation exchange system can therefore operate (Lambert et al., 1977). It may also be that MTA acts as a carrier in the biosynthesis of WTA (Lambert et al., 1977).

TAs are involved in localisation (Boylan, Mendelson, Brooks and Young, 1972) and uptake of cations eg. Mg^{2+}, and in the establishment of the correct cationic environment at the bacterial surface or the cytoplasmic membrane (Heptinstall, Archibald and Baddiley, 1970). They also need Mg^{2+} to bind to the envelope. Autolysins (Section 1.3.1) also need Mg^{2+} for optimal activity and preferentially bind to Mg^{2+}-TA rather than to free Mg^{2+}(Hughes, Hancock and Baddiley, 1973). Therefore it appears that TAs modulate autolysins.

The MTA glycerol phosphate backbone is the major antigenic determinant whilst the alanyl substituents are only minor and not always immunogenic (Aasjord and Haaheim, 1985).

MTA is considered by some (Wicken and Knox, 1975) to be analagous to the lipopolysaccharide, LPS, of Gram-negative bacteria. Although MTA resembles LPS in some physicochemical properties, the former are not pyrogenic in rabbits or lethal for mice (Wicken and Knox, 1977). However, human jaundice due to deposition of MTA in hepatocytes is chronicled (Rose, Lentino, Mavrelis and Rytel, 1982).

The immune system has been implicated in some disease sequelae involving S. aureus antigen-sensitised host tissues (Section 1.5.9)but their manner is not well characterised. Antibody-dependent cellular

cytotoxicity (ADCC; Roitt, 1984) features in the cerebrospinal fluid and mucous membranes, ie. those parts deficient in complement, and it may also be responsible for tissue damage in those sites. MTA is amphiphilic and absorbs spontaneously to many mammalian cells (Wicken and Knox, 1980) and because normal human sera contain anti-TA antibodies, the elements for potential ADCC are present. This has been demonstrated in tissue culture (Lopatin and Kessler, 1985). Hyperimmunisation of rabbits with MTA can produce clinical and pathological signs of encephalitis due to cell-mediated immunity (Section 1.5.8) and this process is thought to be involved in the pathogenesis of multiple sclerosis (Nyland and Aasjord, 1983).

TAs are partially exposed on the cell surface and hence induce specific antibodies although when purified they do not (Knox and Wicken 1973; Wicken and Knox, 1975). They play little part in opsonisation however, thus emphasising that PG has the main role in opsonic recognition, further a teichoic acid deficient mutant S. aureus 52A5, was opsonised as well as its parent strain, and removal of TA from purified walls did not affect opsonisation rates with a variety of human sera (normal, heated, C2-deficient, EGTA-treated) (Peterson et al., 1978a). TAs were found not to be antiphagocytic as judged by their inability to affect both phagocytosis and intracellular killing (Shayegani and Mudd, 1969) but they can precipitate the neutrophil cationic proteins (Section 1.5.7.4b; Gladstone, Walton and Kay, 1974). They feature in the adherence of S. aureus to host tissues (Section 1.5.5). TAs activate the classical C′ pathway (Verbrugh, van Dijk, Peters et al., 1979) and MTA is also capable of activating the alternate C′ pathway (Hummell, Swift, Tomasz and Wink, 1985).

A major extracellular antigen in S. aureus culture supernatants

that is precipitable by naturally occurring human IgG antibodies has recently been isolated. Decomplementation antigen (DA) has been shown to be a homogenous, extracellular form of TA, of molecular weight 70-120 Kd, which when part of an immune complex shows an extraordinary capacity to activate the classical cascade. Thus DA may have an important role in vivo of intercepting host humoral defenses and reducing local opsonic ability (Bhakdi and Muhly, 1985 a and b).

1.3 Enzymes

1.3.1 Autolysins

Strictly speaking, autolysins are endogenous enzymes that can autolyse the intact bacterium. This lysis is often due to hydrolyses of bonds needed for PG integrity. They appear now, to have only a minor role in growth and morphology and this for a small number of strategically placed autolysin molecules. S. aureus autolysins have a major role in envelope turnover and cell separation, and the secondary effects of β-lactam antibiotics (Rogers, 1979; Shockman and Barrett, 1983). Cell separation is an obvious factor in survival and colonisation whilst turnover allows a rapid response to environmental changes and represents a balance between synthetic enzymes and the autolysins. As much as a 25% release rate per generation of old envelope material has been detected (Blümel, Uecker and Gisbrecht, 1978) and this has immunological consequences.

Three autolysins have been found in S. aureus: a β-NAG aminidase, an NAM-L-alanine amidase and an endopeptidase that hydrolysed the peptide chains and bridges between the D-alanyl terminus and the amino group of the adjacent peptide chain (Blümel, Reinicke, Lahav and Giesbrecht, 1983). Autolysins need Mg^{2+} for optimal activity (Tipper,

1969) and thus TA modulates their activity (Archibald, 1974; Brown, Fraser and Young, 1970) and can repress them (Cleveland, Wicken, Daneo-Moore and Shockman, 1976; Höltje and Tomasz, 1975). PG that is free of TA is not as good a substrate for autolysins as "native" PG.

WTA exerts more specific action than MTA upon autolysins because MTA inhibition cross-reacts between bacteria or can be duplicated by compounds such as LPS (Cleveland et al., 1975).

The interaction of TA with autolysins depends upon divalent cationic bridges between them that promote binding of autolysins to the wall (Huff and Silverman, 1968).

Intensive study of the autolysin system in Streptococcus pneumoniae has revealed a profound influence of the environment upon TA composition and therefore upon the autolysins and the secondary effects of penicillin. Replacement of choline by ethanolamine in the TA alters the conformation of the major autolysin, an amidase, so that its molecular weight, catalytic activity and capacity to recognise the correct substrate decreases (Section 1.2.2.5; Höltje and Tomasz, 1976; Tomasz and Westphal, 1971). This underlines the specificity of interaction between TA, autolysins and PG.

Autolysins may act on the outside of the envelope as in some strains a high proportion are excreted (Higgins, Coyette and Shockman, 1973) and it may therefore be significant that MTA is also excreted perhaps as an external control.

1.3.2 Penicillin Binding Proteins (PBPs)

β-lactams disrupt wall synthesis by covalently binding to and inhibiting the carboxypeptidase and transpeptidase enzymes (PBPs) involved in cross-linking the D-alanyl peptides on the PG strands of the growth sites (Kelly, Moews, Frère and Ghuysen, 1982; Waxman and

Strominger, 1983). The PBPs are located in the cytoplasmic membrane facing outwards (Spratt, 1980).

It is generally accepted that the β-lactam ring is a structural analogue of the D-alanyl-D-alanine portion of the nascent PG with the CO-N bond of the β-lactam corresponding to the peptide bond and the D-centre carboxyl group to the carboxyl of the terminal D-alanine (Fig 3a and b; Blumberg and Strominger, 1974; Frère and Joris, 1985; Tipper and Strominger, 1965). Hence the suggestion that the D-alanine carboxypeptidases are ancestors of β-lactamases. However, x-ray crystallography now indicates that the site on the PBPs that catalyses PG cross-linking overlaps the β-lactam binding site.

The amount of penicillin G bound varies according to growth phase and experimental method but is generally 5-6 nanomols per gram of dry weight of S. aureus (Suginaka, Blumberg and Strominger, 1972). The antibiotic acylates the enzyme and the β-lactam ring is broken by a reaction that is similar to the native one, to form an inactive penicilloyl-enzyme intermediate. This is stable enough to ensure that, at saturating concentrations, all the PBPs are affected. PBP activity is slowly restored because the intermediate breaks down and this shows that β-lactams that bind irreversibly are needed in chemotherapy.

The carboxypeptidase and transpeptidase PBP are very similar in activities in that they attack the D-alanyl-D-alanine portion to release a D-alanine (Fig 4a) but the carboxypeptidase leaves the peptide as an uncross-linked tetrapeptide, i.e. the penultimate D-alanine is not transferred to the amino group of the adjacent peptide. S. aureus has a relatively low carboxypeptidase activity.

The various β-lactams have a specific affinity for each of the

Figure 3

a) Generalised representation of the penicillin molecule.

In penicillin G, R = methylbenzene

b) Stereomodels of penicillin G (A) and of the D-alanyl-D-alanine end of the peptidoglycan strand (B).

The arrows indicate the CO-N bond in the β-lactam ring of penicillin and the CO-N bond in the D-alanyl-D-alanine

Fig 3a

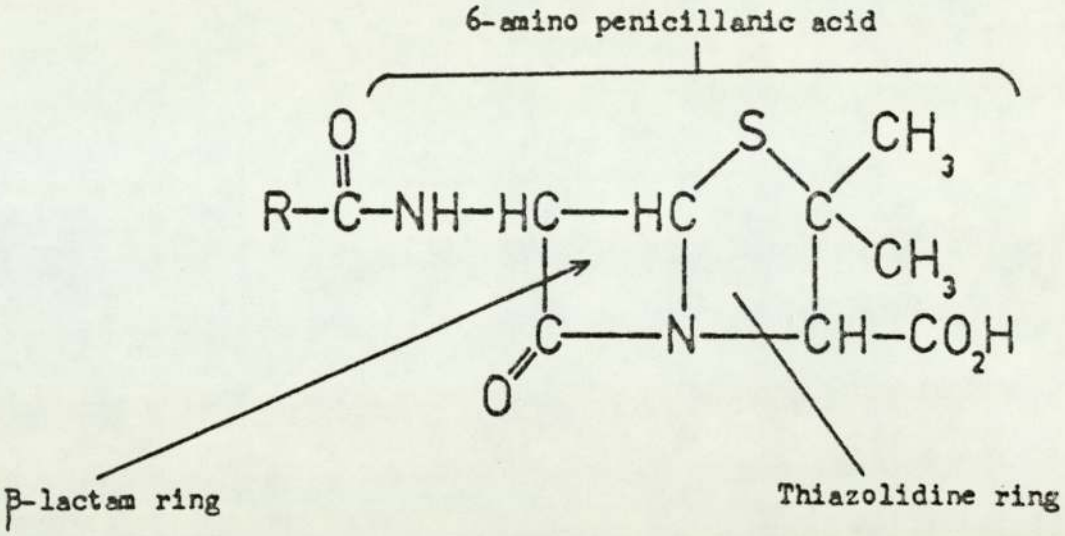

6-amino penicillanic acid
β-lactam ring
Thiazolidine ring

Fig 3b

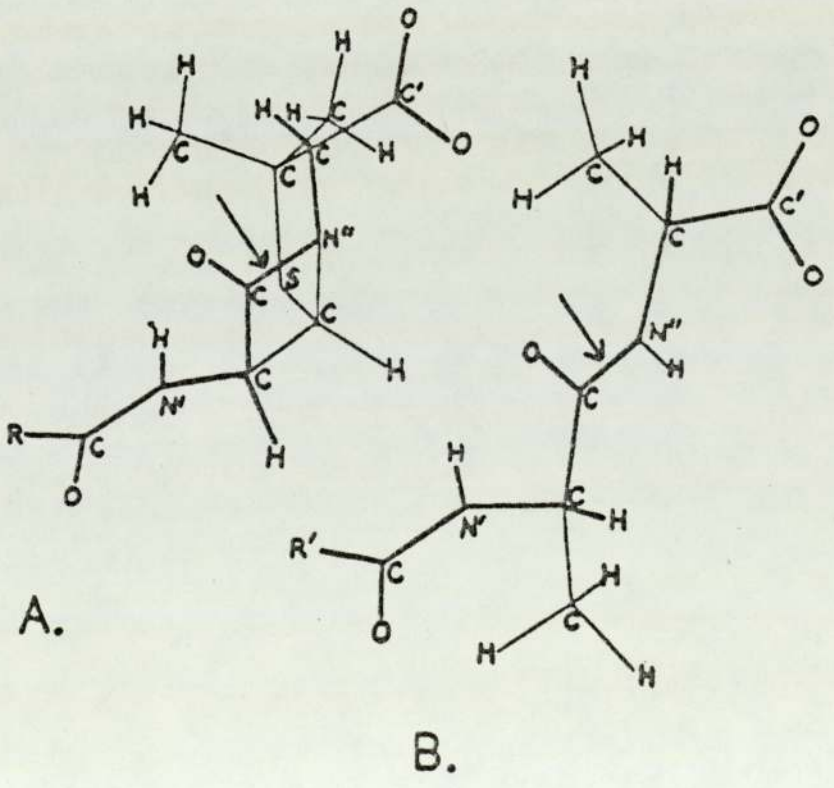

A.
B.

Figure 4

a) The final stage in cell wall synthesis: cross-linking of peptidoglycan polymers.

b) Schematic representation of a section of a Staphylococcus grown in the presence of a sub-MIC (1/3) of penicillin.

1 to 4 are successive stages

(From Lorian, 1980)

Fig 4a

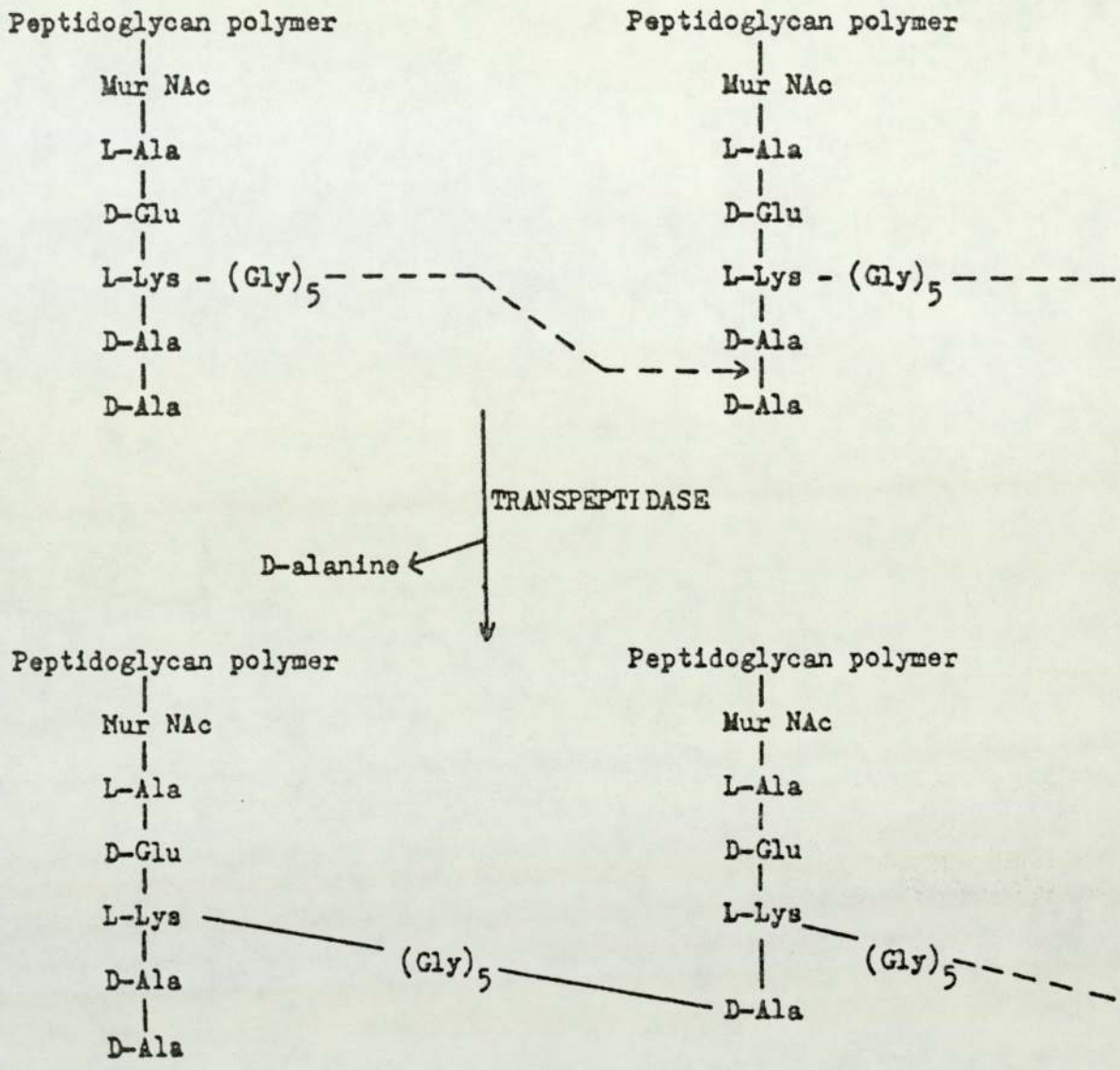
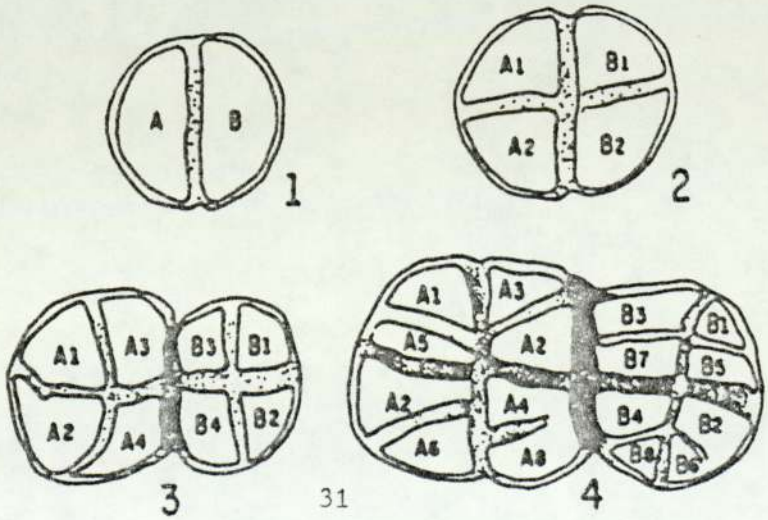

Peptidoglycan polymer
Mur NAc
L-Ala
D-Glu
L-Lys - (Gly)5
D-Ala
D-Ala
Peptidoglycan polymer
Mur NAc
L-Ala
D-Glu
L-Lys - (Gly)5
D-Ala
TRANSPEPTIDASE
D-alanine
Peptidoglycan polymer
Mur NAc
L-Ala
D-Glu
L-Lys
(Gly)5
D-Ala
D-Ala
Peptidoglycan polymer
Mur NAc
L-Ala
D-Glu
L-Lys
(Gly)5
D-Ala

Fig 4b


A
B
1
A1
B1
A2
B2
2
A1
A3
B3
B1
A2
A4
B4
B2
3
A1
A3
B3
B1
A5
A2
B7
B5
A2
A4
B4
B2
A6
A8
4

PBPs, ranging from a very low to almost a total affinity. This is to be expected as each protein has a different function in the maintenance of the bacterium. Penicillin G binds to all PBPs but the drug concentration used decides which are bound (see Section 1.4.2) and at the minimal inhibitory concentration (MIC) those PBPs that are the actual bactericidal targets are affected. Intrinsic resistance has been found to be due to a decrease in affinity of these targets for β-lactams or their replacement by other PBPs with decreased affinity (Brown and Reynolds, 1980; Fontana, 1985; Hayes, Curtis , Wyke and Ward, 1981; Hartman and Tomasz, 1984).

Between four and five PBPs have been detected in S. aureus (Canepari, Varaldo, Fontana and Satta, 1985; Wyke, 1984). PBP 4 has the least affinity for β-lactams whilst PBPs 2 and 3 are most abundantly produced and are the lethal targets and modified in methicillin-resistant strains (Reynolds and Fuller, 1986; Hayes et al., 1981; Wyke, Ward and Hayes, 1982; Rossi, Tonin, Cheng and Fontana, 1985; Ubukata, Yamashita and Konno, 1985; Utsui and Yokota, 1985).

Environmental factors such as growth medium (Fontana, Canepari, Satta and Coyette, 1983), iron deprivation (Turnowsky, Brown, Anwar and Lambert, 1983) affect the detection of PBPs and response to penicillin G. Growth rate also induces such changes (Turnowsky, et al., 1983) and in Streptococcus faecium, PBP 5 which has a low affinity for penicillin G was unaffected by penicillin G when the cocci were growing relatively slowly (Canepari et al., 1986). The bacterium was able to replicate with this one functional PBP. This poses fresh problems in drug design (Fontana, 1985) and modifies the view that growth involves multiple penicillin targets. Indeed, Staphylococcus hyicus, has only one PBP, at 79 Kd (Canepari, Varaldo,

Fontana and Satta, 1985).

1.3.3 Extracellular products

S. aureus can liberate up to 25 extracellular proteins during growth, and these range from proteases to DNase and are capable of degrading most tissues and fluids (Arvidson, 1983). Iso-electric focusing shows that different strains produce distinct protein patterns (Wädstrom, Thelestam and Möllby, 1974) and immunoblotting has revealed many of these proteins to be antigenic and useful in typing S. aureus (Krikler, Pennington and Petrie, 1986).

Examination of these extracelullar products did not feature prominently in this study and as there are extensive reviews (Arvidson, 1983; Freer and Arbuthnott, 1982; Stephen and Pietrowski, 1981; Wiseman, 1975) only a brief account is presented here.

Some of the extracellular proteins are classified into nine toxin types:

α

β

γ

δ

epidermolytic toxins (two types)

enterotoxins A-F

leucocidin

succinic oxidase factor

pyrogenic exotoxin

The majority of the remainder are non-toxic enzymes or enzyme activators involved in degrading macromolecules such as proteins, nucleic acids, lipids and polysaccharides for growth requirements (Arvidson, 1983), or are involved in antibiotic inactivation, eg. the

hydrolysis of β-lactam antibiotics.

1.3.3.1 Staphylocoagulase (A.K.A. free coagulase; tube coagulase)

This is a true excprotein whose molecular weight varies between 40-60 Kd according to the purification and analytical techniques used. It is not yet known whether any carbohydrate is attached. Maximum production in batch culture, is by oxygen-limited cells whilst in continuous culture both oxygen limitation and magnesium depletion give maximum levels. Staphylocoagulase exists in different molecular and therefore antigenic forms. Different strains can produce different forms of the enzyme and the same strain can produce up to four forms (Reeves, Drummond and Tager, 1981). Thus, cross-neutralisation by specific antibodies does not occur. Eight serological types have been recognised to date and antibodies to at least one of these are found in most normal human sera. The enzyme needs a host-specified "coagulase reacting factor", CRF, and the best source is prothrombin. The two react to give a staphylothrombin complex in which the enzyme has acted as an allosteric effector and exposed sites on the prothrombin for fibrinogen to bind to. The staphylothrombin complex then polymerises plasma fibrinogen to a fibrin matrix. The lethal dose of staphylocoagulase in rabbits is 3-5mg Kg^{-1} by the intravenous route which demonstrates it is not as highly active as traditionally thought. Death results from a hypercoagulable state that leads to thrombosis.

S. aureus strains that had lost staphylocoagulase lost their ability to survive in human, defibrinated blood (Spink and Vivino, 1942). The eventual use of deletion mutants showed that there was no significant difference in the ability of these mutants and their wild type parents to produce infection. Also, staphylocoagulase-positive

strains are not more resistant to phagocytosis or killing and anti-staphylocoagulase antibody does not result in an increase in the number of phagocytosed cocci (all references in Jeljaszewicz, Switalski and Adlam, 1983). The deposition of fibrin upon the bacterial surface, as a result of staphylocoagulase action, has been shown to confer resistance to both intracelullar and cell-free neutrophil bactericidins (Kolawole, 1983a and b, 1984). Thus, the role of staphylocoagulase in pathogenesis is slightly more defined now.

Antibodies to staphylocoagulase are moderately protective if the homologous strain is used in challenge. The point has been made though that the majority of studies were performed before present day techniques of purification, and that a modern reappraisal is needed (Adlam and Easmon, 1983).

1.3.3.2 Catalase

It is mainly a stationary-phase enzyme and is inhibited, for example by phenolics which chelate the iron necessary for biosynthesis of co-catalase, the ferriprotoporphyrin prosthetic group (Lin, 1963 a and b). Not surprisingly, the catalase activity of iron-poor S. aureus was much less than that of iron-rich cocci (Schade, 1963). The enzyme's activity is increased by exposure of S. aureus to peroxide or oxygen (Lin, 1963c), and when the environmental oxygen tension drops (Kovacs and Mazarean, 1967). It may therefore counter peroxide poisoning especially at high levels (Wilson and Weaver, 1985), and also is induced to satisfy energy demands if there is insufficient oxygen for the peroxidase system. It confers an advantage against the oxygen-dependent phagocytic microbiocidal systems (Klebanoff, 1975 and 1982) such that there was good

correlation between the level of catalase production and mouse lethality (Mandell, 1975). Elsewhere no correlation existed between catalase production and resistance to intracelullar killing in mice, and catalase was not ascribed an important role in protection of S. aureus (Nishihara, Seiki and Masuda, 1985).

When pathogenicity of virulent and non-virulent strains of S. aureus was compared by the reduction in percent weight gain of neonatal mice, reduced host growth strongly correlated with increased catalase and superoxide dismutase activities in virulent strains (Kanafani and Martin, 1985).

1.3.3.3 Alpha(α)-haemolysin (A.K.A. α-toxin)

It is a major extracellular enzyme, produced mostly in the stationary phase of batch culture, and can constitute up to 2% of the dry weight of cocci in stationary phase (Duncan and Cho, 1971). It interacts with the erythrocyte membrane but it is not known whether it does this as a protease (Wiseman, 1975) or as a surface active agent (Freer and Arbuthnott, 1983). It is cytotoxic and cytolytic especially to rabbit erythrocytes. α-haemolysin is immunogenic and the detection of antibodies to it is the basis of the most common Staphylococcal serological test in Britain. Anti-α-haemolysin antibody does not generally protect but sufficently high titres help to modify development of infection at least in laboratory animals, in that necrotic areas were reduced (Adlam and Ward, unpublished obs. in Adlam and Easmon, 1983, Page 297). In man it may be theoretical if only because toxigenic infections are rare - perhaps because of naturally occurring low levels of α-haemolysin antibody. α-toxoid immunisation is effective though in modifying acute spreading lethal gangerous mastitis of livestock especially sheep where it is a

particular problem, to a chronic localised abscess type of disease (Adlam, Ward, McCartney, Arbuthnott and Thornley, 1977).

1.3.3.4 Penicillinase

Resistance of S. aureus to penicillin G is usually caused by an inducible, extracellular β-lactamase (Barber, 1962) which is generally called staphylococcal penicillinase due to its higher activity against penicillins compared to cephalosporins. It is often plasmid-determined (Novick, 1963) and up to eleven different types of penicillinase plasmids exist classified by the type of penicillinase they produce (Richmond, 1968). Penicillinase can also be chromosomally located (Asheshov, 1966) and constitutive (Rosdahl, 1973). The genetics , clinical significance and mechanism of action of β-lactamases in general has been reviewed elsewhere (Poston and Naidoo, 1983; Sanders and Sanders, 1983).

1.4 Effect of penicillin G

1.4.1 Inhibitory concentrations

The original idea that mechanical or osmotic pressure ruptured a β-lactam weakened wall is not tenable (Tomasz, 1979 a and b). Bulges or pleiomorphy are not trivial results of β-lactam action and simple inhibition of envelope synthesis is not sufficient for lysis (Weidel and Pelzer, 1964). Active protein synthesis, i.e. enzymes, is needed for the lytic effect (Rogers, 1967). Also, cell death and cell lysis are not synonymous since penicillin G lysed Strep. pneumoniae and killed but did not lyse Strep. pyogenes even though it had the same low MIC for both. It was subsequently found that Strep. pyogenes has no major autolytic activity (Horne and Tomasz, 1977). Ethanolamine-containing WTA in Strep. pneumoniae not only conferred autolysin

resistance (Section 1.3.1) but also conferred resistance or "tolerance" to the bactericidal and lytic effects of penicillin G despite not affecting the normal MIC.

S. aureus MTA can be released by β-lactam (Beachey, Nealon, Courtney and Simpson, 1985) action thus derepressing autolysins and inducing lysis (Suginaka, Shimatani, Ogawa and Kotani, 1979; Utsui, Ohya, Takenouchi, Tajima, Sugawara, Deguchi and Suginaka, 1983) whilst addition of exogenous MTA prevented this lysis (Suginaka et al., 1979).

It seemed then that the key event in the lytic action of penicillin G for S. aureus was the deregulation of autolysins. Recent work has forced a reappraisal of this as neither simple inhibition of incorporation of envelope material nor activation of autolysins was needed for penicillin's lytic activity. Penicillin G did reduce cross-linkage in PG by 30% but crucially, did not even at 5x MIC seriously reduce incorporation of ^{14}C-N-acetylgalactosamine up to the start of the lytic sequence. This confirmed previous reports that total PG synthesis in Gram-positives was not affected by sub-MICs or MICs of β-lactams (Fischer and Tomasz, 1984; Shockmann and Barrett, 1983). Incorporation of the label showed that the PG in the envelope was thick, deformed and unorganised. Strikingly, there was a lack of newly synthesised material at the second division plane, i.e. the commencement of the second generation of cocci.

The lytic event started only during or after the second generation of cocci and it lasted for several generations. The well known breakdown of envelope did not occur until later (Giesbrecht, Labischinski and Wecke, 1985). Consequently, the lytic sequence has been suggested to consist of two stages.

Initially, there is penicillin G-induced deposition of new material that favours the first division plane. This has been noted previously (Lorian, 1980). Then, murosomes, that is cytoplasmic membrane-derived vesicles that contain the autolysins act at both first and second division planes to separate daughter cells. However, there are only adequate deposits of new envelope at the first plane. Consequently, minute perforations punched through into the periphery of the envelope occur in the second plane, and lysis occurs.

1.4.2 Sub-inhibitory concentrations

Antibiotic activity is not an all or nothing phenomenon (Rolinson, 1977). Effects at supra-MIC and especially sub-MICs are sometimes overlooked as the MIC is the unit of clinical significance. In fact, increases in the extent or speed of effect can be seen over a wide range of concentrations and the MIC is simply a concentration that produces a particular effect in specific circumstances. The term sub-inhibitory concentration is widely used but it can be a misnomer because such concentrations of some drugs do affect the bacterial cell; accordingly the terms sub-inhibitory and sub-MIC are used synonymously here.

Sub-MIC

Investigations into sub-MICs rather than MICs of antibiotics have shown that the anti-bacterial effects due to sub-MICs of a drug are different to those due to the MIC. The intensity of β-lactam binding is less at sub-MICs and hence the amount of drug available is only sufficient to bind to the protein for which it has the strongest affinity. In for example, E. coli, ampicillin at sub-MICs binds firstly to PBP 3 resulting in long filaments; at concentrations equal to or greater than the MIC ampicillin also binds to PBP 1 the

elongation protein, and causes lysis (Atkinson and Amaral, 1982; Lorian, 1980).

It was known approximately 45 years ago that morphological abnormalities could be induced in S. aureus by sub-MICs of penicillin G (Gardner, 1940). Later, sub-MICs of penicillin were found to reduce the growth of cocci and the rate of bactericidal action of penicillin was concentration-dependent (Eagle and Musselman, 1948).

Systematic work in the 1970's rationalised existing findings and placed sub-MIC effects into two categories.

A. those effects resulting in morphological changes as detected by light microscopy or electron microscopy.
B. those effects resulting in a decrease in number of viable bacteria.

(Greenwood and O'Grady, 1973; Lorian, 1980)

Such definitions led to the concept of a lowest possible concentration of drug which could affect the structure or growth or both, of a bacterium. This is the Minimum Active Concentration or MAC. Accordingly, the MAC can be sub-divided into

A. MAC morphology and ultrastructure
B. MAC inhibition, i.e. the concentration of drug that produces a one log decrease (90%) in a test population when compared to a control population after five h of incubation. A short incubation time was proposed because experiments run in parallel showed that short incubation and long incubation (18-24 h) produced comparable results.

(Lorian and de Freitas, 1979; Lorian, 1980)

MAC morphology and ultrastructure

The only site of growth for Gram-positive cocci is at the equatorial plane where there is a balance between synthesis and lysis (Lorian, 1975). When Staphylococci are treated with penicillin at equal to or greater than the MIC, the septum soon loses its density, the cell wall becomes thinner and lysis results. In contrast, at sub-MICs the septum becomes thicker whilst the cell wall remains normal (Giesbrecht et al., 1985; Warren and Stasny, 1968). In detail, cells of S. aureus 502A became larger and irregularly shaped at 1/3 MIC. Septa present were twice as thick as control cells yet lacked a central dense layer. A medial septum divided the cell into two uneven hemispheres and each hemisphere could show two or more septa, generally at an angle to the central septum (Fig 4b). The surrounding cell wall appeared to be thicker only in localised areas. Two hours after removal to drug-free agar, normal forms of cocci were seen. Many of the abnormal forms had become smaller and with fewer septa than previously. The central septa showed lysis and separation in some instances and the viable count increased significantly (Lorian, 1975).

In the presence of sub-MICs of β-lactam drugs the cells are still able to produce peripheral wall and divide although PG cross-linking is much reduced (Wyke et al., 1982), as evidenced by the large, multinucleate cocci. The drug appears to reversibly inhibit the septum hydrolase and in some instances, pentaglycine subunits accumulate at the septum (Seidl, Zwerenz and Golecki, 1985). It illustrates that peripheral wall growth and septum formation are two separate processes. The actual molecular processes leading to these morphological and ultrastructural effects are still poorly characterised.

MAC inhibition

Whilst a 90% reduction in bacterial load for a wound is not by definition sterility, a decrease in load is recognised as significant in recovery from infection (Kunin, 1970; Louria, 1962; Lyman, Tenery and Basson, 1977), especially if coupled with good patient practice. This form of MAC does not always result in large differences between test and control and can be difficult to determine.

MAC morphology and ultrastructure:

Many experimental conditions have been employed in the study of antibiotic-bacterium-host interactions which can make interpretation and comparison difficult (Gemmell, 1984; Milatovic, 1983).Early studies showed that lipase production in S. aureus was unaffected whilst titres of α-haemolysin were increased by penicillin and methicillin (Hallander, Laurell and Lofstrom, 1966) and not only methicillin but also bacitracin and vancomycin (Kobayasi, Barnett and Sanford, 1966). The latter study demonstrated that lincomycin, chloramphenicol and erythromycin not surprisingly all depressed α-haemolysin production. The means of enhancement by the wall-acting drugs was not stated but possibly they enhanced selective release through a "leaky" wall. Sub-MICs of protein synthesis-inhibiting drugs such as clindamycin or fucidic acid depress levels of extracellular products such as staphylocoagulase, alpha-haemolysin, lipases, with a concommitant decrease in virulence (Gemmell and Shibl, 1976; Shibl, 1983).

Sub-MICs of penicillin G induced a significant increase in the secretion of soluble PG with possible immunogenic consequences (Section 1.2.2.1; Seidl and Schleifer, 1985). They also induce loss of MTA (Beachey et al., 1985; Suginaka et al., 1979; Utsui et al., 1983) which in S. aureus is an adhesin for nasal and buccal mucosa

(Aly et al., 1980; Carruthers and Kabat, 1983). The adherence of a bacterium to a host mucosal surface is the primary event in pathogenesis of many infections (Section 1.5.5). Often the degree of illness produced correlates well with adherence ability, e.g. the capacity to cause endocarditis matched the degree of the causative organisms' adherence to the endothelial surfaces of heart valves (Gould,Ramirez-Ronda, Holmes and Sandford, 1976). Encouragingly, sub-MICs of β-lactams decreased the adherence of Streptococcus sanguis to heart valves in vitro (Ramirez-Ronda and Gutierrez, 1980) whilst pre-treatment of Strep. pyogenes with a sub-MIC of penicillin G also reduced adhesion because of a loss of MTA (Alkan and Beachey, 1978). However, a sub-MIC of oxacillin increased the plasmid-mediated adherence of S. aureus to HeLa cells by unknown mechanisms (Dunkle, Blair and Fortune, 1985).

Fibronectin, a plasma protein, is known to act as a bacterial adhesin (Section 1.5.5; Simpson and Beachey, 1983). A β-lactam-associated increase in fibronectin binding sites, perhaps due to exposure of deep-seated envelope sites has been detected (Beachey et al., 1985; Proctor, Olbrantz and Mosher, 1983). It is possible that the ability of S. aureus to adhere to serum fibronectin probably exposes it for a longer period therefore predisposing to removal by the reticuloendothelial system.

Sub-MICs of penicillin G were shown to increase the susceptibility of Staphylococci to lysozyme/trypsin mixtures (Warren and Gray, 1967) whilst penicillin G treatment rendered S. aureus more sensitive to leucocyte extracts (Ginsberg, Sela, Ne'eman and Lahav, 1983). However, the most significant aspect of sub-MICs of β-lactams is their influence upon bacterial interactions with phagocytes (Eickenberg, Hahn and Opferkuch, 1982; Gemmell, 1984; Lorian, 1980;

Milatovic, 1983; Yourtree and Root, 1982).

β-lactams were said to not to affect S. aureus susceptibility to phagocytosis (Milatovic, 1982) but cultures were grown overnight in the presence of drug before testing so this is not surprising.

Modification of the S. aureus surface by pre-treatment with penicillin G increased susceptibility to killing by intact neutrophils (Root and Metcalf, 1978), more specifically by the non-oxidative mechanism (Section 1.5.7; Root and Metcalf, 1979). This was found to apply to penicillinase-producing strains of S. aureus, and also other wall-active drugs such as vancomycin (Isturiz, Andriole and Root, 1979). It is possible that the S. aureus surface was altered to allow enhanced binding of cationic proteins. The observed killing did not involve the lytic sequence of C′ activity (Yourtree, Metcalf and Root, 1980) and this contrasts the effects of fucidin and lincosamines upon Streptococcus pyogenes ; these antibiotics caused greater opsonisation and ingestion by enhanced activation of C3b on the bacterial surface (Gemmell and O′Dowd, 1983; Gemmell, Peterson, Schmeling, Kim, Mathews, Wannamaker and Quie, 1981; Gemmell, Peterson, Schmeling and Quie, 1982; Milatovic, Braveny and Verhoef, 1983) probably due to a reduction in Pr A (Gemmell and O′Dowd, 1983). As a result, lower concentrations of serum could be used for opsonisation (Milatovic et al., 1983) and neutrophil chemotaxis was enhanced (Gemmell, 1985). When neutrophil phagolysosome (phagosome) formation was inhibited by cytochalasin B,enhanced killing of penicillin pre-treated S. aureus was still detected, the mechanism of the action was not elucidated but opsonising serum had to be present in order to bind the organism onto opsonic receptors on the cytochalasin-treated neutrophil surface and penicillin-enhanced killing was dose-dependent. Vancomycin, but not gentamicin, gave

similar results (Root, Isturiz, Molavia, Metcalf and Malech, 1981).

Pre-treatment with sub-MICs of cloxacillin and nafcillin also rendered S. aureus more susceptible to killing by bovine neutrophils (Craven, Williams and Anderson, 1982).

Viable count-based assays of phagocytosis of pre-treated S.aureus can be misleading. This is because the mass of these morphologically-altered cocci is bigger than that of normal controls. Hence, assays based upon determination of mass reveal that large staphylococci induced by pre-treatment with β-lactams are better phagocytosed than controls (Lorian, 1985).

1.5 Interactions with the host

1.5.1 Pathogenesis

Pathogenesis is multifactorial and pathogens are usually capable of infecting external surfaces (skin, mucosa) and entering the host where they then multiply and resist or interfere with host defences, with resultant damage to host tissues (Hammond et al., 1984; Smith, 1984).

Staphylococci initiate disease in mammals by still poorly understood mechanisms. Humans and animals seem to have a high, natural immunity to staphylococcal disease. This is apparent because these organisms are commonly found in, e.g. the nares or axillae without necessarily causing morbidity. Indeed "man becomes the habitat of a very rich microbial flora. The skin, the mucous membranes, the intestines and the genital organs offer a feeding ground for bacteria of all kinds" (Metchnikoff, 1905). There are though host species differences in natural immunity (Gladstone and Glencross, 1960) due for example to the efficiency of neutrophils

(Donnelly and Stark, 1985; Williams, Craven, Field and Bunch, 1985) or speed of the inflammatory response (Donnelly and Stark, 1985).

As a reflection of this immunity, there is a rapid host response to most foci of S. aureus infection and localisation by neutrophils. In fact, a typical severe S. aureus sepsis consists of an acute infection with neutrophil infiltration and neutrophil leucocytosis in peripheral blood.

As a further reflection of this immunity attempts to induce disease usually need large inocula, e.g. approximately 10^6 cfu in 0.1ml volumes were needed to produce localised skin infections in humans (Elek and Conen, 1957). There was no reduction in the pus-forming dose for intradermal, subcutaneous and full thickness infections nor for different S. aureus strains (carrier, clinical isolates and epidemic strains). It was concluded that because natural infections due initially to this high dose are rare, S. aureus must be able to delay host defences and reach the critical number of cfu.

The presence of a foreign body dramatically reduces the number of S. aureus cells required to cause a clinical infection. Thus 10^2 cfu on cotton sutures caused abscesses in man (Elek and Conen, 1957) whilst in mice, 10^1 cfu of certain S. aureus strains induced disease when implanted subcutaneously on cotton dust (Noble and White, 1983) and as little as 15 cfu of S. aureus produced sero-purulent exudate and erythema in humans when introduced onto human skin whose stratum corneum had been broken by scraping. Milk contains several protein and lipid antibacterial substances derived from the teat canal tissue but if challenge organisms are inserted past the teat canal, only very few S. aureus are needed for infection (Anderson, 1983). Even less cfu are needed if the inocula is previously grown in vivo (Section 1.6.2) and this is often overlooked.

However, if the non-specific host resistance is high, then the specific immunity to re-infection is low. Hence the incidence of spots or boils amongst the general population.

The lack of a suitable animal model of the classical human infection, the abscess, has hampered the determination of the host and bacterial factors that allow S. aureus to become a pathogen. It is reasonable to suppose that as S. aureus diseases are superimposed on an already immunised host, they circumvent or inhibit host defences. The evolution from infection, i.e. colonisation, to the disease state seems invariably to involve local or generalised immunocompromisation. This can be caused by several factors, commonly extremes of age, existing infection, foreign bodies, metabolic disturbances or trauma which result in a lowering of the size of the inoculum to cause infection. But even so, relatively large numbers can still be needed, e.g. at least 10^5 cfu per ml of gram of host tissue were needed in a porcine burns model (Wadström, Björnberg and Hjertén, 1985). It is probable then that the enzymes and cell envelope factors involved in pathogenesis of S. aureus exert their effects by synergistic interactions and that what is clinically observed is the sum result.

1.5.2 Primary and Secondary defences

The primary defence against S. aureus is the intact integument hence the common place observation that cuts and scratches and even mild scraping such as shaving predisposes to infection. Infections frequently begin around hair follicles but the exact mechanisms are poorly characterised.

The role played at the skin surface by factors such as lysozyme or immunoglobulins is still unknown. On the face of it, lysozyme is presumed not to affect S. aureus (but see section 1.5.7.4b). Skin-

localised IgG, IgM, IgA and IgE have been detected in man and animals and active local production occurs as a response to introduction of antigen.

Once S. aureus has penetrated the skin or mucosa, it faces a second line of host defence known generally as inflammation (Mims, 1977). The outcome of the inflammatory lesion is usually decided within three to five h. There are obvious signs of reddening and elevated temperature with transient vasoconstriction and then vasodilation. Local release of histamine by mast cells is followed by infiltration of various leucocytes into the damaged tissues. Usually infection resulting from skin trauma is localised and phagocytes assume the role of defence. Neutrophils arrive as early as one h post-infection and peak at approximately three h post infection.

Various antibacterial substances are released during inflammation. These include iron-binding proteins (Section 1.5.3), progesterone, platelet-derived proteins, neutrophil-derived cationic proteins (Section 1.5.7.4b) and acute phase proteins. Clotting also occurs and this helps to restrict the spread of infection (Roitt, 1984). Host differences such as a more rapid inflammatory response in rats, c.f. mice and guinea pigs, allow faster elimination of S. aureus (Donnelly and Stark, 1985).

The major staphylococcal pathogenic determinant in this instance is its ability to inhibit the inflammatory response (Agarwal, 1967) and DOCR has already been mentioned (Section 1.2.2.1) but it didn't supress the response in immune as distinct from normal mice. α-haemolysin, when present, is anti-inflammatory in that it is vasoconstrictory. C′ consumption can be very high at the site of an infection and it can lead to eventual reduced opsonic activity. Also,

the chemotaxigenic product C5a may diffuse and cause aggregation of neutrophils away from the site (Verhoef and Verbrugh, 1981).

1.5.3 Importance of iron

Iron is one of the most common elements on Earth. In biological systems, it is involved in storage and transport of oxygen, electron transport and concommitant generation of energy, in decomposition or utilisation of hydrogen peroxide, and also in non-haem iron systems such as ferredoxin. Despite being essential, it is also toxic to virtually all life forms (Neilands, 1972 and 1974).

Utilisation of environmental iron by organisms is difficult because iron exists as insoluble ferric hydroxide polymers at physiological pH and under aerobic conditions. The trend to polymerisation is so great that the equilibrium concentration of free ferric iron cannot exceed approximately 10^{-18}M, which is too low to allow free iron to participate in biochemical reactions (Aisen, 1977). Thus, iron has to be solubilised and complexed for biological reactions.

Evidence was obtained early on that the inhibitory activity of serum and egg white for micro-organisms was due to their iron-binding properties and could be overidden by excess iron (Schade and Caroline, 1944 and 1946). This helped to delineate the role of iron in host-parasite relationships (Finkelstein, Sciortino and McIntosh, 1983) and lead to the "nutritional immunity" concept whereby host mechanisms complex iron for host useage, thereby denying pathogens.

Animals contain iron far in excess of the 0.4-4.0 μm iron needed by Gram-positives (Weinberg, 1974), but it is mostly located intracellularly, as ferritin, haemosiderin, haemoglobin and myoglobin and is generally unavailable to bacteria. The concentration of free

iron in body fluids is approximately 10^{-18}M Fe^{3+} (Weinberg, 1978; Bullen, 1981).

This low amount of iron is tightly bound to a family of host carrier and transport glycoproteins that have association constants of about 10^{36}M for iron (Bullen, 1981; Griffiths, 1983). Transferrin is present in the blood and lymph (Aisen and Listowski, 1980), lactoferrin is found in mucosal secretions (Masson, Heremans and Dive, 1966) and neutrophils (Bullen and Armstrong, 1979). These glycoproteins have two iron binding sites, each capable of reversibly binding one ferric ion and simultaneously, one bicarbonate ion. Lactoferrin is normally highly unsaturated and thus a more avid iron scavenger than transferrin which is used more for transport. Also lactoferrin differs from transferrin in that it can function at lower pH.

Inflammation triggers synthesis of transferrin (Beumier, Caldwell and Holbrin, 1984) and release of lactoferrin from neutrophils (Weinberg, 1978). Iron-saturated lactoferrin is removed from circulation by the macrophages of the reticuloendothelial system (van Snick, Masson and Heremans, 1974) whilst iron moves from transferrin to the iron stores, i.e. a "hypoferraemia of infection" (Griffiths, 1983).

Effective pathogens are thus likely to be capable of sequestering iron more effectively than host systems. Bacterial iron uptake mechanisms have been well characterised but only for Gram-negative bacteria (Griffiths, 1983; Neilands, 1981 and 1982). Very little is known about those in Staphylococci although they exhibit iron-dependent characteristics so that iron deprivation reduced growth rate and final yield, catalase, staphylocoagulase and toxins including haemolysins (Schade, 1963; Schade, Myers and Reinhardt, 1968; Theodore

and Schade, 1965a) and pigmentation was lost (Schade, 1963). The MIC for penicillin G was also reduced by iron deprivation (Schade et al., 1968) and aerobic metabolism was affected as iron-poor S. aureus was limited in its ability to oxidise glucose and was unable to use pyruvate, lactate or formate, i.e. it had increased dependence on glycolytic rather than oxidative energy production (Schade, 1963; Theodore and Schade, 1965 b; Schade et al., 1968).

The Staphylococcal chelator(s) and those of the Enterobacteriaceae can be used by each other but those from the Gram-negatives are more effective at stimulating Staphylococcal growth than vice versa (Marcelis, den Daas-Slagt and Hoogkamp-Korstanje, 1978; Maskell, 1980; Miles and Khimji, 1975). However, the Staphylococcal receptors are sufficiently specific not to be able to use spermidine catecholamide iron chelators to overcome iron unavailability (Bergeron, Elliott, Kline, Ramphal and St. James III, 1984). S. aureus resisted the inhibitory effects of transferrin far better than coagulase-negative Staphylococci (Marcelis et al., 1978; Valenti, De Stasio, Mastromerino, Seganti, Sinibaldi and Orsi, 1981) whilst lactoferrin inhibited toxigenic S. aureus but only at between 0.02-2.0 mg ml^{-1} (Batish, Chander, Zumdegni, Singh and Bhatia, 1984). Ovotransferrin also had a bacteriostatic effect not only because of iron witholding but possibly because of direct interaction with the Staphylococcal envelope or slow deprivation of the internal iron pool (Valenti, Antonini, Rossifanelli, Orsi and Antonini, 1982).

In other Gram-positive bacteria, an ATP-dependent non-specific cation uptake system exists in Strep. mutans. This organism lacks cytochromes but its growth needs or is stimulated by iron (Evans, Arcenaux, Martin, Aranha and Byers, 1985). Although,

Listeria monocytogenes exhibits iron-stimulated growth it too lacks a high affinity iron uptake system and removes iron from transferrin and ferritin by a largely uncharacterised 8-10 Kd soluble reductant. There was no direct interaction between transferrin and the envelope surface (Cowart and Foster, 1985).

Iron not only features in nutritional immunity but also has a role in the conventional immune defences. Hypo- and hyperferraemia predispose to infections (Weinberg, 1974, 1978 and 1984) and the balance between the opposing effects influences the host-parasite relationship. Iron-deficency has been linked to impaired intracellular killing of S. aureus by human neutrophils (Chandra, 1973) which was corrected by parenteral administration of iron. But, the earliest record of the use of iron in human therapy dates from 1500 BC. Prince Iphyclus of Thesally was cured of sexual impotence by Melampus a physician. Melampus scraped the rust from a knife which had been embedded in an oak tree for an unknown period, into some urine which Iphyclus drank. After ten days of such treatment, Iphyclus was cured (Frazer, 1935).

Iron-overloading saturates the transferrins and nullifies their bacteriostatic effect (Bullen, 1981; Weinberg, 1978). Additionally, excess iron impaired killing of S. aureus (van Asbeck, Marx, Struyvenberg, van Kats and Verhoef, 1984) and reduced ingestion (Waterlot, Cantinieaux, Hariga-Muller, De Maertelaere-Laurent, Vanherweghem and Fondu, 1985). It interfered with the neutrophil cationic proteins (Gladstone and Walton, 1970 and 1971) specifically with the myeloperoxidase activity (Waterlot et al., 1985) and also catalysed the formation of oxygen species toxic to neutrophils (van Asbeck et al., 1984).

1.5.4 Role of surface hydrophobicity (SH) and surface charge (SC)

Hydrophobic properties of protein molecules, are primarily attributed to side chains of phenylalanine, tyrosine and tryptophan residues, and to folding of the polypeptide chain to give tertiary and quartenary structures (England, 1986). In MTA or lipopolysaccharide (LPS), hydrophobicity is conferred by the presence of lipid moieties.

SH can be assayed by contact angles (CA) using saline drops (van Oss, Gillman and Neuman, 1975) or with air bubbles and immersed, inverted bacterial layers (Fattom and Shilo, 1984). Other methods include partitioning between hydrocarbons and an aqueous buffer (Lichtenberg, Rosenberg, Sharfman and Ofek, 1985; Rosenberg, 1984; Rosenberg, Gutnick and Rosenberg, 1980), hydrophobic interactions between non-polar groups on both bacteria and resins (Jonsson and Wadström, 1983; Smyth, Jonsson, Olsson, Söderlind, Rosengren, Hjertén and Wadström, 1978; Wadström, Hjertén, Jonnson and Tylewska, 1981), precipitation or "salting out" by ammonium sulphate (Lindahl, Faris, Wadström and Hjertén, 1981; Rozgonyi, Szitha, Ljungh, Baloda, Hjertén and Wadström, 1985), and the partitioning of radioactive fatty acids between bacteria and an aqueous buffer (Malmqvist, 1983).

All these assays measure different aspects of SH yet insufficient comparative studies have been done. Hydrophobic interaction chromatography (HIC) is relatively sensitive and revealed degrees of polarity for less hydrophobic bacteria whilst bacterial affinity to hydrocarbons (BATH) revealed degrees of difference between relatively hydrophobic strains only; presumably because the interfacial tension is high in the first place (Kjelleberg and Hermansson, 1984). BATH also interacts with membrane phospholipids, MTA or the inner core of LPS, whilst salting out interacts with hydrophobic surface components

and these differences resulted in a lack of correlation between the two methods when applied to E.coli (Ferreirós and Criado, 1984). Elsewhere BATH correlated well with CA and HIC when benthic and planktonic bacteria were studied (Fattom and Shilo, 1984), and CA and partioning in a polyethylene glycol (PEG) system correlated for enteric bacteria (Magnusson, Dahlgren, Maluszynska, Kihlström, Skogh, Stendahl, Söderlund, Öhman and Walan, 1985). Radioactive fatty acid partitioning into S. aureus was consistent with HIC and PEG (Malmqvist, 1983). Thus, not only is the choice of SH assay important but at least two should be used (Ferreirós and Criado, 1984; Kjelleberg and Hermansson, 1984; Magnusson, Dahlgren, Maluszynska, Kihlström, Skogh, Stendahl, Söderlund, Öhman and Walan, 1985).

A correlation was noted between SH and SC in that envelope constituents that conferred an increased SH also determined a strongly negative SC (Stendahl, 1983; Xiu, Magnusson, Stendahl and Edebo, 1983). Similar precautions apply to SC assays as, for example, ionised PEG or electrostatic interaction chromatography (EIC) measure localised charges due to interactions between parts of the bacterial envelope and substrate, whilst particle electrophoresis registers movements of cells together in an electrical field, i.e. the sum of all bacterial surfaces (Hermannson, Kjelleberg, Korhonen and Stenström, 1982; Magnusson et al., 1985).

Environmental factors such as nutrition, growth phase or the presence of antibiotics alter SH. Iron deprivation is known to decrease both SH and SC of Neisseria gonorrhoea (Magnusson, Kihlström, Norquist, Davies and Normark, 1979). SH of S. aureus was supressed by unknown factors in blood agar but enhanced by brain heart infusion agar (Ljungh, Hjertén and Wadström, 1985; Wadström, Brown, Ljungh, Rozgonyi and Hjertén, 1985). Exponential-phase S. aureus were more

hydrophobic than in stationary phase (Miörner, Albertsson and Kronvall, 1982; Wadström et al., 1985b). It was shown that just before onset of exponential phase growth there was an abrupt 4-5 fold increase in SH. As stationary phase was approached, SH reached a plateau level and then slowly decreased throughout stationary phase (Malmqvist, 1983). The SH of low Pr A-producing S. aureus strains was increased by sub-MICs of penicillin G by uncharacterised mechanisms (Wadström et al., 1981).

Approximately 90% of clinical isolates of S. aureus had a high SH relative to non-clinical isolates (Ljungh et al., 1985; Wadström et al., 1985b). SH appeared to be related to the Pr A content as Pr A-rich S. aureus strains Cowan I and 17970, had higher relative SH than did S. aureus strains that produced less Pr A. However, other surface structures such as the fibronectin-binding protein and other protease-sensitive components probably contribute to SH because enzyme digests drastically reduced SH (Ljungh et al., 1985) and because some Pr A-negative S. aureus strains had the same high relative SH values as high level Pr A-producers (Jonsson and Wadström, 1983; Wadström et al., 1981). Possession of a capsule rendered S. aureus strains hydrophilic (Jonsson and Wadström, 1983; van Oss et al., 1975).

The knowledge of the SH properties of S. aureus has been exploited in the production of a hydrophobised wound dressing that actively removes S. aureus from wounds thereby speeding up healing (Wadström, Bjornberg and Hjertén, 1985).

The phagocytic process (Section 1.5.7) is a multi-step surface phenomenon that can be described in physico-chemical terms (Mudd and Mudd, 1933; Stendahl, 1983; van Oss, 1978). The first step results in attachment of the bacterium to phagocyte membrane receptors via

an energy-dependent process.

There are receptors that undergo specific ligand-ligand or antibody-C′ interactions that are saturable and possibly represent evolved mechanisms. There are also receptors for non-specific interactions such as SH and SC which can be regarded as an earlier, more primitive system (Magnusson, et al., 1985). It is possible that specific binding mechanisms may have evolved to modulate the predicted sequences of phagocyte-prey interactions governed by genetically more ancient mechanisms such as SH or SC.

In non-opsonised systems, the virulent encapsulated S. aureus M was less prone to phagocytosis and had a lower CA than avirulent noncapsulated strains. Most importantly, CAs for virulent bacteria were lower than those of phagocytes (van Oss et al., 1975), i.e. the relative interfacial tensions of the bacterium and phagocyte were important. The CA of human neutrophils is approximately 18^{o} (van Oss et al., 1975; Tufano, Romano-Carratelli, Sommese, Bentivoglio and Galdiero, 1985) and bacteria with a lower CA, i.e. a lower interfacial tension, were not easily ingested. Other human blood cells had the same CA as the neutrophils, thankfully. Binding of specific antisera during opsonisation increased S. aureus SH to give CA greater than 18^{o} and the presence of C′ ensured maximum attainable SH. C′ alone had no action on the SH (van Oss et al., 1975). The increase in SH was also caused by normal human serum and purified IgG and was dose-dependent for IgG (Miörner et al., 1980). SH was increased by fibrin and fibrinogen (Miörner et al., 1980; van Oss et al., 1975) which made ingestion easier (van Oss et al., 1975). Thus, one result of successful opsonisation is an increase in bacterial SH relative to that of the phagocyte. Penicillin G increased the SH of S. aureus (Wadström et al., 1981) but did not affect neutrophil SH (van Oss

et al., 1975). IgA reduced S. aureus SH which is significant as IgA's role may be to protect mucosal surfaces by reducing bacterial adherence (van Oss et al., 1975). Perhaps the reported interference of opsonisation by Pr A (Section 1.2.2.2) is because it binds IgG at F_c exposing the hydrophilic F_{ab} portion to the neutrophil. Significantly, abnormally high neutrophil SH values have been reported in diabetics prone to recurrent infections (Galdiero, Romano-Carratelli, Folgore and Nuzzo, 1983; van Oss et al., 1975) and a group of children with recurrent middle ear and upper respiratory tract infections (Gillman, Bernstein and van Oss, 1976).

In natural environments, hydrophobic adhesins were partly responsible for adherence and also for scavenging surface-localised nutrients (Kefford, Kjelleberg and Marshall, 1982; Kjelleberg, Humphrey and Marshall, 1983). The in vivo significance of adherence due to SH is apparent in that expression of high SH (in BATH) correlated with adherence of oral streptococci to saliva-coated hydroxylapatite (Nesbitt, Doyle and Taylor, 1982; McBride, Song, Krasse and Olsson, 1984). The degree of SH of various bacteria correlated positively with the degree of adherence to different types of animal cells (Magunsson et al., 1985; van Oss et al., 1975; Xiu et al., 1983. However, it can be misleading to ascribe adherence solely to SH because factors such as SC (Craido, Ferreirós and Sainz, 1985) or other interactions (Section 1.5.5) modulate adherence.

1.5.5 Adherence

The attachment of bacteria to host tissues is the initial step in the pathogenesis of many infections. The process, affinity or rate of this process is known as adhesion whilst adherence can be regarded as the actual state or end result (Vosbeck and Mett, 1983). Many

interactions that allow bacteria to become adherent to host tissues involve very specific receptors, of a proteinaceous or carbohydrate nature, that are found on both host and parasite envelopes (Beachey, 1981; Feingold, 1986; Jones and Isaacson, 1983; Ofek and Beachey, 1980; Pistole, 1981).

Neutrophils especially, have an evolved system capable of recognising F_c, C3b, carbohydrates such as NAG, glucose, galactose, mannose and lectins. Binding of the latter is not as strong as C′ mediated binding and not as potent in mediating ingestion but lectins co-operate with the non-specific system to promote attachment to host tissues.

Lectins are protein or glycoprotein molecules that include enzymes, structural components, hormones, toxins, whose main characteristic is their ability to bind sugars by virtue of having several binding sites on each molecule. Lectin-like proteins have only one site and fall outside the definition (Weir, 1980).

Bacteria are also bound to phagocytes by the recognition of bacterial envelope carbohydrates by lectin-like receptors on the phagocyte surface (Weir, 1980; Symington, Klebanoff, Waltersdorph, 1984) and this can trigger the phagocytic sequence (Section 1.5.7; Symington et al., 1984).

The mechanism by which S. aureus colonises some people and not others remains obscure. Similarly, little is known about how S. aureus adheres to and colonises host tissues especially the intact skin. S. aureus adhered better to the skin and nasal epithelium of atopic dermatitis patients (Bibel, Aly, Shinefield and Maibach, 1983) and it showed greater adherence for atopic corneocytes than those from psoriasis or normal controls. Pr A interactions were implicated and other skin organisms did not show this preference (Cole and

Silverberg, 1986). Adherence to canine kidney cells did not correlate with SH determined by BATH and was mediated by both heat-sensitive and heat-resistant adhesins depending upon the S. aureus strain. These adhesins were a diverse group of minor proteins. Influenza A virus infection increased host receptors for the heat-resistant adhesins (Sanford, Davison and Ramsay, 1986). S. aureus exhibited preferential uptake to and ingestion by human endothelial cell lines. Although uptake of and invasion by S. aureus could not be distinguished, evidence suggested the phenomena had a role in endovascular colonisation and subsequent metastatic events (Ogawa, Yurberg, Hatcher, Levitt and Lowy, 1985).

It is known that the HLA host type is influential either by coding for specific receptors or for uncharacterised immune mechanisms (Kinsman, McKenner and Noble, 1983), whilst host tissue maturation influences bacterial binding as receptors for MTA were not found on human adult buccal epithelial cells but on embryonic and foetal cells (Nealon and Mattingly, 1985).

S. aureus is able to adhere specifically to the ductular epithelial cells of the bovine mammary gland (Frost, Wanasinghe and Woolcock, 1977) and to pharyngeal cells. In the latter case, adherence was increased as a result of viral infection, smoking or bronchitis (Fanstein, Musher and Cate, 1980).

Adherence of S. aureus to human buccal epithelial cells is dependent upon the lipid portion of MTA and non-specific SH interactions were implicated. However, adherence suffered after trypsinisation of cocci so it may be that an MTA-protein configuration is needed for full adherence (Carruthers and Kabat, 1983).

S. aureus adheres better to nasal mucosal cells of human

carriers. WTA was involved in this by virtue of binding to fibronectin (see later) of the epithelial surface. Two host receptors featured, one found on immature and keratinised cells that was not blocked by exogenous TA and was responsible for the increased binding of S. aureus to atopic cells. It could be that this receptor is similar to or the same as the receptor for Pr A mentioned earlier (Cole and Silverberg, 1986). The second receptor was present only on keratinised cells and was blocked by WTA (Aly et al., 1980; Bibel et al., 1983; Bibel, Aly, Shinfield, Maibach and Strauss, 1982). Some adhesins, non-characterised and for HeLa cells, are plasmid-mediated (Dunkle, Blailand and Fortune, 1985 and 1986).

Non-specific host mechanisms that protect against adhesive staphylococci include the ciliated epithelial cells of respiratory and genital tracts, the anti-adhesive effect of mucus production and the witholding of essential nutrients (Magnusson et al., 1985; Sugarman and Epps, 1985).

IgG and IgA binding to bacteria altered their envelope carbohydrate topology so that galactose, mannose, and NAG residues became exposed. It could be that these are then recognised by lectin-like receptors on surfaces of the components of the reticulo-endothelial system, eg. Kuppfer cells, and more rapidly eliminated (Magnusson et al., 1985).

Tissue tropism can also be mediated by interactions between S. aureus envelope receptors and host serum, epithelial and matrix proteins. Pr A and clumping factor are well known but there is growing awareness of other, poorly-characterised receptors on S. aureus for laminin (Lopes, dos Reis and Brentani, 1985; Vercelloti, McCarthy, Lindholm, Peterson, Jacob and Furcht, 1985), collagen (Vercellotti et al., 1985), the human serum spreading factor

glycoprotein (Fuquay, Loo and Barnes, 1986) and fibronectin (FN). Of these, the FN interaction is most characterised. FN can act as an effective opsonin for S. aureus (Eriksen, Esperson and Clemmensen, 1984) and indeed seems to be necessary for optimum opsonisation (Lanser and Saba, 1982). FN aids adhesion of endothelial cells and in that situation it may have a role in binding Gram-positive bacteria, especially S. aureus, to host vascular surfaces (Vercellotti, Lussenhop, Peterson, Furcht, McCarthy, Jacob and Moldaw, 1984). Adherence of S. aureus to FN is altered by cultural conditions, eg. increased by penicillin G (Beachey et al., 1985; Proctor, Mosher and Olbrantz, 1982; Proctor et al., 1983) and is dependent on an envelope protein (Beachey et al., 1985; Proctor et al., 1983), that is not Pr A (Switalski, Rydén, Rubin, Ljungh, Höök and Wadström, 1983; Vercellotti et al., 1984). The degree of effective masking of the FN receptor on the envelope surface depends upon capsular thickness (Switalski et al., 1983; Vercellotti et al., 1984). No single species of Staphylococcus is uniformly positive or negative in binding FN but this phenomenon is very common amongst those Staphylococci associated with infection. The ability to adhere to FN correlated with the ability to cause endocarditis in rabbits (Scheld, Strunk, Balian and Calderone, 1985) and aided S. aureus in adherence to wound fibrin deposits (Toy, Lai, Drake and Sande, 1985).

1.5.6 Humoral Immunity

Once S. aureus is established, antibodies are produced both to extracellular products and envelope antigens (Adlam and Easmon, 1983; Verhoef and Verbrugh, 1981). These have little role in prevention of infection as they do not lyse S. aureus, phagocytosis is efficient in both normal and immune serum and antibodies are not involved in

intracellular killing but opsonisation prior to ingestion.

Serum itself has little or no effect on S. aureus because envelope polysaccharides and TA protect against lysozyme (Ginsburg, 1979). The TA may also "intercept" and inactivate neutrophil-derived cationic proteins present in the serum (Section 1.5.7.d).

1.5.7 Phagocytosis and intracellular killing

Once the outer epithelial barrier is breached and inflammation occurs, phagocytosis is the most important part of the host defences (Stossel, 1975; Verhoef, 1982). Phagocytosis is a co-ordinated, multi-step process by which cells transport particles from the extracellular environment into intracellular vacuoles or phagosomes. It most commonly occurs in "professional" phagocytes such as neutrophils and macrophages.

1.5.7.1 Chemotaxis

The histological hallmark of an acute inflammation is the accumulation of neutrophils in the tissues. Inflammation opens the area for neutrophil invasion and chemotaxis is thus a positive response to a concentration gradient of a mediator(s) or chemotaxin(s) and is induced by both host and microbial factors. Chemotaxins however can be direct, indirect and even negative.

The S. aureus wall, especially the PG and TA components, seems to be the most important chemotaxin. It operates optimally in the presence of both the classical C′ pathway because C5 is split to the chemotaxigenic C5a, and antibody if the latter has a specificity for the D-alanyl-D-alanine of the pentapeptide. Protease production either by S. aureus or from damaged host tissues, can split C3 and C5 to produce chemotactic C3a and C5a. Thus, the major neutrophil

chemotactic response to S. aureus infection results through the activation of complement (Schmeling, Peterson, Hammerschmidt, Youngki, Verhoef, Wilkinson and Quie, 1979).

Several S. aureus products e.g. the α-haemolysin and leucocidin inhibit neutrophil migration. Perhaps long-range chemotaxins act to attract neutrophils to a local site but then short-range negative chemotaxins are used by S. aureus to protect itself (Russel, Wilkinson, McInroy, McKay, McCartney and Arbuthnott, 1976).

1.5.7.2 Opsonisation and association (adherence) with phagocytes

Greek OPSONOS "to prepare food for". Opsonisation is necessary for the efficient phagocytosis of S. aureus (Wright and Douglas, 1904). The ability to distinguish foreign particles is inherent in the specific receptors of neutrophils and some micro-organisms have surface receptors that directly attach to neutrophil receptors. However opsonisation enhances this and allows selective engulfment and optimal phagocytosis. Receptors on the neutrophil surface detect the F_c of immunoglobulins especially IgG 1 and 3, as well as C3b of C′. Opsonic antibodies not only act as bridges or ligands and bind S. aureus with F_{ab} and the neutrophil receptor with F_c but also neutralise antiphagocytic factors on S. aureus. Antibody and C′ either both feature in subsequent attachment and ingestion or C3b features mainly in the attachment phase and bound antibody acts as the trigger for ingestion (Verhoef and Verbrugh, 1981).

Opsonisation characteristics are strain dependent so that S. aureus can be satisfactorily opsonised in the presence or absence of antibody and with or without the classical or alternate pathway (Verhoef, Peterson, Kim, Sabath and Quie, 1977). But a pattern exists such that in normal serum, attachment of S. aureus is mainly to the

neutrophil C3b receptor whilst in immune serum it is via the neutrophil IgG receptor (Verhoef, Peterson and Quie, 1977). Paradoxically, strains associated with disseminated intravascular coagulation tended to be very potent activators of the C′ pathways (Tuazon, Sheagren and Quie, 1981).

Opsonisation via the classical C′ pathway takes up to 5 min, whilst it can take approximately 60 min via the alternate C′ pathway. Once C3b is attached engulfment is very efficient; bacteria opsonised without C′ are far less effectively attached to neutrophils (Verhoef, et al., 1977b). C′ can itself be fixed without the need for IgG. The alternate C′ pathway can opsonise S. aureus in the absence of both classical pathway and antibody but it can only be activated by PG, i.e. not by capsulated strains.

Physicochemically, C3b and F_c attachment can be said to alter SC to a more negative state and increase SH so that engulfment is enhanced (Verhoef, 1982; van Oss, 1978).

There is growing awareness that bacteria in vivo especially in chronic infections are adherent to surfaces or in a focus of infection such that phagocytosis occurs against solid surfaces, eg. in abscesses, clots, or the epithelial tissues (Section 1.6). In vitro "surface phagocytosis" (Wood, Smith and Watson, 1946) and subsequent killing of S. aureus did not require opsonisation in contrast to cell and suspension assays although it was enhanced by it (Hayashi, Lee and Quie, 1986; Lee, Holdal, Clawson, Quie and Peterson, 1983; Vandenbroucke-Grauls, Thijssen and Verhoef, 1985). Surface phagocytosis is dependent on the density of cells and bacteria rather than their actual ratios, and increased with increasing density (Gladstone and Walton, 1971).

Cytophilic antibody systems obviate normal opsonisation processes

involving Ab and C´. IgG2 specific to S. aureus has been found on neutrophils (Watson, 1975 and 1976). Also, it is now apparent that opsonisation can be due to non-immunological factors such as the extracellular matrix proteins (Section 1.5.5), cationic proteins and cationic polyamino acids such as protamine which acts in a dose-dependent manner (Peterson, Gekker, Shapiro, Freiberg and Keane, 1984).

The role of anticapsular antibodies and the S. aureus capsule in opsonisation has already been mentioned (Section 1.2.1). PG is a potent C´ activator and there is a direct parallel between the kinetics of this activation and the kinetics of opsonisation (Verhoef, et al., 1977b). Pr A´s role is not straightforward. Cell wall-bound Pr A acts as a promoter or inhibitor of engulfment depending on the availability of C´ or antibody. Pr A activates C´ directly and combines with F_c thereby competing with neutrophil receptors and thus potentially reducing opsonisation. Accordingly, high Pr A-producing S. aureus are best opsonised in IgG-deficient sera (Peterson, Verhoef, Sabath and Quie, 1977). Cell-free Pr A activates C´ in the host´s fluid phase thereby potentially causing an abortive cascade (Dossett, et al., 1969).

1.5.7.3 Engulfment

The interaction between bacteria, neutrophil receptors and opsonins if present, results in an increase in neutrophil membrane fluidity and pseudopod formation which ultimately engulfs the coccus. The flow of pseudopods around the target is due to calcium-dependent regulation of the actinomyosin microfilament network (Stendahl, 1983; Verhoef, 1982), and incorrect activation of this does not result in ingestion. When the pseudopods meet, they fuse with lysosomes to form

phagolysosomes (phagosomes) and the phagocytic process is strictly, over. Phagosome formation is the start of intracellular killing and digestion of the micro-organism.

1.5.7.4 Intracellular killing

Inside the phagosome, lysosomally-derived granules discharge their contents and a very high concentration of microbiocidal proteins builds up on the microorganism's surface. This degranulation represents the oxygen-independent killing system of neutrophils. Intracellular killing also depends on oxygen-dependent systems (Beaman and Beaman, 1984; Elsbach and Weiss, 1983; Klebanoff, 1975). Antibody is not involved in killing of S. aureus but rather its opsonisation. There does seem to be a role for complement in that killing by human adult neutrophils is less efficient in complement-depleted serum (Wheat, Humphreys and White, 1974) and in the absence or in the presence of inadequate concentrations of whole serum. C3b was implicated possibly as an efficient trigger of the oxygen-dependent mechanisms (Leijh, van den Barsellaar, Daha and van Furth, 1981).

a) Oxygen-dependent mechanism

Phagocyte membrane perturbation such as contact with ingestible particles, triggers increased oxygen consumption. This "respiratory burst" (Klebanoff, 1975) is cytochrome b-associated (Beaman and Beaman, 1984; Klebanoff, 1982) and accompanied by a simultaneous increase in glucose consumption via the hexose monophosphate shunt to produce reducing moieties such as NADH or NADPH. It is probably the NADH or NADPH oxidase, located in the membrane, that is responsible for the burst (Babior, 1984). This

burst can be fifteen times greater than the basal rate of neutrophil respiration and most of the oxygen consumed is converted by the NAD(P)H oxidase to superoxide, O_2^-. This can act as an oxidant and is reduced to hydrogen peroxide, H_2O_2, either spontaneously or by superoxide dismutase, SOD. H_2O_2 and O_2^- are only weakly microbiocidal thus they are used to generate two classes of microbiocidal oxidants, oxidised halogens and oxidising radicals (Babior, 1984).

Myeloperoxide, MPO, is present in the primary granules or lysosomes. It is a very basic protein and attaches to the negatively charged S. aureus surface thereby accelerating the process. MPO uses H_2O_2 and catalyses chlorine to the prototype oxidised halogen, hypochlorite, OCl^-. Oxidising radicals such as the hydroxyl radical, $OH^{\cdot}$, are made in the iron or copper-catalysed Haber-Weiss reaction that uses O_2^- and H_2O_2. Singlet oxygen, $^1O_2$, is also produced by the MPO system and this emits energy or chemiluminescence (Allen, 1977; Klebanoff, 1982). This can be used as an assay of phagocyte function. Spontaneous chemiluminescence is emitted by human neutrophils upon their adherence to surfaces. This may be linked to the non-opsonic surface phagocytosis and killing mentioned earlier (Yanai and Quie, 1981). The H_2O_2 — MPO — halide complex is most potent (Klebanoff, 1982) because of its interaction with iron-sulphur components of the electron transport chain in the microbial cytoplasmic membrane (Rosen and Klebanoff, 1985). Additionally, the complex may decarboxylate amino acids resulting in peptide bond breakage, and generate bactericidal aldehydes from free amino acids (Beaman and Beaman, 1984).

In patients, sub-populations of neutrophils exist that exhibit approximately five times more oxidative metabolism than the remaining neutrophils. This sub-population varied widely (0-80% of

total neutrophils) but on average was 40% of the total (Bass, Olbrantz, Szejda, Seeds and McCall, 1986). Most phagocytes have sufficient quantities of catalase, SOD and antioxidants such as ascorbic acid, vitamin E or a glutathione system to protect themselves. Again, most of the cytotoxic effects are manifest in the phagosome whose "wall" consists of the original external membrane where the NAD(P)H oxidase responsible for the burst is located. However, this is obviously not foolproof. The inflammatory process causes host tissue damage due largely to the destructive agents released from neutrophils which themselves can be damaged (Babior, 1982). The contribution by catalase and SOD to S. aureus pathogenesis has been referred to (Section 1.3.3).

b) oxygen-independent mechanisms

These are not as understood as the oxygen-dependent system, because of their inherent complexity and because they are modulated by the many chemicals present in the inflammatory exudate (Ginsburg, Sela, Newman and Lahav, 1983).

The granules or lysosomes in neutrophils are of two types. The specific granules are the first to fuse with the phagosome and contain lactoferrin, lysozyme, phospholipase and vitamin B12-binding protein. The primary (azurophilic) granules contain MPO, lysozyme, cationic proteins, acidic hydrolases, and neutral proteases. These are not particularly bactericidal as isolated factors, e.g. MPO, but together in vivo they potentiate killing and may be involved in degradation of bacterial debris. The low phagosome pH may also amplify their activities, e.g. the acidic hydrolases. It could be that factors such as the cationic proteins represent an evolutionary earlier defence system (Elsbach and Weiss, 1983).

Cationic proteins can be precipitated by TAs whose presence was implicated in resistance to the proteins. Killing of S. aureus by cationic proteins depends on an energy-dependent binding to the bacterial surface and a functional bacterial cytochrome system, i.e. aerobic respiration (Gladstone et al., 1974; Walton and Gladstone, 1976). They are found in inflammatory exudate and can also act as opsonins (Ginsberg et al., 1983).

Various cationic proteins including lysozyme and RNase have a catalytic activity at least in vitro as they released MTA and activated the Staphylococcal autolysins (Ginsburg and Lahav, 1983; Sela, Lahav and Ginsberg, 1977). This activation differs from that induced by penicillin G in that large parts of the envelope are degraded (Giesbrecht et al., 1985). Lysozyme was also shown to have muralytic activity for S. aureus. Although it does not lyse S. aureus, early reports showed that it could do damage by dissolving the outer envelope without loss of viability (Kern, Kingkade, Kern and Behrens, 1951). It was recently shown that increase in O-acetylation of the muramic acid in PG resulted in an increase in lysozyme resistance. Levels of O-acetylation are very low and constant up to mid-logarithmic phase and then increase rapidly to reach a high plateau coincident with stationary phase onset. Chloramphenicol, erythromycin or co-trimoxazole also increased O-acetylation, whilst significantly penicillin G reduced it. Levels were also influenced by complex media (Burghaus, Johannsen, Naumann, Labischinski, Bradaczek and Giesbrecht, 1983; Johannsen, Labischinski, Burghaus and Giesbrecht, 1983). Thus lysozyme can possibly synergise in vivo with the S. aureus autolysins (Ginsburg et al., 1983). The situation is complex however. Inflammatory exudate, pus, etc. can also contain

serum proteins, sulphated mucopolysaccharides and breakdown products of connective tissues. Anionic polyelectrolytes such as chondroitin sulphate and hyaluronic acid inhibited the S. aureus autolysins in vitro. This supression also occurred in penicillin-treated S. aureus which thus could not lyse (Ginsburg and Lahav, 1983). Both anionic polyelectrolytes and cationic proteins can be pinocytosed, or phagocytosed simultaneously with a bacterium (Ginsburg et al., 1983). Consequently, autolysis in the phagosome may be determined by the balance between the opposing factors as well as by S. aureus.

Inevitably, a malfunctioning phagocyte defence predisposes to S. aureus infections (Quie, Mills, Cates, Abramson, Regelmann and Peterson, 1983). Such deficiencies can occur at every step of the phagocytic and intracellular killing sequence. Job's syndrome or hyperimmunoglobulin E syndrome can cause a chemotactic flaw that results in chronic skin lesions and "cold" abscesses (Hill, Quie, Pabst, Ochs, Clark, Klebanoff, and Wedgwood, 1974). Defects in serum complement also result in poor chemotactic response (Quie et al., 1983).

Chronic granulomatous disease, CGD, is characterised by repeated infections with S. aureus but not catalase-negative, H_2O_2-producing bacteria such as the various Streptococci (Quie et al., 1983). Consequently granulomas consisting of mononuclear cells and suppurative material occur in the lymph nodes, skin, lungs, liver, bone and gastrointestinal tract. Persistent, viable intracellular bacteria contribute to these prolonged lesions. The chemotactic and engulfment responses of CGD neutrophils are normal but intracellular killing is very poor because of defective triggering of the respiratory burst and consequent lowered production of H_2O_2. They are thus chemiluminescence negative. Other flaws in neutrophil oxidative

bactericidal defences are seen in MPO production, the hexose monophosphate shunt and the glutathione system (Quie et al., 1983).

Faults in the non-oxidative system are also recognised. Decreased levels of the vitamin B12-binding protein and lactoferrin resulted in decreased intracellular killing of S. aureus and recurrent pyogenic infections even though remaining degranulation and neutrophil functions were normal (Ambruso, Sasada, Nishiyama, Kubo, Komiyama and Allen, 1984).

1.5.8 Cell mediated immunity (CMI)

This is mediated by T-lymphocytes whose reaction with antigen releases macrophage-activating lymphokines (Roitt, 1984). S. aureus PG contains the major determinants for CMI (Easmon and Glynn, 1978) but because of the non-specific aspect of this immunity (Roitt, 1984), S. aureus can also be attacked by macrophages activated by other stimuli. However, CMI defects do not predispose to S. aureus diseases (Adlam and Easmon, 1983) and although CMI to S. aureus is common in humans (Mudd, Taubler and Baker, 1970) it does not appear to prevent chronic or recurrent infections (Cluff, 1965).

1.5.9 Chronic response and hypersensitivity

S. aureus is better able than many pathogens to survive inside phagocytic cells but many still die, leaving a small number of persisting intracellular survivors. However, unlike Mycobacterium tuberculosis or Brucella abortus, S. aureus is not a classical intracellular pathogen in that it does not multiply significantly inside the phagocyte. Ineffective elimination of S. aureus can lead to chronic inflammation which is so termed if the condition persists one year or more (Downie, 1979). Continued attempts of fibroblasts to

lay down collagen eventually gives tissues a scarred appearance which restricts blood flow and exudation. There can also be a chronic CMI response with accumulation of densely packed macrophages which release fibrogenic factors and also stimulate the formation of granulation tissue and ultimately fibrosis. This is called a granuloma and is an attempt to seal off a focus of disease.

Killing of S. aureus does not necessarily involve its degradation, i.e. bactericidal activity is not necessarily bacteriolytic activity (Elsbach, 1980; Ginsberg et al., 1983). Also, very little is known about the degradation of S. aureus in vivo (Section 1.5.7.4.b; Ginsberg et al., 1983). This has clinical implications because undegraded bacterial envelope, PG or TA can persist in macrophages for example and can cause chronic inflammatory sequelae such as the granulomae seen in CGD (Section 1.5.7) or chronic arthritis with erosion of bone and cartilage (Ginsberg et al., 1983). These are examples of hypersensitivity reactions that S. aureus or its components take part in and that can lead to tissue damage if there is sufficient antigen and if the host immune system is in a heightened state (Roitt, 1984).

Three hypersensitivity reactions are antibody mediated. The type I anaphylactic response is seen in, e.g. Job's disease (Section 1.5.7) and TA can elicit this response. Type II antibody-dependent cytotoxic hypersensitivity has been mentioned (Section 1.2.2.5).

Type III immune complex or Arthus reaction hypersensitivity occurs when S. aureus antigen-antibody complexes form with activation of C'. Lysosomal release from recruited neutrophils causes tissue damage. These complexes can stay localised as in eczema or move to the peripheral vascular system, joints or kidneys. S. aureus

endocarditis can cause immune complex glomerulonephritis. TA can also precipitate these reactions. Pr A can combine non-immunologically with F_c, trigger the C´ cascade and cause Arthus-like reactions. The amount of tissue destruction depends on the focus of infection and the antigen concentration and the reactions are reduced when the infections are controlled (Adlam and Easmon, 1983).

Type IV is cell mediated or delayed hypersensitivity and occurs when antigen bound to macrophages is presented to T-lymphocytes. These either release lymphokines which attract and activate new macrophages or they differentiate and kill antigen-bearing macrophages. Animal models have been well chronicled (Adlam and Easmon, 1983). Essentially, it was only seen in cyclophosphamide-free mice after repeated skin injections and the presence of a humoral response prevented harmful manifestations (Easmon and Glynn, 1975b). In cyclophosphamide-treated mice it was shown that B-supressor cells probably act on its expression whilst factors in immune serum act on the induction of the response. Repeated infections override these controls (Easmon, 1981).

Delayed hypersensitivity is common in normal humans (Mudd et al., 1979) possibly because of nasal carriage, etc. but seems not to prevent chronic or recurrent infection (Cluff, 1965). Apart from an inability to degrade S. aureus other defects in neutrophil function can also result in delayed hypersensitivity (Hill et al., 1974).

1.5.10 Serodiagnosis

It is well known that most adult human sera contain a variety of antibodies that combine with S. aureus. This is generally due to the carrier state, coupled with repeated subclinical or minor infections. However, these antibodies may be non-specific and due to exposure to

other bacteria that contain common or similar antigens (Oeding, Wergeland, Endresen, Natås and Aasjord, 1983). Conversely, antibodies that combine with Staphylococci are found in patients with non-staphylococcal illnesses (Oeding et al., 1983). Accordingly, the patient's response depends on earlier exposure plus stimulation by common and exclusive antigens of the new infecting strain.

Thus a problem in S. aureus serology is the demonstration of an antibody rise rather than simply its detection. This problem is encapsulated by the fact that serious S. aureus infections rarely result in a strong and regular booster affect whereas carriage and minor infections cause a seemingly normal response. It may be that the strong tendency of S. aureus infections to abscess formation and consequent "walling in" means that insufficient antigen reaches the antibody forming centres to act as a boost.

The most commonly used tests are the anti-alpha (α) haemolysin, anti-TA and anti-PG antibody assays. Anti-TA antibody assays are very popular if only because their initial drawbacks were poorly understood. Early assays used double diffusion (DD) and counter-immunoelectrophoresis (CIE). DD is sufficiently insensitive and therefore more specific and likely to overcome the problem posed by low levels of anti-teichoic acid antibody found generally. CIE is more sensitive and therefore less specific and thus used to titre out positive reactions from the DD screen. However, a survey of the literature reveals a morass of different preparative methods for antigen and gels and therefore confusing results arise and potentially good techniques are wasted (White, Wheat and Kohler, 1983).

The anti-α haemolysin antibody test is the oldest of the three and illness produces measurable antibodies approximately two weeks into the infection against a component peculiar to S. aureus.

However, it lacks sensitivity and can give variable results (Abramson, 1983).

The significance of ELISA tests for anti-TA and anti-PG antibodies and for anti-α haemolysin antibodies has recently been re-evaluated (Verbrugh, Peters, Goessens and Michel, 1986). The anti-PG antibody assay was the most sensitive of the three and specific for differentiating complicated and uncomplicated infection. The anti-TA antibody assay was somewhat less sensitive than the anti-PG antibody test whilst the anti-α haemolysin test, the oldest of all, was as sensitive as the rest but had a lower specificity for the complicated and uncomplicated infections. Endocarditis patients produced the highest titres and usually to all three tests. All patients without endocarditis were usually only positive in one or two tests. Cross-reacting antibodies to all three were found in controls and most commonly in the anti-PG assay.

The serological tests correlated well with the class of illness, i.e. whether complicated or not, but they did not correlate with clinical severity of the bacteraemia in that the antibody response of those with or without a clinically significant bacteraemia were similar. So the underlying illness or its class and not the bacteraemia decided the antibody response.

Newer tests such as ELISA are much more sensitive and thus cause a "dislocation" of levels in that higher titres result in both patients and controls. It is thus important to determine the level of antibody response by titration especially where anti-TA antibodies are concerned (Dziarski, 1985). Diagnosis can only be reliable if several, serial samples are tested by at least two of the above three tests (Dziarski, 1985; Verbrugh et al., 1986).

The future may lie in using an antigen of synthetic pentaglycine as not only is this unique to S. aureus but antibodies to it have been detected in patients (Oeding et al., 1983).

1.5.11 Vaccines

These have been reviewed (Adlam and Easmon, 1983). There is little need for a vaccine for the general population but it would be useful for patients at risk and in the veterinary field (Brückler and Blobel, 1984). Also, the need to know why no single S. aureus structure or enzyme has proved to be the immunising and protective factor fuels interest. Certainly, no strong, long-lasting immunity follows a Staphylococcal infection, and vaccination with whole cells or subcellular fractions, or passive transfer of immune serum are only partially protective (Sheagren, 1984). Previous infection did though confer the ability to mount a brisk, protective, early inflammatory response in a mouse dermonecrotic model, possibly by antibody-mediated hypersensitivity (Easmon and Glynn, 1975a).

Toxoid vaccines are not particularly relevant in human Staphylococcal disease and results have anyway been disappointing. Besides which, the extracellular products are produced late in the growth phase. Alpha-toxoid immunisation has limited the severity of ovine gangrenous mastitis (Adlam and Easmon, 1983) and enabled a rapid inflammatory response in murine dermonecrosis (Easmon and Glynn, 1975a).

Killed whole cell vaccines or envelope constituents elicit a humoral response only, but S. aureus is readily phagocytosed in both normal and immune sera and specific antibody seems not be be involved in C′-associated killing or in intracellular killing. Vaccines here have given mixed results. Whole cells only produced incomplete

protection, whilst envelope-capsule preparations gave good homologous protection because of effective opsonisation. Lysed cell vaccines were equivocal but they did desensitise individuals with delayed hypersensitivity whose lesions subsequently improved (Mudd et al., 1970).

Living vaccines elicit both a humoral and cellular response. However, repeated S. aureus infection does not result in immunity to disease. Thus, vaccine-elicited protection was good or even complete but strain specific. To date, no vaccines are reported that interfere with adhesion of S. aureus.

1.6 S. aureus in vivo

Bacterial pathogenicity is not only dependent on extracellular products but reflects the interaction of the host immune system and host tissues with the bacterial surface. This surface is altered by the environment that bacteria grow in (Smith, 1977; Brown and Williams, 1985a) so that they are typical of it. There are thus differences between in vivo and in vitro grown S. aureus and this reflects both genotypic and phenotypic changes (Brown and Williams, 1985a; Dalhoff, 1985; Gemmell, 1985; Smith, 1980).

It is not often appreciated that it takes at least several generations before a bacterium becomes typical of its environment (Brown and Williams, 1985a) and conversely it is emphasised that in vivo characteristics such as virulence, antigenicity, morphology both at coccus and colony level, response to host defences and antibacterial agents and metabolism can be lost in vitro even after one subculture (Beining and Kennedy, 1963; Brown and Williams, 1985a and b; Costerton, Irvin and Cheng, 1981; Dalhoff, 1985; Karakawa and

Young, 1979a and b). Thus, more attention to *in vivo* characteristics would enhance current understanding of disease (Brown and Williams, 1985a; Smith, 1980) and where ethics, ease or economy dictate *in vitro* experiments, their design must approach *in vivo* (Lewis, Reeves, Wiedmann and Zinner, 1985).

1.6.1 Morphology and ultrastructure

Host tissues can be covered with a fibrillar or amorphous glycocalyx overlying the plasma membrane (Beveridge, 1980). Electron microscopy shows that the growth of *S. aureus* *in vivo*, whether in acute (Speers and Nade, 1985) or chronic (Marrie and Costerton, 1985) infections in humans or animals, is very frequently as a biofilm, i.e. cocci or microcolonies also embedded in a glycocalyx (Costerton and Marrie, 1983). Bacterial glycocalyx is a polysaccharide-containing structure that lies outside the envelope. There are two types, S layers which consist of regular arrays of glycoprotein subunits at the envelope surface (Costerton *et al.*, 1981) and capsules (Wilkinson, 1983). Thus, *S. aureus* *in vivo* is surrounded by a thick continuous, ordered, hydrated, polyanionic, polysaccharide matrix that influences the access of a variety of molecules and ions to the cell envelope. The glycocalyx also acts as an ion-exchange barrier. Once established, the glycocalyx contributes to adhesion and generally, to the ability to cause localised or disseminated illness. If host tissues are colonised by saprophyte biofilms then invading potential pathogens have to compete to find host receptors (Costerton *et al.*, 1981).

The adherent mode of growth allows *S. aureus* to colonise various types of catheter (Marrie and Costerton, 1984) and *S. aureus* on tissues, PVC catheters or plastic materials generally has elevated MBCs (Costerton and Marrie, 1983; Sheth, Franson and Sohnle, 1985), is

less efficiently phagocytosed and has enhanced resistance to killing by neutrophils (Vaudaux, Zulian, Huggler and Waldvogel, 1985). This is not surprising as S. aureus forms microcolonies on such implants (Bayston, 1984; Costerton and Marrie, 1983) and these "biofilms" are refractory to host defences and antimicrobial therapy (Costerton and Marrie, 1983). Sloughing of infected cells or mucociliary clearance does not function very well against adherent microcolonies thus perpetuating persistence.

This can lead to cryptic infections and inflammatory sequelae. Patients with such illnesses can have very high levels of antibody and as the biofilm is not cleared it seems that the glycocalyx is protective. Clinical results also suggest that the glycocalyx presence means that especially high antibiotic concentrations are effectively needed in vivo to exhaust the binding capacity of the glycocalyx (Costerton and Marrie, 1983).

The ultrastructure of individual cocci in vivo has been studied. S. aureus in rabbit and rat endocarditis and osteomyelitis models were unlike broth-grown counterparts as the cocci had very thick peripheral walls and occassional thick cross walls. Those S. aureus from the animals' peritoneal fluid were like those grown in broth. However, S. aureus from animals and from patients with respiratory infections and treated with β-lactams, were most unlike their counterparts grown in drug-containing broth. The former were very large and had multiseptate walls. This could be reproduced in vitro by growth on membranes on drug-containing agar but not in drug-containing broth (Lorian, Atkinson, Waluschka and Kim, 1982; Lorian, Zak, Kunz and Vaxelaire, 1984; Lorian, Zak, Suter and Bruecher, 1985). β-lactams may not penetrate to the central cocci in these multiseptate forms and this has implications for experimental design. The in vitro

phagocytosis of these large multiseptate cocci is however as efficient as more normal forms (Lorian, 1985).

Bacterial structures called "dense bodies" have been isolated from osmotically-lysed patients' blood. These particles can be less than 0.22 μm in diameter and may represent adaptation to *in vivo*. They reverted upon subculture to Gram-positive bacteria, some of which were Staphylococci (Domingue and Schlegel, 1977).

1.6.2 Virulence

The enhanced virulence of *in vivo* rather *in vitro* grown *S. aureus* for inoculation purposes is appreciated (Gladstone and Glencross, 1960; Watson, 1982). *S. aureus* virulence characteristics were enhanced after growth in rabbits as they resisted killing by neutrophil bactericidal extracts and whole neutrophils (Adlam, Pearce and Smith, 1970a and b). This could be mimicked by growth in plasma. Both *in vivo* grown and plasma grown *S. aureus* had a fibrin or fibrinogen-derived deposit on their surfaces which was lost in ordinary media with concomittant return to full sensitivity to the above killing. Staphyloccagulase was more efficient at deposition than the clumping factor (Kolawole, 1983a and b, 1984). Both human plasma and fibrinogen had an enhancing effect upon *S. aureus* murine intraperitoneal infections and in this instance the clumping factor and not staphylocoagulase, was implicated (Espersen, Clemmensen, Frimodt-Møller and Jensen, 1984). Non-toxigenic *S. aureus* had reduced deposition of fibrin and granulomatous material surrounding them compared to toxigenic strains and this possibly reflected extracellular toxin production (Arbuthnott, 1981). However, extracellular toxins are maximally produced in the later stages of growth *in vivo* and so may help to consolidate rather than initiate

disease (Gladstone and Glencross, 1960; Hauser and Berry, 1961).

Evidence was obtained from a murine intraperitoneal model that a virulence-enhancing effect of added IgG could be due to the formation of IgG-Pr A complexes that depleted host C′ (Espersen et al., 1984).

The virulence of in vivo grown inocula in mice and rabbits was enhanced compared to in vitro equivalents because of increased output of DNase, α-haemolysin, leucocidin and hyaluronidase (Beining and Kennedy, 1963). S. aureus strains originally considered negative for α-haemolysin and leucocidin production were found to produce these in vivo (Gladstone and Glencross, 1960).

There is in vivo evidence that sub-MICs of β-lactams are effective. Pre-treatment with cloxacillin and nafcillin rendered S. aureus more susceptible to killing in a murine chronic mastitis model (Craven, Williams and Anderson, 1982). They caused a significant reduction in the multiplication of S. aureus in the rabbit peritoneal cavity (Zak and Kradolfer, 1979) and also decreased the virulence of S. aureus in mice in which the host cellular defences had already been recruited to the site of inoculation. Here, the hypersensitivity to killing was as great in vivo as in vitro (Lam, Georgopoulous, Laber and Schütze, 1984). Sub-MICs also reduced S. aureus toxin synthesis and lesion size in murine subcutaneous infections (Gemmell, 1978).

1.6.3 Phagocytosis

Penicillin G penetrates neutrophils but poorly (Prokesch and Hand, 1982) whilst the highly acid pH of the phagosome required to kill S. aureus (Styrt and Klempner, 1985) reduced its growth rate (Lam and Mathison, 1982) and decreased the efficiency of β-lactam molecules bound to or ingested simultaneously with the cocci (Craven and Anderson, 1982; Lam and Mathison, 1982). This in vivo reduction in

growth rate results in greater resistance to degradation (Ginsburg, 1979). The need for β-lactam antibiotics that can lyse slow growing or even stationary S. aureus is implicit.

Microbial synergism features in vivo and antecedent viral infection temporarily suppressed phagocytosis (Larson and Blades, 1976) and intracellular killing, and predisposed to disseminated S. aureus infections (O'Driscoll, Crawford and Biggar, 1985).

S. aureus passaged several times in mice needed more extensive C'-mediated opsonisation compared to in vitro grown controls, before being properly phagocytosed in vitro. However, the in vivo grown phenotype was just as susceptible to sub-inhibitory concentrations of clindamycin and fucidin, and these rendered the in vivo phenotype as sensitive as the drug-free in vitro phenotype to ingestion and killing, by a reduction in surface-associated Pr A (Gemmell, 1985). It is conceivable that this exposed antibody and C' binding sites on the S. aureus surface.

1.6.4 Degradation

S. aureus mediates hypersensitivity reactions. Even partly intact cocci or envelope components can trigger chronic inflammatory sequelae. However, very little is known about the control of the degradative processes in vivo. S. aureus was not degraded in a rat model of arthritis even beyond 72 h postinfection (Ginsburg et al., 1983). Other in vivo evidence suggests that as degradation is sufficiently slow to allow pyrogenic products to exist, the balance in inflammatory exudate of the cationic proteins which activate autolysis and the anionic polyelectrolytes which inactivate autolysis is in favour of the latter (Sections 1.5.7.4.b and 1.5.9; Ginsberg, 1979).

β-lactam antibiotics can induce release of MTA and therefore remove the negative control on autolysis (Section 1.4). This can also result in accumulation in tissues of S. aureus envelope components thus offsetting the advantages of drugs.

1.6.5 Metabolism and doubling times

Generally, S. aureus grown in vivo have an increased metabolic rate than when in vitro either because they need to offset nutrient depletion or because they are manufacturing factors needed for growth at the host's expense (Dalhoff, 1985). Aerobic rates of respiration in S. aureus grown in guinea pigs were much higher than in vitro (Beining and Kennedy, 1963; Gellenbeck, 1962).

In vivo, and despite the increased metabolic rate, S. aureus doubling times are usually reduced. S. aureus had doubling times in mouse blood in vitro of 36 min whilst in a mouse sepsis model it was between 46 min and 2 h 30 min and in a rat osteomyelitis model between 8 and 24 h (Zak and Sande; modified in Dalhoff, 1985, page 179). S. aureus NCTC 6571 had a doubling time of 12 to 15 min in cellophane sacs held in rabbit serum whilst it was 30 to 40 min when the sacs were placed in rabbits and mice, and 68 min when in guinea pigs (Gladstone and Glencross, 1960). S. aureus in subcutaneous diffusion chambers in rabbits had a doubling time of 42 min which was over twice as slow as in vitro (Lam et al., 1984). An increase in in vivo growth rate compared to in vitro has been noted but S. aureus was held in dialysis tubing in sheep and it is possible that dialysis occurred to benefit the cocci (Watson and Prideaux, 1979).

1.6.6 Antigenicity

In vivo-induced antigenic changes are now well characterised in Gram-negative bacteria. The effect of iron deprivation by the host

causes the derepression of various iron-regulated outer membrane proteins which are antigenic and can be detected by immunoblotting. Thus, evidence has been obtained directly from humans, that Gram-negative bacteria at least, grow under iron deprivation in the cystic fibrotic lung (Anwar, Brown, Day and Weller, 1984; Brown, Anwar and Lambert, 1984), in the urine (Lam, Turnowsky, Schwartzinger and Neruda, 1984; Shand, Anwar, Kadurugamuwa, Brown, Silverman and Melling, 1985) and in burns (Anwar, Shand, Ward, Brown, Alpar and Gowar, 1985).

The antigenic status of S. aureus in vivo is poorly characterised. Differences were noted in the ability of antisera to agglutinate in vivo and in vitro grown S. aureus and two extra antigens were detected in the in vivo phenotype in gel diffusion (Beining and Kennedy, 1963). In vivo grown S. aureus exhibited substantial differences in amino acid composition whilst gel diffusion revealed that the in vivo cocci had an extra antigen that was recognised by specific antisera (Watson and Prideaux, 1979).

S. aureus in vivo is probably encapsulated (Section 1.2.1). The composition of the capsule of a chronic osteomyelitis strain was changed in vitro in the presence of antibody so that variants emerged. Titres of patients' sera were highest against this variant indicating similar in vivo alterations (Karakawa and Young, 1979b). Freshly isolated strains were antigenically dissimilar to in vitro cultured derivatives because of antigenic drift in particular antigens (Pereira, 1961).

The above data suggests that not only is S. aureus in vivo antigenically dissimilar from the common laboratory culture but in vivo there is potential for antigenic variation. This may partly explain the failure to develop a successful S. aureus vaccine.

A killed S. aureus vaccine was not as effective as a live vaccine in immunising sheep because the latter resulted in neutrophils with enhanced activity in vitro at least, to S. aureus. This was initially attributed to cytophilic IgG2 on the neutrophil membrane, but because it was also present on neutrophils from sheep immunised with dead vaccine or non-immunised sheep, the enhancement was presumed to be opsonic and due to the antibody effect of IgG2 (Watson, 1975 and 1976; Watson and Lee, 1978). This is further corroboration that S. aureus growing in vivo manifests antigens not seen in vitro.

MATERIALS and METHODS

2.1 Materials

2.1.1 Bacteria

Staphylococcus aureus NCTC 6571 (OXFORD Strain; National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London, NW9 5HT.) was used throughout. S. aureus Hopewell, an endocarditis isolate that was kindly supplied by Doctor I. Farrell (Public Health Laboratory, East Birmingham Hospital, Bordesley Green, Birmingham B9 5ST.) became available for immunological analysis at the end of this study. Both strains were stored in liquid nitrogen and also maintained on nutrient agar slopes at 4°C by subculture at monthly intervals.

2.1.2 Chemicals and Media

Sodium benzylpenicillin, Penicillin G, was obtained from Glaxo Laboratories Limited, Greenford, Middlesex.

Chemicals and reagents used and not specified in the text were Analar grade (BDH Chemicals Ltd., Poole, Dorset). All solutions were made up in double distilled water.

Tryptone soya broth (TSB), Nutrient agar (NA) and Diagnostic Sensitivity Test Agar (DSTA) were obtained from Oxoid Ltd. (Basingstoke, Hampshire). All were prepared and sterilised according to the manufacturer's instructions.

2.1.3 Blood and serum

2.1.3.1 Non-immune blood and sera

These were taken from two male and two female apparently healthy volunteers ages 23 to 45 years. Serum from subject one was tested for anti-alpha-haemolysin and anti-nuclease antibodies because this was to be the most commonly used subject (Central PHLS, Colindale Avenue,

London, NW9 5HT.).

2.1.3.2 Hyperimmune sera

These were raised in male New Zealand White rabbits with an average body weight of 4.5 Kilogrammes.

The gift by Dr I. Farrell of a small quantity of antiserum from a S. aureus endocarditis patient at East Birmingham Hospital is acknowledged.

2.1.4 Equipment

Manufacturers' addresses are only cited once. Equipment and apparatus used and not specified in the text were as follows:

Balances - macro, Oertling HC 22; Oertling Ltd., Orpington, Kent
- micro, Sartorius 1702; Baird and Tatlock Ltd./ BDH Chemicals Ltd., Atherstone, Warwickshire.

Blood sample tubes - lithium heparin-coated and plain, stoppered, 10 ml.; Searle and Co., High Wycombe, Buckinghamshire.
- plain, screwcap, 5ml.; LIP Ltd., Shipley, Yorkshire.

Capillary blood tubes - plain, 1604; Gelman-Hawksley Ltd., Lancing, Sussex.

Centrifuges - Beckman J2-21; Beckman Ltd., High Wycombe, Buckinghamshire.
- Eppendorf 5412; Baird and Tatlock Ltd.
- MSE Super Minor; MSE Ltd., Crawley, Sussex.

Chart recorder - model 28000; Bryans Southern Instruments Ltd., Mitcham, Surrey.

Chemiluminometer - model 1250, non-heated, non-automated; LKB Instruments Ltd., Selsdon, Surrey.

ELISA plate washer - model 120; Flow Laboratories Ltd., Irvine, Scotland.

ELISA plate reader - Titertek Multiscan model 310 C; Flow Laboratories Ltd.

Filtration system - clamped, glass funnel; Millipore UK Ltd., London.
- filterpads, 47mm, 0.2 μ, cellulose acetate; Millipore UK Ltd.
- filterpads sterile, pyrogen free, 25mm, 0.2 μ, Acrodisc; Gelman-Hawksley Ltd.

Gel electrophoresis apparatus - large (250 mm x 200 mm) made in house by Aston Services.

- mini (100 mm x 80 mm): Model 360
mini vertical slab gel and Model II - mini Protean 125 BR vertical slab gel; Bio-Rad Laboratories Ltd., Watford, Hertfordshire.

Gel electrophoresis power pack - model 500/200; Bio-Rad Laboratories Ltd.

Gel slab dryer - model 224; Bio-Rad Laboratories Ltd.

Glass beads - for homogenising, Type 1, 75-150 microns; Sigma Ltd., Poole, Dorset.

Homogeniser - Mickle Engineering Company Ltd., Gomshall, Surrey.

Immunoblotting apparatus - Trans Blot Cell; Bio-Rad Laboratories Ltd.

Immunoblotting power pack - model 250/2.5; Bio-Rad Laboratories Ltd.

Incubators - shaking waterbath; Mickle Engineering Company Ltd.
- orbital incubator; Gallenkamp Ltd., Loughborough, Leicestershire.

Ion-exchange columns - 1 inch internal diameter, 500ml volume; Pharmacia Ltd., Milton Keynes, Buckinghamshire.

Laser densitometer - model 2202 Ultrascan; LKB Ltd., London.

Microscopes - Wild M20; Microscopic Instruments Ltd., Oxford, Oxfordshire.

Osmometer - Knauer Regler, Berlin, West Germany.

pH meter - Model PT1-15; FSA Ltd., Loughborough, Leicestershire.

Pipettes - Gilson Pipetman, P-200, P-1000, P-5000; Anachem Ltd., Luton Bedfordshire.
- Digital multi-channel pipette; Flow Laboratories Ltd.

Spectrophotometers - atomic absorption, model 560 manually loaded spectrophotometer fitted with:

Deuterium background corrector, model HGA 74 graphite furnace with argon gas flow, model HGA 500 programmer, model 56 chart recorder; Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire.

- model 292, digital, ultraviolet; Cecil Instruments Ltd., Cambridge, Cambridgeshire.

2.2 Experimental methods

2.2.1 Preparation of glass and plastic ware

The following procedure was employed:

1. 18h immersion in 2% (V/V) Extran 300 (BDH Ltd.).
2. One rinse in single distilled water.
3. One rinse in 1% (V/V) HCl.
4. Six rinses in single distilled water.
5. Three rinses in double distilled water.
6. 18h immersion in 0.01% ethylene diamine tetra-acetic acid (BDH Ltd.).
7. Six rinses in double distilled water.

Glass ware was sterilised in an oven at 180°C for 4h whilst plastic ware was sterilised by autoclaving at 115°C for 20 min.

2.2.2 Preparation of iron-depleted TSB

Two ion-exchange columns were each packed with 200 ml bed volumes of Chelex 100 resin (Bio-Rad Laboratories Ltd.) that had been conditioned to pH 7.4 according to the manufacturer's instructions (Bio-Rad Technical Bulletin No. 2020, 1981). 1.4 l of double strength TSB was reconstituted in chilled, double distilled water. It was then passed down each column, in the cold, at a rate of 2 ml min^{-1}. The first 200 ml of fluid from each pass was discarded to avoid dilution by the water retained on the columns due to the conditioning. The treated TSB and also double strength control (untreated) TSB that had been reconstituted with double distilled water were sterilised together, by autoclaving at 115°C for 20 min.

Atomic absorption spectroscopy was used to determine pre- and post-treatment levels of Fe, Ca, Mg, Zn and Cu and double distilled water washings from glass and plastic ware were regularly checked for potential contamination. The levels of each cation were determined

from a calibration curve constructed using commercial standards (Spectrosol grade; BDH Ltd.). The operational conditions of the spectrophotometer were as specified by the manufacturer for the cation in question. Cations including or excluding Fe were added back to treated TSB to restore original levels (Fe+ and Fe- TSB respectively).

The osmolarities of Fe+ and Fe- TSB, untreated TSB and pooled, normal human serum were compared in an osmometer using a calibration curve constructed with commercial standards (Fiske Associates Incorporated, Uxbridge, Massachusets). Sufficient amounts of a vitamin supplement (biotin, 0.003 $\mu g\ ml^{-1}$; pantothenic acid, 0.5 $\mu g\ ml^{-1}$; nicotinic acid, 0.5 $\mu g\ ml^{-1}$; thiamine, 0.5 $\mu g\ ml^{-1}$ (Miller and Fung, 1973; Sigma Ltd., Poole, Dorset) were added so that the Fe+ TSB was the equivalent of control TSB in morphological, cultural and biochemical tests (Section 3.1).

2.2.3 Measurement of bacterial growth by optical density

Measurements based upon optical density (OD) values are the most appropriate for following changes in bacterial number during growth (Greenwood, 1977) and within limits the OD is directly proportional to the bacterial concentration. This relationship is expressed by the Beer-Lambert law:

$$OD \times \log_{10}(I_o/I)$$

Where I_o = incident light

I = emergent light

This relationship does not apply at higher bacterial concentrations due to secondary scattering of light (Meynell and Meynell, 1970) and in this study, the linear relationship for optical density and bacterial number was lost above an OD of 0.25. All suspensions with an OD exceeding this were thus diluted to restore linearity. An absorption/wavelength scan of cell-free supernatants of iron-rich and

iron-poor stationary phase cultures showed that absorption by pigments and metabolic products was negligible at 470 nm. This wavelength was thus used for spectrophotometric growth measurements.

2.2.4 Growth measurements

Bacterial growth was monitored by observing the change in OD with time. Inocula were always taken from stationary phase, iron-poor cultures. Unless otherwise stated, 20 ml of pre-warmed Fe+ and Fe- TSB in 100 ml flasks was inoculated and agitated at 120 strokes per min at 37°C. Samples were removed at appropriate intervals and diluted whenever necessary for OD measurements.

2.2.5 Minimal inhibitory concentration (MIC) and sub-minimal inhibitory concentration (sub-MIC) of Penicillin G

The MIC was determined by a tube dilution method (Reeves, Phillips, Williams, and Wise, 1978) using 1 ml volumes of untreated, Fe+ and Fe- TSB and an inoculum of 10^6 colony forming units (cfu) of overnight, iron-poor bacteria. The tubes were incubated for 18 and 36h at 37°C. The lowest concentration of penicillin that inhibited visible growth after incubation was regarded as the MIC. Stock penicillin G (600 $\mu g\ ml^{-1}$) was stored at -18°C and checked for potency by frequent MIC testing.

The sub-MIC was chosen by growth in untreated, Fe+ and Fe- TSB in the presence, from zero time, of different sub-MIC's of penicillin. Samples were taken along the growth curve and transferred to 5% (V/V) formalin for morphological analysis. The sub-MIC chosen was one which did not alter the growth rate as judged by OD_{470} results, but did induce morphological changes visible upon light microscopy.

2.2.6 Viable counts

Clumping of samples was minimised by vortexing for 2 min and two methods of plate counting were used:

a) spread plate method (Crone, 1948)

b) method of Miles and Misra, 1938.

Reproducibility was tested by performing five replicate counts for method a) and eight replicate counts for method b). DSTA plates were incubated for 18 hours at 37°C.

2.2.7 Growth conditions

Iron-rich and iron-poor cocci were grown with a relatively slow doubling time of 48 min (t_d = 48 min) by using an inoculum size that gave an initial absorbance at 470 nm of 0.5 (E_{470} 0.5) in 250 ml of pre-warmed TSB in a 500 ml flask with an orbital shaking rate of 200 revolutions per min (rpm) at 37°C. Relatively fast growth (t_d = 24 min) was obtained by the use of an inoculum size that gave an initial E_{470} 0.05 in 250 ml of pre-warmed TSB in a 1 l flask and 300 rpm at 37°C. The sub-MIC of Penicillin G was added, when required, to mid- to late-logarithmic cells. The addition was at an approximate E_{470} 3.0 for slow-growing cocci and E_{470} 0.5 for fast-growing cocci. Bacteria were then harvested after they had been exposed to penicillin for a further generation.

2.2.8 Surface hydrophobicity (SH)

a) contact angle method

this was assayed by the method of van Oss, Gillman and Neuman, 1975 as modified below. Cells were harvested, washed three times in 0.85% (W/V) NaCl and resuspended in the same to a final volume of 25ml and E_{470} 20.0. They were then vacuum filtered onto 0.2 μ cellulose acetate filter pads (Sartorius Ltd./ Baird and Tatlock Ltd.) that had previously been washed three times in hot, double distilled water. After filtration, the pads were glued onto microscope slides with water-soluble glue (Gloy; Henkel Chemicals Ltd., London). They were then placed in petri dish damp chambers and left overnight at 4°C.

Next day the chambers were uncovered and the pads and adherent bacteria left to dry for three hours at 22°C. This period had previously been determined to give a plateau of constant contact angles. After drying, the pads were placed in position opposite a goniometer (Ealing Scientific Ltd., London) and the angle of contact made between the bacterial lawn and a 20 µl drop of 0.85% (W/V) NaCl measured. A minimum of 10 observations, ie. 10 contact angles, were obtained for each cultural condition and each experiment was performed a minimum of three times.

b) bacterial affinity to hydrocarbons (BATH)

the method of Rosenberg, Gutnick and Rosenberg, 1980 was used with modifications. Cocci were harvested, washed twice in phosphate-urea-magnesium buffer, pH 7.0 (PUM buffer, $K_2HPO_4.3H_2O$ 22.20 g, KH_2PO_4 7.26g, urea 1.80 g, $MgSO_4.7H_2O$ 0.20 g and distilled water to 1 l) and resuspended in the same to an E_{470} 0.3. 1.2 ml of suspension were added to 0.2 ml of either octane or hexadecane and then incubated for 10 min at 30°C. The mixtures were then vortexed for 2 min and the aqueous and hydrocarbon phases allowed to separate for 15 min at 22°C. The aqueous phase was removed and its E_{470} determined and compared to the pre-treatment extinction.

c) hydrophobic interaction chromatography (HIC)

this was according to the methods of Smyth, Jonsson, Olsson, Söderlind, Rosengren, Hjertén and Wadström, 1978 and Jonsson and Wadström, 1983. It was performed on octyl-sepharose and sepharose (Sigma Ltd.) using different molarities of sodium chloride in 10 mM sodium phosphate buffer, pH 6.8. The sepharose stock solutions were washed repeatedly in double distilled water until all visible traces of detergent and preservative were gone. They were loaded to a height of 4cm (1.0 ml packed gel volume) into pasteur pipettes that had been

fitted with glass wool (BDH Ltd.) sinters at their constrictions. The columns were conditioned prior to immediate use by washing with 10 ml of the appropriate molarity of buffered sodium chloride. Cocci were harvested, and washed and resuspended in the appropriate buffered sodium chloride solution to E_{470} 0.5. 1 ml of the suspension was applied to each column followed by 2 ml of buffered sodium chloride. The E_{470}s of the eluates were compared to those of eluates obtained from control columns of sepharose and to the E_{470}s of 1 ml of the original suspensions diluted in 2 ml of buffered sodium chloride solution.

2.2.9 Surface charge (SC)

The electrostatic interaction chromatography (EIC) method of Pederson, 1980, was used with the following modifications. The procedure followed was exactly that for HIC except that pasteur pipettes were filled with the strongly basic, anion exchange resin Dowex 1 (chloride form, 8% cross-linked, mesh size 100-200; Sigma Ltd.).

2.2.10 Opsonisation

The amount of serum required for opsonic experiments far exceeded that obtainable from the four subjects used. Pooled time-expired, serum, a gift from the Haematology Laboratory, General Hospital, Steelhouse Lane, Birmingham was checked for antibacterial activity by agar diffusion assay and added to the pool of subjects' serum (2.2.11).

a) BATH

this was chosen to resolve the opsonic requirements of S. aureus NCTC 6571 in pooled, non-immune serum as the method is economical and serum was scarce. 4 ml of cocci in PUM buffer (E_{470} 1.0; approximately 2 x

10^8 cfu ml^{-1}) were incubated for 15 min at 37°C with 1.0 ml volumes of varying dilutions of whole serum, neat complement-depleted serum (56°C for 30 min) or absorbed serum (see below). After the cocci had been incubated in the sera, they were washed twice in PUM buffer and resuspended to E_{470} 0.3 for BATH.

The absorption of serum was performed as follows. 100ml of stationary phase, iron-rich cocci were divided into three, harvested and washed once in 0.85% (W/V) NaCl and centrifuged. The first pellet was resuspended in 10 ml of serum and incubated for 15 min at 37°C. The serum was then recovered by centrifugation and the absorption repeated with the remaining pellets. Finally, the serum was sterilised by filtration through an Acrodisc (Gelman - Hawksley Ltd.).

b) contact angles

cocci were harvested, washed twice and resuspended to 25 ml and E_{470} 20.0 in 0.85% (W/V) NaCl. They were then centrifuged (10 min; 10,000 rpm) and resuspended in 100 ml of 20% (V/V) pooled, non-immune human serum and left for 15 min at 37°C. After two washes in 0.85% (W/V) NaCl the cocci were resuspended to their original volume and OD and the contact angle procedure followed. Control cocci were treated as above except that they were incubated in 0.85% (W/V) NaCl.

c) chemiluminescent studies.

cocci were suspended in 0.85% (W/V) NaCl (E_{470} 1.0) and 4 ml were incubated for 15 min at 37°C with 1 ml of 20% (V/V) serum prepared from the same subject as the blood for leucocyte preparation (2.2.12), and separated at the same time. They were then washed twice in 0.85% (W/V) NaCl to remove the serum.

2.2.11 Whole blood and serum bactericidal assays

This was in heparinised whole blood according to the methods of Al-Hadithy, Addison and Goldstone, 1981 and Jones, Roe and Gupta,

1979. Fresh human blood taken from the volunteers was analysed by Coulter Counter (Coulter Electronics, Bedfordshire), the number of neutrophils was taken to be 66% of the leucocytes (Diem, 1962), and the sample was used in the challenge within one hour of collection. 500 µl of blood were added to 500 µl of cocci in 0.85% (W/V) NaCl in a 5 ml sterile, capped polypropylene tube. The final coccus to neutrophil ratio was approximately 50:1 (high challenge ratio) or 10-20:1 (low challenge ratio). The blood-bacteria mixture was shaken at 120 rpm at 37°C. 100 µl samples were removed at intervals and added to 900 µl aliquots of sterile, ice-cold distilled water and allowed to stand for at least ten min to lyse the blood cells. Dilutions were then made to allow viable counts and results were expressed as a percentage of the original cfu ml^{-1}. At approximately 20 to 30 min into the experiment, smears were made for subsequent May-Grünwald-Giemsa staining (Dacie, 1950). The serum assay was essentially as above. 500 µl of the high or low number of cocci were challenged with 500 µl of serum taken and separated at the same time as the blood.

Equal aliquots of serum from each subject were pooled and held at -18°C for opsonic experiments (Section 2.2.10).

2.2.12 Leucocyte separation

This was after the method of Babior and Cohen ,1981 . 17 ml of fresh whole blood were added to 3.0 ml of acid-citrate-dextrose (2.2% W/V tri-sodium citrate, 0.8% W/V citric acid, 2.5% W/V D-glucose). 10 ml of 6% (W/V) Dextran 75 (FSA Ltd.) in 0.85% (W/V) NaCl was added and the mixture left in a conical, polypropylene, graduated centrifuge tube (Baird and Tatlock Ltd.) for ninety minutes at 4°C for cell separation. The supernatant was removed and centrifuged for 12 min at 900 rpm and 4°C. The resultant supernatant was discarded and 600 µl of ice-cold distilled water added to the pellet to lyse the

erythrocytes; the mixture was then gently vortexed for 25 sec and 200 µl of 0.6 M NaCl added to restore isotonicity before vortexing for five sec. Finally, centrifugation was performed for 4 min at 1500 rpm and 4°C. If erythrocytes were still visible in the resultant pellet then the lysing and subsequent steps were repeated because traces of whole or lysed erythrocytes depress chemiluminescence (Easmon, Cole, Williams and Hastings, 1980). The leucocyte pellet was washed three times in ice-cold 0.85% (W/V) NaCl using 4 min at 1500 rpm and 4°C and finally held at 4°C in 0.85% (W/V) NaCl. A cell count was performed in an Improved Neubauer counting chamber and the number of neutrophils present taken to be 66% of the total (Diem, 1962).

2.2.13 Chemiluminescence (CL)

The luminometer was zeroed by using the built-in standard to give a baseline of 5mV. 200 µl of 2.0×10^{-4} luminol (Sigma Ltd) in Eagle's Minimum Essential Medium modified with 2mM glutamine (EMEM, Flow Laboratories Ltd.) was placed in a polypropylene cuvette. This concentration had previously been found satisfactory for the experimental conditions (LKB Wallace Application Note 513; Williams, Lambert, Haigh and Brown, 1986). 100 µl of opsonised or non-opsonised cocci that were held at 4°C between runs, and 100 µl of EMEM were added to each cuvette (Sarsted Ltd., Beaumont, Leicestershire). This mixture was incubated for five min at 37°C in a waterbath before the addition with mixing of 200 µl of leucocytes (equivalent to 1.65×10^{6} neutrophils ml^{-1}) at time zero. The ratio of cocci to neutrophils gave the high and low challenges as in Section 2.2.11. 100 µl of EMEM replaced the bacteria or leucocytes in the leucocyte or bacterial controls respectively. CL measurements were made, without mixing, at regular periods for one hour. The removal of cuvettes from the waterbath to the luminometer and their replacement was prompt and timed. All cuvettes were run at least in triplicate

for any sample and CL recorded as peak heights. Control runs at the beginning and end of each experiment confirmed that when the neutrophils were held at 4°C, their activity remained relatively constant for up to ten hours after the blood was taken.

2.2.14 Catheter adhesion

The method used represents modifications of published work (Franson, Sheth, Rose, and Sohnle, 1984; Sheth, Franson and Sohnle, 1985). Cocci were harvested, washed twice and resuspended in 0.85% (W/V) NaCl to E_{470} 1.0. 20 ml of suspension was placed in sterile, 100 ml culture flasks that each contained six 1 inch lengths of polypropylene IVAC catheter (IVAC UK Ltd., Basingstoke, Hants.). The lengths had been aseptically cut to avoid a need to re-sterilise and therefore possibly alter the surface properties of the plastic. Also, the pieces were cut along their lengths to aid in the removal of air bubbles.

The flasks were agitated for 60 min at 100 rpm and 37°C. Catheter pieces were recovered, drained carefully onto sterile filter paper and placed in 5 ml of sterile 0.85% (W/V) NaCl. In early experiments, the pieces were vortexed for 30 sec before viability counts were performed, and then re-vortexed for 2 min and 4 min with viability counts at both stages. This was to detect possible sub-populations with differences in adhesive properties. Later, vortexing was carried out for 2 min only. Results were expressed as the number of cfu attached to the catheters as a percentage of the cfu ml^{-1} in the supernatant. Experiments were carried out a minimum of three times.

2.2.15 Protein A (Pr A) estimation

The method was modified from those of Sjöquist and Stålenheim, 1969, Gemmell and O´Dowd, 1983 and the manufacturer´s instructions

regarding the antiserum. Sheep blood (50% whole blood in 50% Alsevers solution; Gibco Ltd., Paisley, Scotland) was washed twice and resuspended to 2% (V/V) in phosphate buffered saline, pH 7.4 (PBS, KH_2PO_4 0.2g, $Na_2HPO_4.2H_2O$ 2.9g, KCl 0.2g, NaCl 8.0g, distilled water to 1 l) in a conical centrifuge tube. 100 µl aliquots of the 2% (V/V) blood and 100 µl of different dilutions (control, 1:50, 1:100; 1:150; 1:200; 1:250; 1:300; 1:400) of dog anti-sheep erythrocyte antiserum (Miles Laboratories Ltd., Stoke Poges, Slough) were mixed in 12 x 75 mm tubes and left for 30 min at 37°C to sensitise the erythrocytes (RBCs). The tubes were read for the lowest dilution in which no haemagglutination occurred. This was then regarded as the optimum dilution or the sub-agglutinating dose (SAD) of the serum for the RBCs in this batch of blood.

2% (V/V) blood washed as above was combined with an equal volume of the SAD and left for 120 min at room temperature with occasional agitation. After two washes in PBS the blood was resuspended to 2% (V/V) in Alsevers solution (Gibco Ltd.) and held at 4°C. This stock of sensitised RBCs was used within a week.

For the test proper, cocci were harvested and cell-free culture supernatants prepared by centrifugation (10 min; 10,000 rpm) and filtration through an Acrodisc (Gelman-Hawksley Ltd.). 50 µl of cocci that had been resuspended to E_{470} 4.0 in PBS, or the supernatants, were serially diluted in PBS in V-shaped microtitre plates (model M25A; Dynatech Ltd., Billingshurst, Sussex). The sensitised RBCs were washed twice and resuspended back to 2% (V/V) in PBS and 50 µl added to each well. Sensitised RBCs were also challenged with PBS and known Pr A concentrations (salt-free; Sigma Ltd) as controls. After 30 min incubation at 37°C, plates were left at room temperature for 60 min before reading. The endpoint for samples and standards was the highest dilution giving haemagglutination. Viability counts allowed

results to be expressed as Pr A content per cfu ml^{-1}. Determinations were in triplicate.

2.2.16 Alpha-haemolysin (α-haemolysin) assay

The method used was that of Saleh and Freer, 1984, except that 2% (V/V) blood was used and values were related to cfu ml^{-1} and not dry weights. Fresh rabbits' blood in Alsevers solution (supplied in house) was washed three times and resuspended to 2% (V/V) in PBS. 50 µl of the cell-free supernatants from Section 2.2.15 were serially diluted in PBS in V-shaped microtitre plates. 20 µl of the blood was added to each test well and to control wells containing PBS. Plates were incubated for 30 min at 37°C and then left overnight at 4°C. The 100% lysis endpoint was recorded and related to cfu ml^{-1}. Determinations were in triplicate.

2.2.17 Staphylocoagulase assay

This was after the method of Cowan and Steel, 1974. 0.2 ml volumes of the supernatants from 2.2.15 were serially diluted in 0.85% (W/V) NaCl in 12 x 75 mm tubes. 0.2 ml of citrated rabbit plasma (Gibco Ltd.) were added to each dilution and the tubes incubated for four hours at 37°C with regular inspection. They were then left overnight at room temperature. The enzyme titre was read as the highest dilution with visible clot or fibrin formation and related to cfu ml^{-1}. Determinations were in triplicate.

2.2.18 Protein estimation

This was after Lowry, Rosebrough, Farr and Randall, 1951. Pyrex, screw-capped boiling tubes (Baird and Tatlock Ltd.) were used and all glassware was prepared as in Section 2.2.1, steps 1-4.

Stock solutions were as follows:

A = 5% (W/V) Na_2CO_3 in distilled water

B = 0.5% (W/V) $CuSO_4$ in 1% Na D-tartrate.$4H_2O$ (FSA Ltd)

These solutions were stored at room temperature.

C = 50 ml of A + 2ml of B

This was only mixed when required and discarded after use. A stock solution of bovine serum albumin (BSA, 1 mg ml^{-1} in distilled water; Sigma Ltd.) was made and standards prepared from 0 μg ml^{-1} to 200 μg ml^{-1} in distilled water to a final volume of 500 μl. 200 μl of sample diluted as appropriate, were also made up to 500 μl in distilled water. 500 μl of 1N NaOH was added to standards and samples and tubes heated at 100°C for 5 min. Solutions were cooled to room temperature and 2.5 ml of solution C added to all tubes which were then left at room temperature for 10 min. Folin-Ciocalteau reagent (BDH Ltd.) was diluted to 1N with distilled water and 500 μl added and the reaction left for 30 min at room temperature. Results were recorded at 750 nm using the 0 μg BSA ml^{-1} standard as the blank.

2.2.19 Lysostaphin digest of whole cells

This was one method used to prepare cocci for gel electrophoresis. 1 ml of washed cocci (E_{470} 6.0 in distilled water) were incubated for 120 min at 37°C with 100 μl of lysostaphin (0.5 μg ml^{-1} in 10 mM Tris-HCl, 0.85% W/V NaCl, pH 7.4, TBS; Sjöguist, Meloun and Hjelm, 1972). The digests were then held at -18°C until needed.

2.2.20 Preparation of cell envelopes

Cocci were harvested, washed three times in ice-cold 0.85% (W/V) NaCl and resuspended in the same to E_{470} 60-70. 10 ml of this suspension were loaded in a Mickle disintegrator vial and an equal volume of acid-washed glass beads added. Balanced vials were given three ten min cycles of shaking and 5 min cooling periods in between. Whole cells were removed by centrifugation twice (10 min; 3,000 rpm), with intermediate and final centrifugations (10 min; 10,000 rpm). The

deposit from the latter was checked by Gram stain to ensure breakage, and was regarded as the "cell wall-membrane containing" or cell envelope preparation. Cell envelope preparations were also prepared from iron-rich and iron-poor S. aureus Hopewell. All preparations were stored at -18°C until required.

2.2.21 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Cell envelope preparations were subjected to SDS-PAGE by the Lugtenberg system (Lugtenberg, Meijers, Peters, van der Hoek and van Alphen, 1975) using a 12% (W/V) acrylamide concentration in the separation gel. Complete heat modification of cell envelope components was achieved by denaturing samples at 100°C for 10 min in an equal volume of sample buffer (10% W/V sodium dodecyl sulphate 5.0 ml; 0.5M Tris-HCL, pH 6.8 2.5ml; glycerol 2.5 ml; 2-mercaptoethanol 0.25 ml; 5% W/V Bromophenol blue 200 µl; distilled water 5 ml).

Electrophoresis was performed in electrode buffer (Tris HCl 6.0 g, glycine 28.8g, 10% W/V SDS 20 ml, distilled water to 2 l, pH 8.3) at room temperature and 40mA for the large gels and 16mA for paired mini-gels. Electrophoresis was stopped when the dye front had moved 120 mm for large gels or simply to the end for the mini-gels. Gels were stained with Coomassie Brilliant Blue R-250 for proteins (0.1% W/V in 50% V/V methanol, 10% V/V glacial acetic acid in distilled water; Sigma Ltd.) and destained (5% V/V methanol, 10% V/V glacial acetic acid in distilled water). Alternatively, they were subjected to immunoblotting (Section 2.2.23). Molecular masses were determined from calibration curves constructed with standard protein markers: bovine serum albumin, ovalbumin, pepsin, trypsinogen, lysostaphin, bovine β-lactoglobulin (Sigma Ltd.) and outer membrane proteins of Pseudomonas aeruginosa PAO1 (in house preparation) and Ps. aeruginosa EW serotype 04 (courtesy of K.H. Ward).

2.2.22 Raising of hyperimmune rabbit antisera

S. aureus NCTC 6571 was grown to late logarithmic-early stationary phase in Fe+ and Fe- TSB with or without the sub-MIC of penicillin. After harvesting, they were treated with a 5% (V/V) final volume of formaldehyde for 60 min at 22°C, washed twice in 0.85% (W/V) NaCl and resuspended to E_{470} 1.0 in the same.

Paired rabbits were injected intravenously with 1 ml aliquots of the bacterial suspensions at weekly intervals for six weeks. Blood was taken from the marginal ear veins before immunisation and ten days after the last injection and a significant increase in titre established. The titre was determined by the method of Live, 1972. 20 µl volume serial dilutions of serum were made in 0.85% (W/V) NaCl on microscope slides and cocci emulsified into the drops. A control for spontaneous agglutination omitted the serum. Slides were agitated manually and placed in damp chambers with observation and agitation, for 4 h at room temperature. Most agglutination reactions took place within a few sec to an h. The rabbits were then bled by cardiac puncture and serum separated and stored at -18° in 1 ml aliquots.

2.2.23 Immunoblotting (IB) and lectin blotting (LB)

This was by a modification of the method of Towbin, Stachelin and Gordon, 1979. Proteins separated by SDS-PAGE were transferred to nitrocellulose paper (pore size 0.45 µm; Bio-Rad Ltd.) in the presence of ice-cold transfer buffer (Tris-HCl 15.0 g, glycine 72.0 g, methanol 1 l, distilled water to 5 l, pH 8.3) initially for 1 h at 80 mA and then overnight at 50 mA. After transfer, the blots were either stained with Amido Black (1% W/V in destain, Section 2.2.21; Sigma Ltd) and de-stained as in Section 2.2.21 to confirm protein transfer, or the antigens were visualised as follows. The blots were incubated for 2 h at 37°C in TBS-Tween, ie. Tris-buffered saline (10 mM Tris-

HCl, 0.85% W/V NaCl pH 7.4, TBS), that contained 0.3% (W/V) Tween 20 to saturate non-specific protein binding sites (Batteiger, Newhall and Jones, 1982). After thorough rinsing in TBS the blots were cut into strips (strip blotting) some of which were then held at -18°C overnight whilst others were incubated in a 1:50 dilution of the appropriate antiserum in TBS-Tween for 4 h at 37°C with gentle shaking and subsequently left overnight at 4°C. Some of the frozen strips were next treated with a 1:300 dilution of rabbit-raised anti-rat IgG-peroxidase conjugate (Miles Laboratories Ltd.) to probe for Pr A whilst others were probed with plant lectin-peroxidase conjugates to detect glycosylated residues:

Concanavalin A (Con A) for glucose and mannose (Reeke, Becker, Cunningham, Gunther, Wang and Edelman, 1974)

Soya Bean Agglutinin (SBA) for N-acetyl galactosamine (Lis Sela, Sachs and Sharon, 1970)

Asparagus Pea Lectin (APL) for fucose (Pereira and Kabat, 1974)

Wheat Germ Agglutinin (WGA) for N-acetyl glucosamine and N-acetyl muramic acid (Nagata and Burger, 1974)

(all at 20 $\mu g\ ml^{-1}$ in TBS-Tween; Sigma Ltd.).

Finally, Avidin-peroxidase conjugate (10 $\mu g\ ml^{-1}$ in TBS-Tween; Sigma Ltd.) was used as a probe for an avidin receptor site (Korpela, Salonen, Kuusela, Sarvas and Vaheri, 1984). Simultaneously and after another rinse in TBS, the antiserum-probed strips were treated with a 25 $\mu g\ ml^{-1}$ solution of Pr A-peroxidase conjugate in TBS. All strips were incubated for 120 min at 37°C with gentle shaking. The Pr A-peroxidase binds to the F_c portion of I_gG and was preferred to goat anti-rabbit I_gG peroxidase conjugate which reacted with staphylococcal

antigens in the absence of rabbit serum. All strips were then washed thoroughly in TBS, and antigenic and other sites visualised with 4-chloro-1-napthol (25 μg ml^{-1}) and H_2O_2 (0.01% V/V) in Tris-HCl (10 mM, pH 7.4). The reaction was stopped after 30 min by washing with distilled water.

S. aureus Hopewell preparations were treated as above but blotted with serial dilutions of the patient's antiserum as a means of determining the "titre" and this was done in parallel with an enzyme linked immunosorbent assay (ELISA, Section 2.2.25).

2.2.24 Labelling of surface proteins of whole cocci with ^{125}I-lactoperoxidase

The radiolabelling method of Booth, 1980 was used. S. aureus NCTC 6571 (t_d = 48 min) was harvested, washed three times in PBS, resuspended in the same (E_{470} 10.0) and split into 1.0 ml aliquots. 500 μl of lactoperoxidase (0.1 mg ml^{-1} in PBS, purified grade, 100 IU per mg protein; Sigma Ltd.) and 10 μl of carrier-free $Na^{125}I$ (10 mCi ml^{-1} in water, Radiochemical Centre, Amersham) were added to each aliquot. Four 100 μl volumes of H_2O_2 (10mM in distilled water) were added at 2.5 min intervals. The iodination of proteins was stopped by the addition of 8 ml cysteine (10 mM in distilled water, Sigma Ltd.) 10 min after the first addition of H_2O_2. Control cocci received the same treatment but the lactoperoxidase was omitted. All cocci were washed four times in PBS and resuspended in 500 μl of distilled water. The radioiodinated and control cocci were subjected to SDS-PAGE as in Section 2.2.21. Gels were dried and labelled surface proteins visualised autoradiographically by taping X-ray film (CEA Singul-X RP grade, Ceaverken, AB, Strangnas, Sweden) on top of the gel. The film was developed after seven days and contact printed to give a reverse image.

2.2.25 Enzyme-linked immunosorbent assay (ELISA)

This was based upon the method of Jacob, Arendt, Brook, Durham, Falk and Schaberg, 1985, and that of Engvall, Johnson and Perlman, 1971, as modified by Shand, Anwar, Brown, Tranter, Modi and Melling (manuscript submitted). S. aureus Hopewell cell envelopes from Section 2.2.20 were resuspended to 0.1 mg protein ml^{-1} in coating buffer (0.05 M sodium carbonate-sodium bicarbonate, pH 9.6). Wells of cobalt-irradiated microtitre plates (model M 129B; Dynatech Ltd.) were loaded with 200 µl of the suspensions. Every reagent used was sequentially replaced in controls with blanking solution which was also controlled. Plates were gently shaken for 60 min at 37°C and left overnight at 4°C.

Plates were washed four times with PBS containing 0.05% (V/V) Tween 20. Non-specific binding sites were blanked by adding 300 µl per well of 1% (W/V) BSA in RPMI 1640 tissue culture medium (with L-glutamine; Gibco Ltd.) that contained 10% (V/V) foetal calf serum (Gibco Ltd.). Plates were shaken for 30 min at room temperature and then 120 min at 37°C. Plates were washed as above and 200 µl added of either Hopewell antiserum or the pooled, non-immune serum (Section 2.2.11), that had been serially diluted in gelatin-phosphate buffer (0.07 M sodium phosphate containing 0.2% W/V gelatin, pH 6.5). Plates were then incubated for 120 min at room temperature with gentle shaking and left overnight at 4°C. After further washing, 200 µl of Pr A-peroxidase (Section 2.2.23) was added and plates were incubated for 120 min at 37°C. The use of this conjugate to detect bound antibody rather than a second antibody conjugate provided a comparison with the immunoblotting. After a final wash, 100 µl of substrate was added to all wells. The substrate had been freshly made by dissolving 10 mg of 3,3′, 5,5′—tetramethylbenzidine (TMB; Sigma Ltd.) in 1 ml of dimethylsulphoxide (DMSO) and adding sodium acetate-sodium citrate

buffer (0.1 M, pH 6.0) to a final volume of 100 ml. 8 µl of H_2O_2 was added to the substrate and 100 µl of this added to all wells. Plates were left for approximately 60 sec at room temperature until a blue colour developed. The reactions were stopped by adding 50 µl of 2 N H_2SO_4 and the absorbance of the wells read at 450 nm. Each determination was in duplicate.

RESULTS and DISCUSSION

3. Characteristics of growth of S. aureus NCTC 6571 in chelex-treated TSB : influence of a sub-MIC of penicillin G

3.1. Reconstitution of chelex-treated TSB

Linear regression analysis of the atomic absorption calibration curves always gave correlation coefficients > 0.9500. Pre-treatment and post-treatment cation levels are in Table 1. Double distilled water washings of glassware were free of contaminating cations and the contribution to iron levels by the vitamin and cation stock solutions was negligible (Table 1).

Linear regression analysis of osmometer calibration curves gave correlation coefficents > 0.9500 and both Fe+ TSB and Fe- TSB (Section 2.2.2 for definitions) were isotonic (280 milliosomoles) with untreated (control) TSB and pooled, normal human serum.

The Fe+ TSB was the equivalent of the untreated TSB in terms of growth kinetics (Fig 5), colonial morphology, Gram stain, clumping factor (CF) and staphylocoagulase enzyme (Cowan and Steel, 1974) alpha (α)-haemolysin and biochemical tests (API Staph; API Ltd., Basingstoke, Hampshire).

Cultures grown in Fe- TSB had a more rapid onset of stationary phase and a lower plateau (Fig 5). They also had less pigment, looser pellets upon centrifugation and reduced catalase activity (Fung and Petrishko, 1973). When the API Staph test was modified to use cell-free supernatants of Fe+ TSB and Fe- TSB stationary phase cultures, the ability to reduce nitrate was only detected in Fe- TSB supernatant. The staphylocoagulase test was positive in both types of culture but approximately four times as much was present in Fe+ TSB culture supernatants when titres were related to 10^8 cfu ml^{-1}. (Fe+ TSB = 1:1.48; Fe- TSB = 1:0.40). The CF reaction was positive only in Fe+ TSB cultures. The α-haemolysin titre related to 10^8 cfu ml^{-1} was

Table 1

The cation content of Chelex-treated and untreated TSB and iron content of vitamin and cation stock solution.

	MOLARITY x 10^{-5}			
	TSB			
Cation	Untreated	Treated	Vitamins(10^{3}x stock)	Cation(250x stock)
Fe	5.73	0.125	0.0125	0.107
Cu	0.0998	0.0281	---	---
Mg	39.20	0.0417	---	---
Ca	28.30	0.218	---	---
Zn	5.91	1.720	---	---

Table 2

Percentage of S. aureus NCTC 6571 showing morphological changes in the presence of 1/16 MIC of penicillin G.

Fe+ TSB		Fe- TSB	
No antibiotic	1/16 MIC	No antibiotic	1/16 MIC
4.00	56.98	2.80	62.12

Figure 5

Growth of S. aureus NCTC 6571 in TSB of varying iron content.

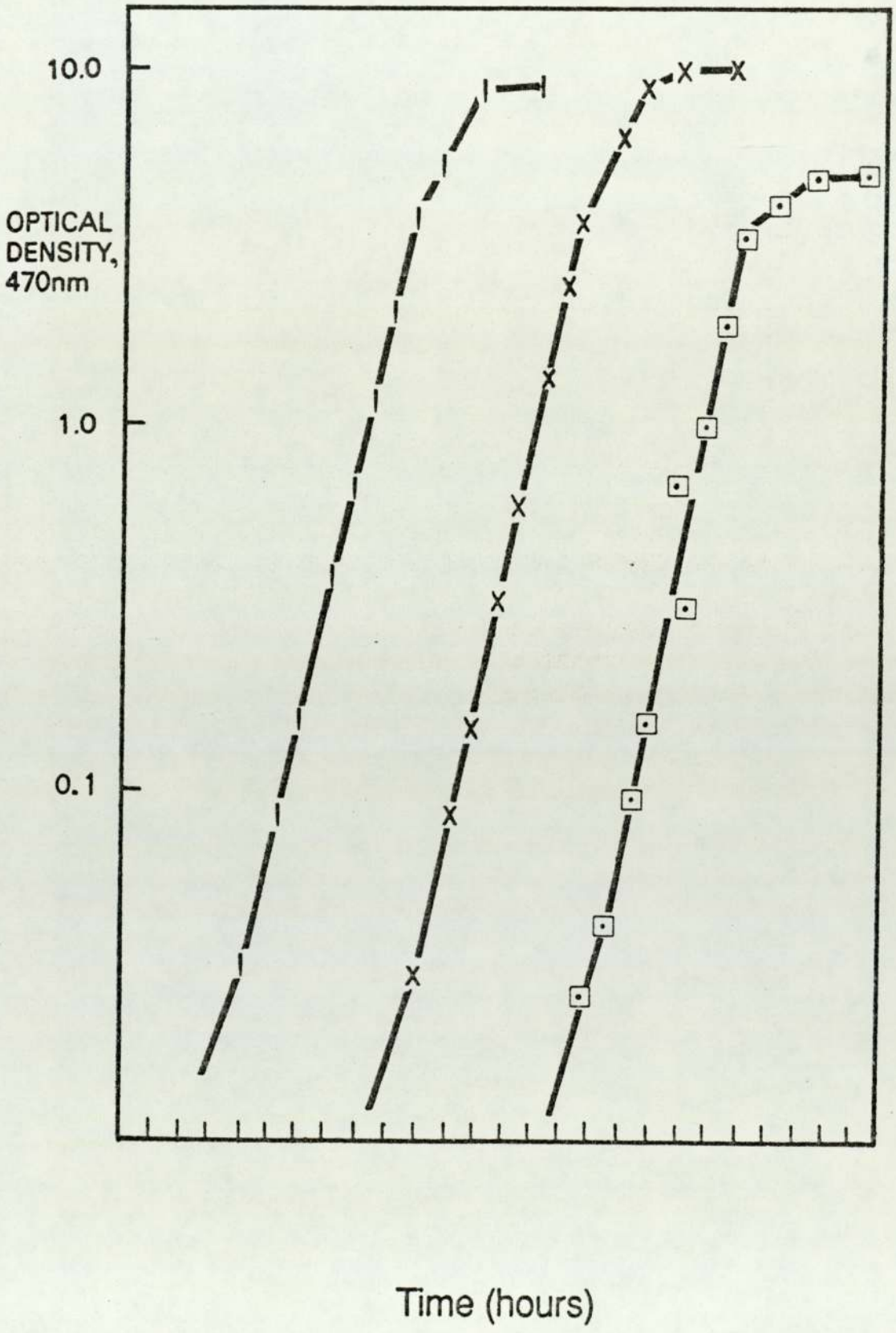
| untreated TSB

× Fe+ TSB

⊡ Fe- TSB

The curves are superimposable but have been separated for clarity and do not imply different lag phases.

Fig 5



very similar for both (Fe+ TSB = 1:0.53; Fe- TSB = 1:0.72).

3.2 Choice of a sub-MIC of penicillin G

The MIC of penicillin G at 18 and 36 h was 0.047 $\mu g\ ml^{-1}$ for untreated TSB, Fe+ TSB and Fe- TSB cultures of S. aureus NCTC 6571.

Growth in the various TSBs and in the presence of graded sub-MICs of penicillin, from zero time, is shown in Fig 6 and 7.

No morphological changes (Lorian, 1980) were detected at 1/20 MIC and lysis occured at 1/8 MIC. Morphological changes evident as giant cocci or incompletely divided cocci were present in 1/16 MIC 6 h after inoculation and were quantified microscopically (Table 2). 1/16 MIC did not alter the growth rate and therefore this sub-MIC was used throughout the study.

3.3 Iron uptake by S. aureus NCTC 6571 ; correlation between optical density (OD) and viable count

There was no difference between untreated TSB and Fe+ TSB cultures when uptake was determined by atomic absorption analysis of culture supernatants. Uptake was apparently immediate and reached a peak in late-logarithmic phase. There then appeared a plateau period before further uptake of iron in early stationary phase. Fe- TSB cultures, in contrast, did not take up iron from the supernatant until late-logarithmic phase and there was no plateau period nor a second uptake phase (Fig 8 and 9; Table 3).

The sub-MIC of penicillin G did not visibly affect iron uptake in any of the cultures. The correlation coefficents (Table 4) showed that regardless of the sub-MIC or iron deprivation, there was a straight line relationship between OD and the number of log-phase cocci.

Figure 6

Growth of S. aureus NCTC 6571 in the absence and presence of graded sub-MICs of penicillin G.

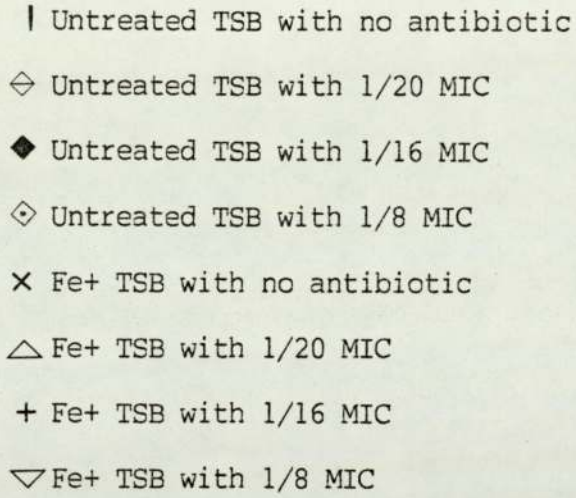


Fig 6

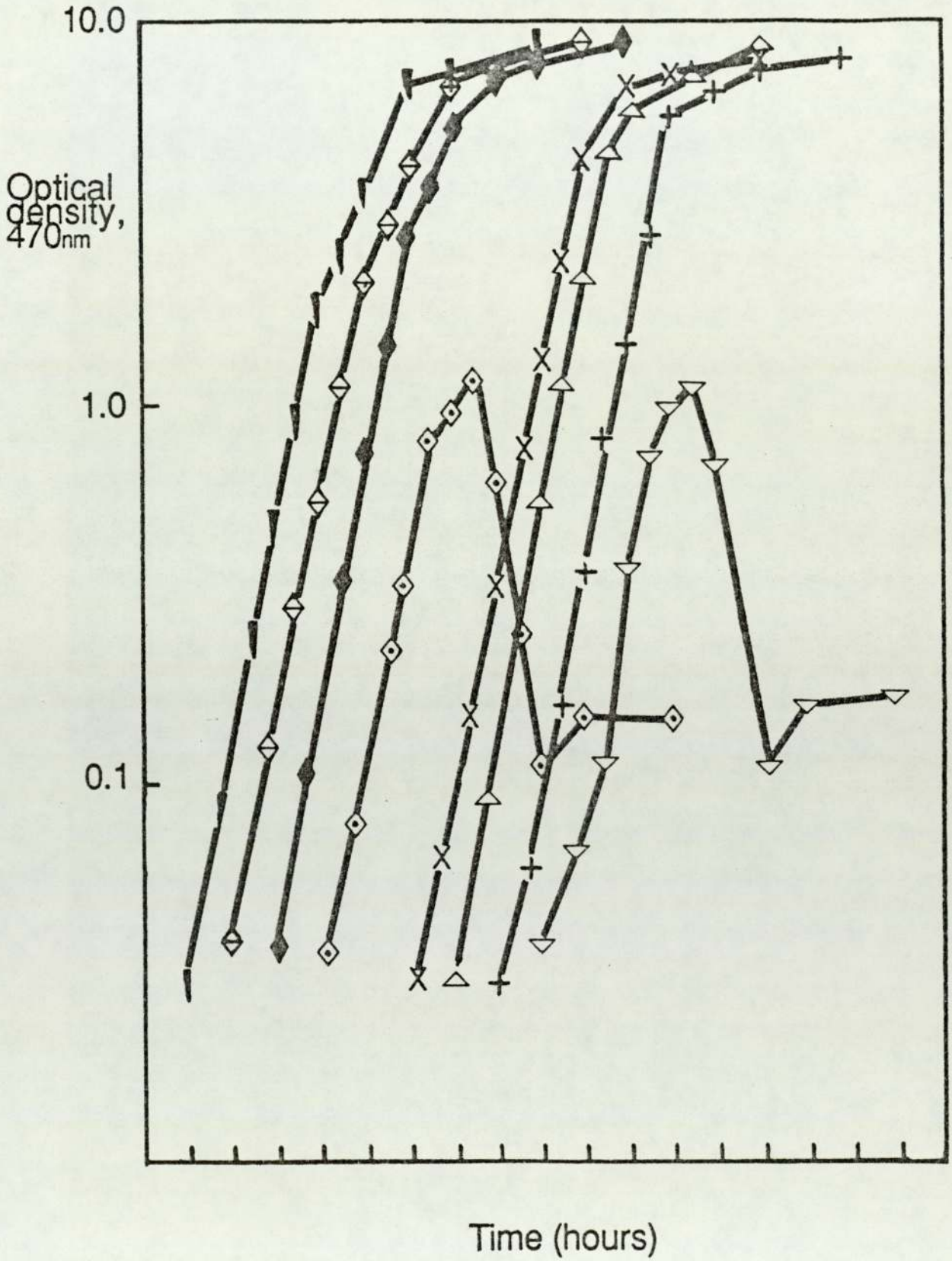


Figure 7

Growth of S. aureus NCTC 6571 in the absence and presence of graded sub-MICs of penicillin G

× Fe+ TSB with no antibiotic

△ Fe+ TSB with 1/20 MIC

+ Fe+ TSB with 1/16 MIC

▽ Fe+ TSB with 1/8 MIC

⊙ Fe- TSB with no antibiotic

▩ Fe- TSB with 1/20 MIC

⊡ Fe- TSB with 1/16 MIC

▧ Fe- TSB with 1/8 MIC

Fig 7

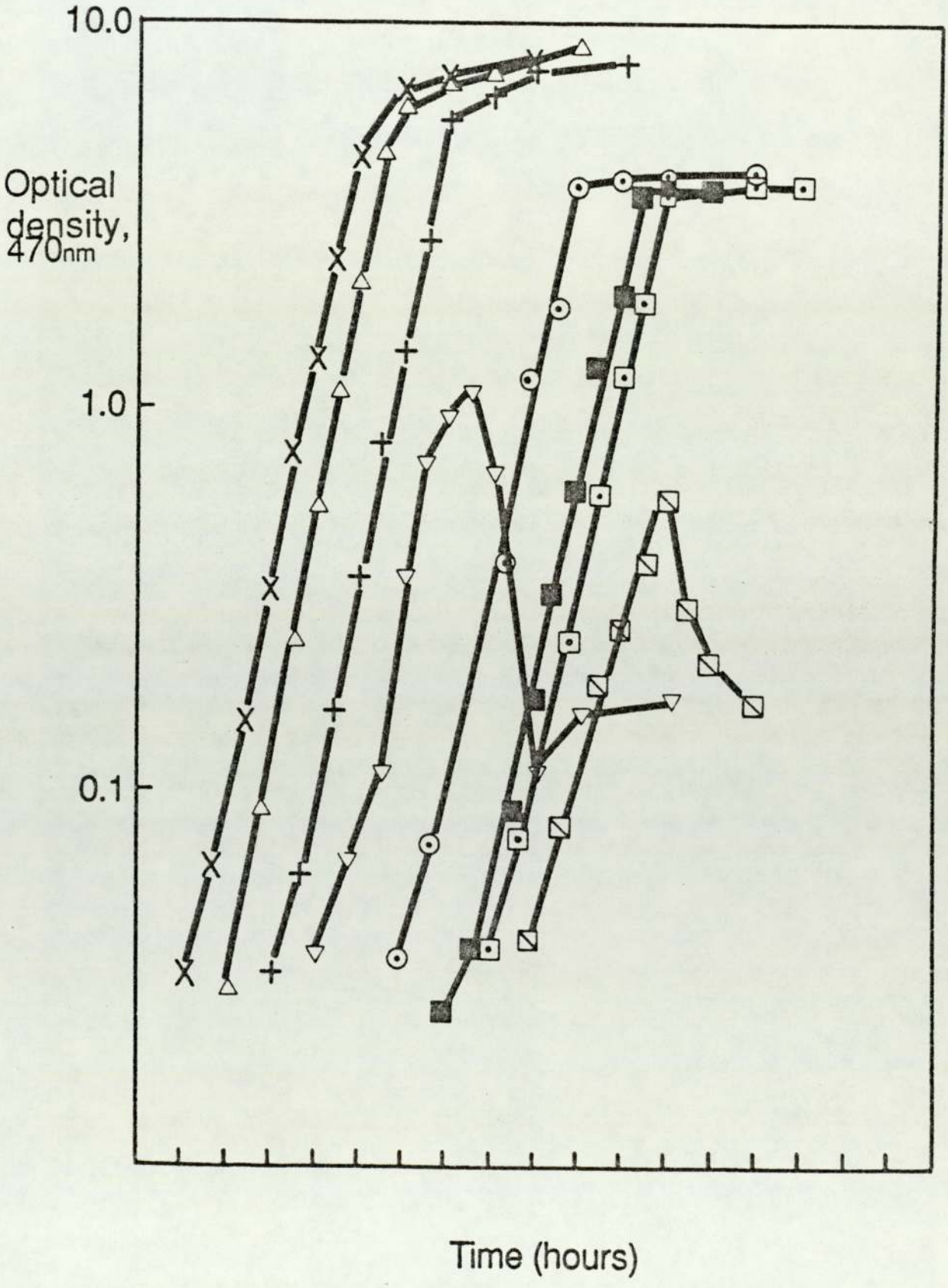


Figure 8

The growth of S. aureus NCTC 6571 and uptake of iron in the absence and presence of 1/16 MIC of penicillin G, in untreated TSB and Fe+ TSB.

| Untreated TSB with no antibiotic

◆ Untreated TSB with penicillin G

× Fe+ TSB with no antibiotic

+ Fe+ TSB with penicillin G

Fig 8

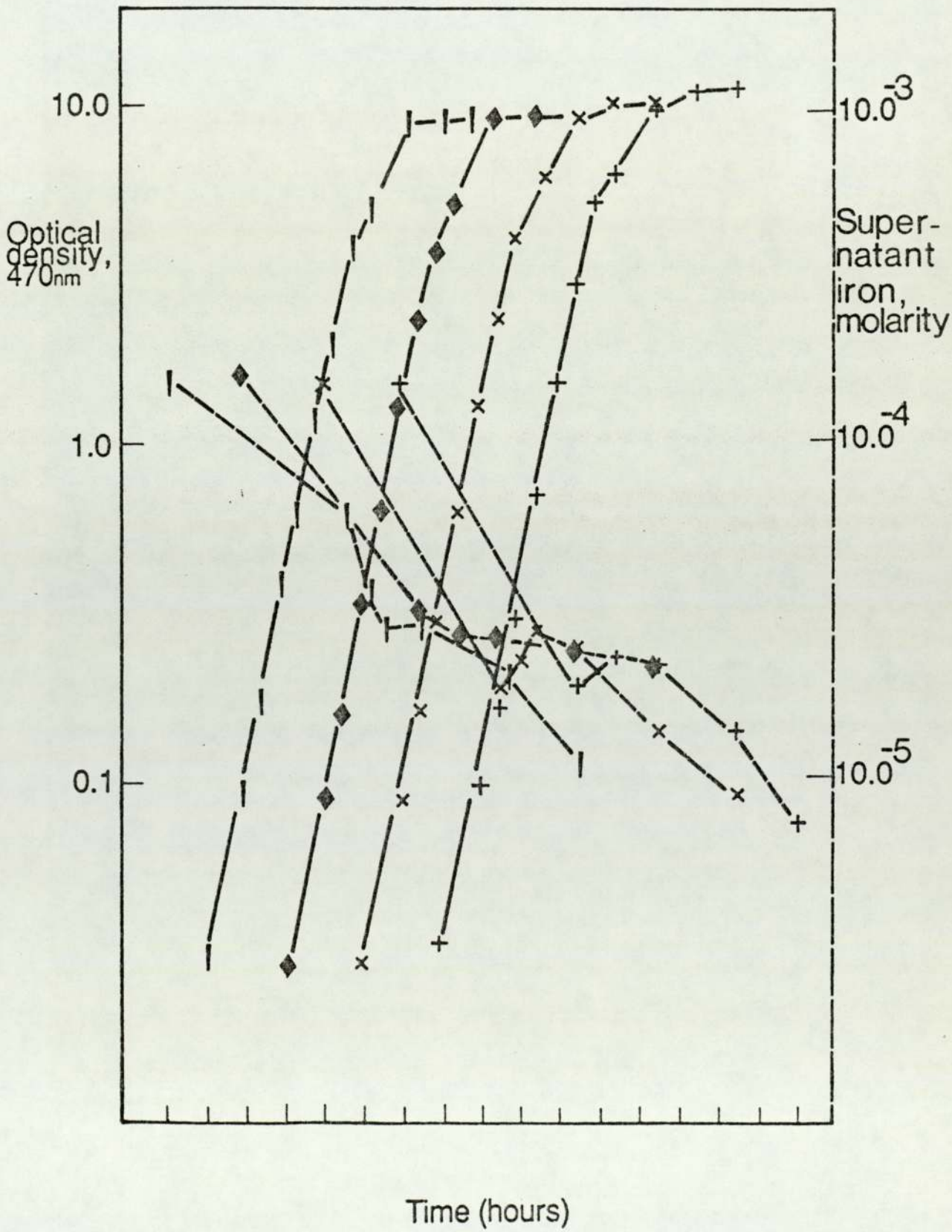

10.0
1.0
0.1
Optical density, 470nm
10.0-3
10.0-4
10.0-5
Super-natant iron, molarity
Time (hours)

Figure 9

The growth of S. aureus NCTC 6571 and uptake of iron in the absence and presence of 1/16 MIC of penicillin G, in Fe+ TSB and Fe- TSB.

× Fe+ TSB with no antibiotic

+ Fe+ TSB with penicillin G

⊙ Fe- TSB with no antibiotic

⊡ Fe- TSB with penicillin G

Fig 9

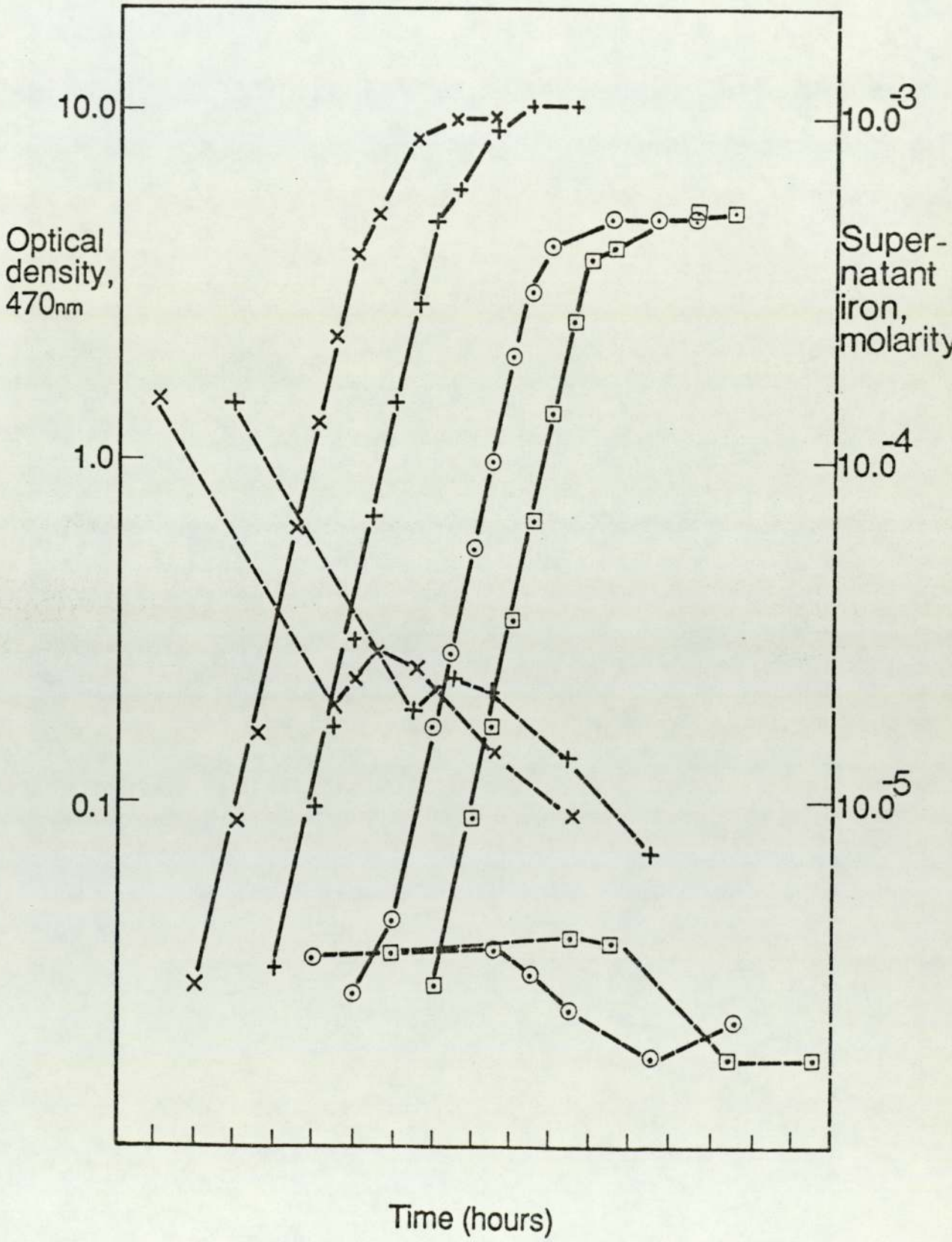

10.0
1.0
0.1
Optical density, 470nm
10.0^-3
10.0^-4
10.0^-5
Super-natant iron, molarity
Time (hours)

Table 3

Percentage of iron remaining in the supernatant during growth of S. aureus NCTC 6571 in the presence of a sub-MIC of penicillin G (results are means of duplicate flasks).

Time after inoculation, hours	Control TSB no antibiotic	Control TSB 1/16 MIC	Fe+ TSB no antibiotic	Fe+ TSB 1/16 MIC	Fe- TSB no antibiotic	Fe- TSB 1/16 MIC
0	100.00	100.00	100.00	100.00	100.00	100.00
3.5	22.67	20.00	12.40	12.40	100.00	100.00
4.5	18.90	16.70	17.70	15.90	90.00	90.00
5.5	19.27	16.70	16.70	14.30	72.00	61.00
7.5	14.00	15.60	9.52	9.52	50.00	50.00
9.5	7.56	13.80	6.20	5.00	65.00	50.00

Table 4

Correlation coefficents for the relationship between optical density and viable count (cfu ml^{-1}) for log-phase S. aureus NCTC 6571 grown in various TSBs in the presence of 1/16 MIC of penicillin G.

	r
Control TSB with no antibiotic	0.928
Control TSB with 1/16 MIC	0.995
Fe+ TSB with no antibiotic	0.999
Fe+ TSB with 1/16 MIC	0.995
Fe- TSB with no antibiotic	0.993
Fe- TSB with 1/16 MIC	0.968

3.4 Discussion

Chelex 100 has previously been shown to be efficent at removing cations from brain heart infusion broth (Cowart, Marquardt and Foster, 1980). The method developed here for the production of iron-poor TSB was successful in that Fe+ TSB supported growth of S. aureus NCTC 6571 indistinguishable from that in untreated TSB in terms of growth kinetics and iron uptake, enzymic and morphological tests and a simple biochemical screen. Yet, the omission of iron in the reconstituted medium had a significant effect. Alterations in growth parameters, the loss of pigment and decrease in catalase activity for iron-poor cocci have been previously noted (Schade, 1963; Theodore and Schade, 1965 a and b).

There are several methods of determining a sub-MIC (Atkinson and Amaral, 1982). The one chosen here was one that induced significant morphological changes compared to controls but neither lysis nor a change in growth rate.

Iron uptake in excess iron conditions (untreated TSB, Fe+ TSB) was rapid and efficent with regard to residual supernatant iron. Iron uptake in Fe- TSB lagged appreciably. This could be because of the use of an iron-poor inoculum in that the organism's metabolism was already adjusted to conditions of low iron. Only approximately 50% of iron was taken up from Fe- TSB implying that iron was not needed or more likely, that the cocci could not take up any more. However, very little is known about mechanisms of iron uptake in Staphylococci. Certainly the experiments indicate that there is a rapid switch in metabolism that allows iron-poor cocci quickly to store iron when it is freely available. The sub-MIC of penicillin G did not influence the kinetics of iron uptake for any of the coccal types.

4.1 Opsonic requirements of S. aureus NCTC 6571

Overnight Fe+ TSB and Fe- TSB cultures were examined in the BATH assay using hexadecane and octane to determine which dilution of pooled, normal human serum (Section 2.2.10) was effective in opsonisation as judged by an increase in hydrophobicity.

No difference was found between the two hydrocarbons although withdrawal of samples from octane-containing tubes was less prone to hydrocarbon contamination. Fe- TSB cultures were always less hydrophobic but 10% (V/V) and 20% (V/V) serum rendered both types of culture completely hydrophobic (Fig 10). 20% (V/V) serum was chosen for use throughout this study.

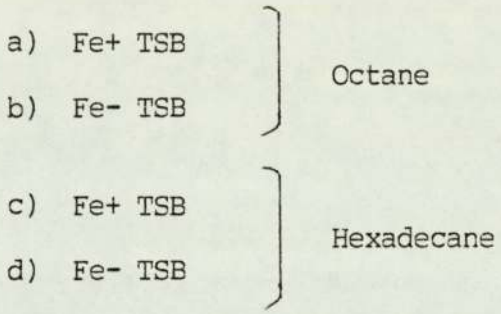
Significantly more opsonisation was conferred by antibody than by complement, although both were needed for effective opsonisation, ie. maximum hydrophobicity in BATH. No differences in opsonic needs were detected between Fe+ TSB and Fe- TSB cultures (Fig 11 and 12).

4.2 Discussion

S. aureus has been shown to exhibit strain differences in opsonic requirements in phagocytic assays (Verhoef et al., 1977a). In the BATH assay, S. aureus NCTC 6571 was less efficently opsonised, ie. its surface hydrophobicity was increased less, by 20% (V/V) complement-depleted serum than by 20% (V/V) antibody-depleted serum. Neither was as efficient as 20% (V/V) whole serum at opsonising. A dose-response relationship exists for opsonising serum concentrations and phagocytosis of S. aureus (Leijh, van der Barselaar, van Zwet, Dubbeldeman-Rempt and van Furth, 1979), and for opsonising serum concentrations and chemiluminescence provoked by S. aureus (Easmon, Cole, Williams and Hastings, 1980; Gemmell and O'Dowd, 1983; Williams, Hastings, Easmon and Cole, 1980). The indications are that opsonisation with 20% (V/V) whole serum is satisfactory and that

Figure 10

The effect of graded dilutions of pooled, normal human serum upon the surface hydrophobicity of S. aureus NCTC 6571 as determined in the BATH assay.



1 PUM buffer (Section 2.2.8)

2 5% serum

3 10% "

4 20% "

Fig 10

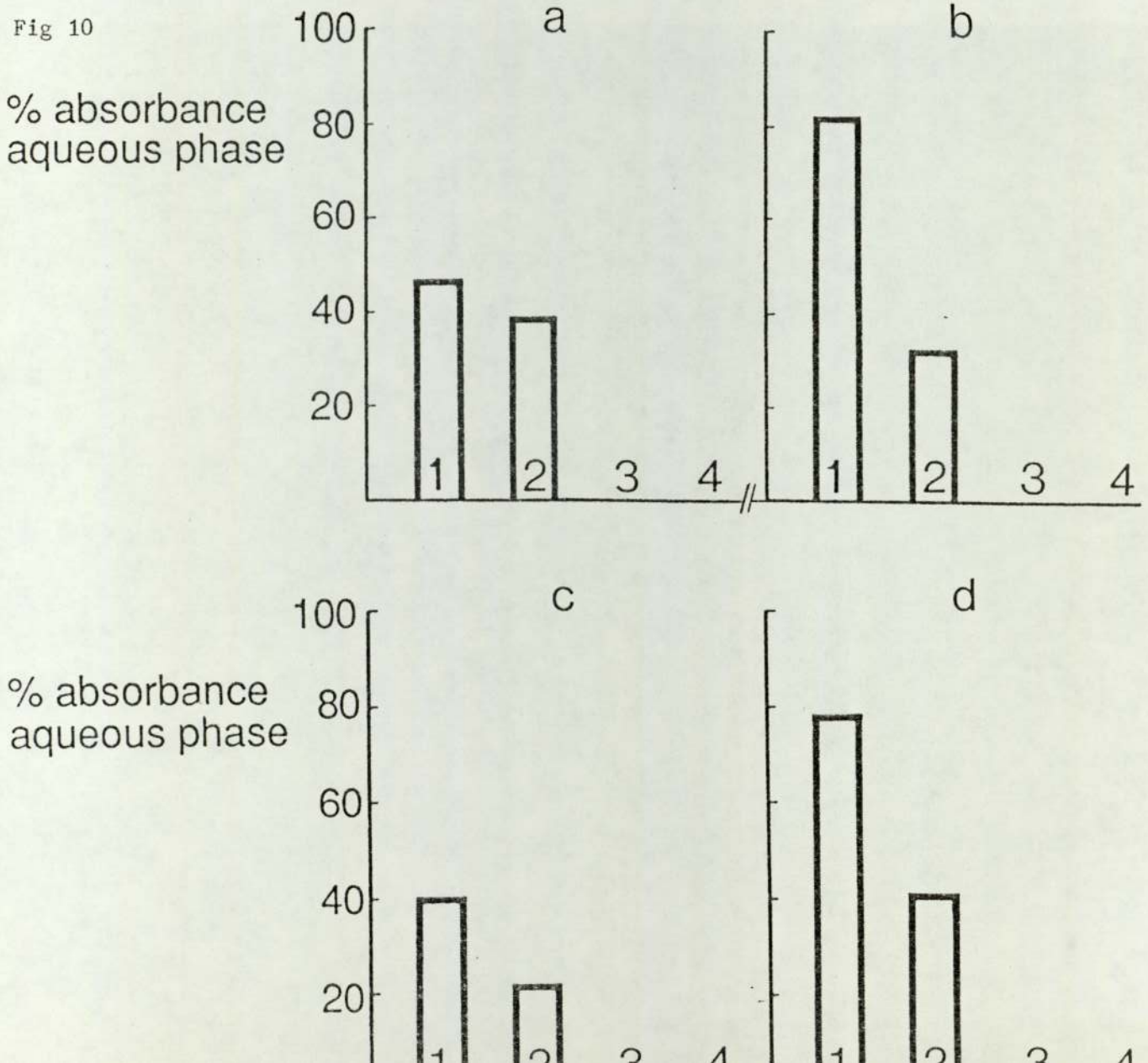

a
b
c
d
% absorbance aqueous phase
% absorbance aqueous phase
100
80
60
40
20
1
2
3
4

Figure 11

The effect of 20% (V/V) treated, pooled normal human serum upon the surface hydrophobicity of S. aureus NCTC 6571 as determined in the BATH assay.

a) Fe+ TSB
b) Fe- TSB
} Octane

c) Fe+ TSB
d) Fe- TSB
} Hexadecane

1 PUM buffer (Section 2.2.8)

2 Absorbed 20% serum

3 Heated " "

4 Whole " "

Fig 11

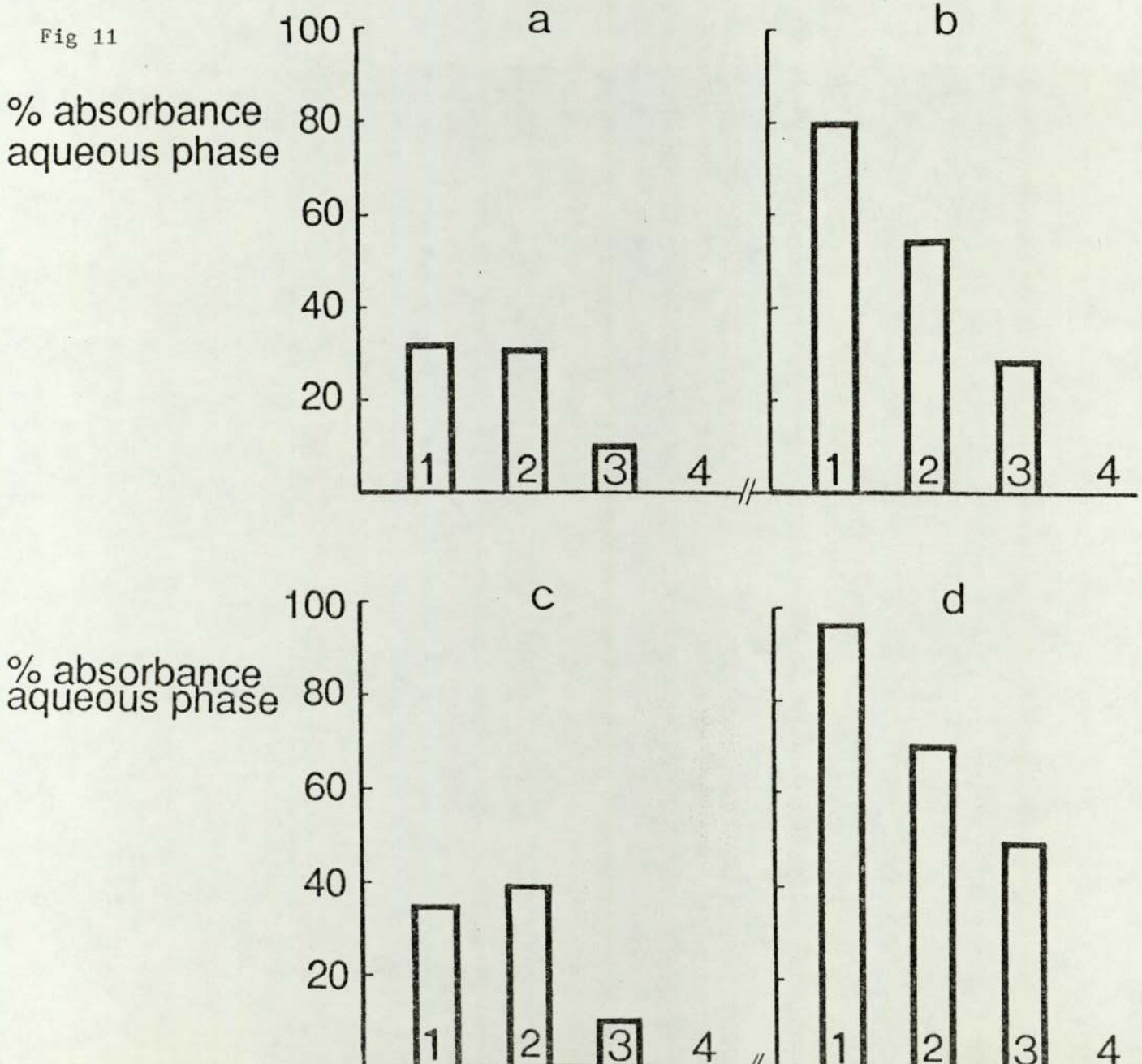


Figure 12

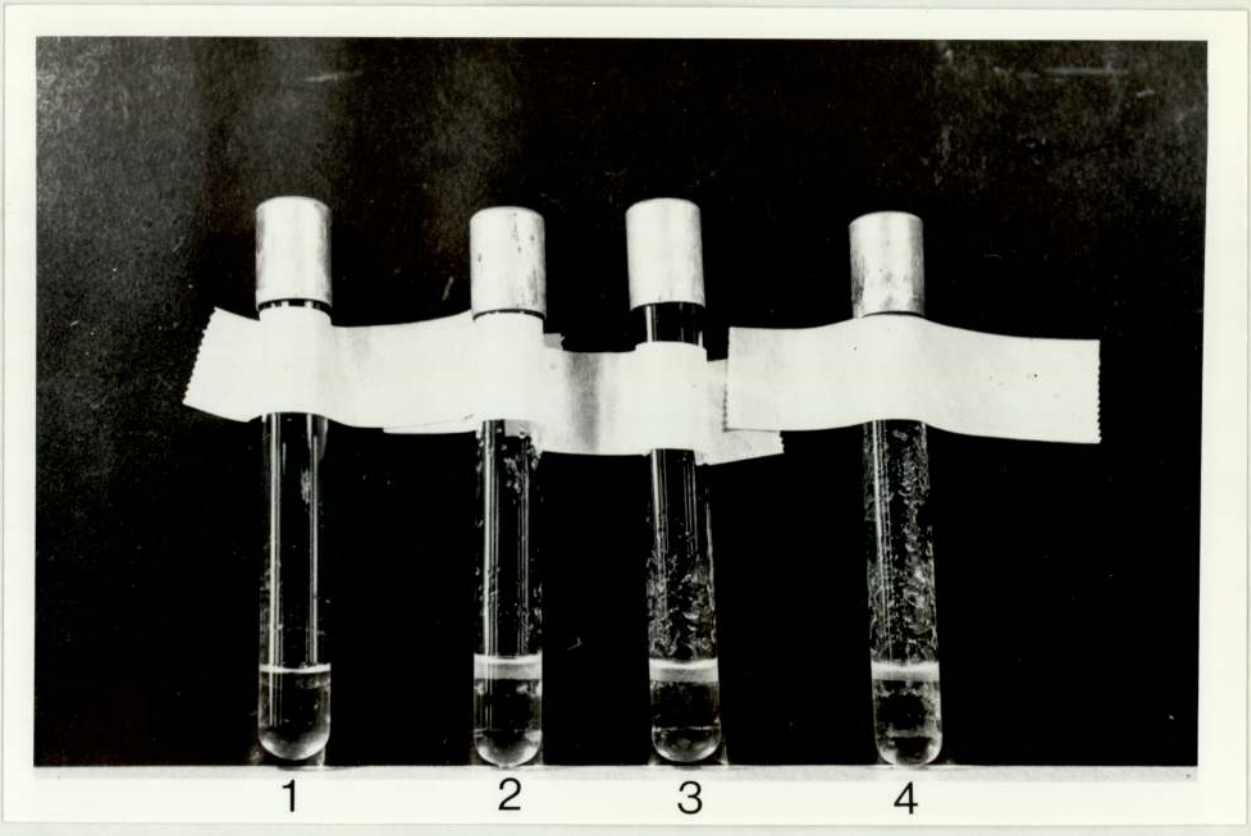
The effect of 20% (V/V) treated, pooled normal human serum upon the surface hydrophobicity of S. aureus NCTC 6571 grown in Fe- TSB as determined in the BATH assay with OCTANE.

1 PUM buffer (Section 2.2.8)

2 Absorbed serum

3 Heated "

4 Whole "



primarily, antibody is involved for both iron-rich and iron-poor S. aureus NCTC 6571.

5. CHARACTERISTICS OF S. AUREUS NCTC 6571 WITH DIFFERENT DOUBLING TIMES IN Fe+ TSB AND Fe- TSB : INFLUENCE OF A SUB-MIC OF PENICILLIN G

5.1 Growth Kinetics

Different initial ODs, broth volume: flask size ratios and shaking rates resulted in relatively slow (t_d = 48 min) and relatively fast (t_d = 24 min) growing cocci harvested in mid-log to early stationary phase (Fig 13). Growth rate was not affected by the penicillin G but iron deprivation caused a lower stationary phase onset for the slow growing cocci.

Alterations in growth rate and environmental iron (Fe+ TSB, Fe- TSB) and adding the sub-MIC of penicillin G for one generation produced eight phenotypes that were used throughout the remainder of this study.

5.2 The surface hydrophobicity (SH) of S. aureus NCTC 6571

a) BATH

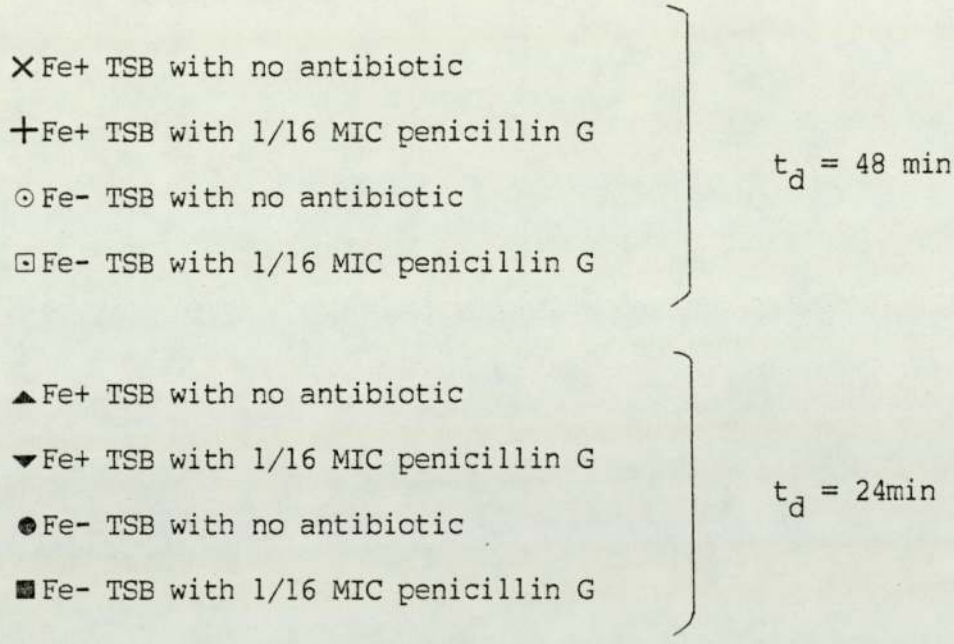
No significant differences were noted between the eight phenotypes as all were relatively hydrophobic. Those in hexadecane were slightly more hydrophobic overall than those in octane (Fig 14).

b) HIC

All eight coccal types were indistinguishable and totally hydrophobic with 4M and 2M NaCl as eluate (not shown). Slight differences were detected with 1M and 0.5M NaCl (Fig 15). In 1M NaCl, the fast grown Fe+ TSB cocci treated with 1/16 MIC were rather less hydrophobic than the others, whilst in 0.5M NaCl cocci were overall less hydrophobic.

Figure 13

Slow (t_d = 48 min) and fast (t_d = 24 min) growth of S. aureus NCTC 6571. Different growth rates were induced by alterations in initial optical densities and aeration (see text).



Arrows denote time of addition of penicillin G

Fig 13

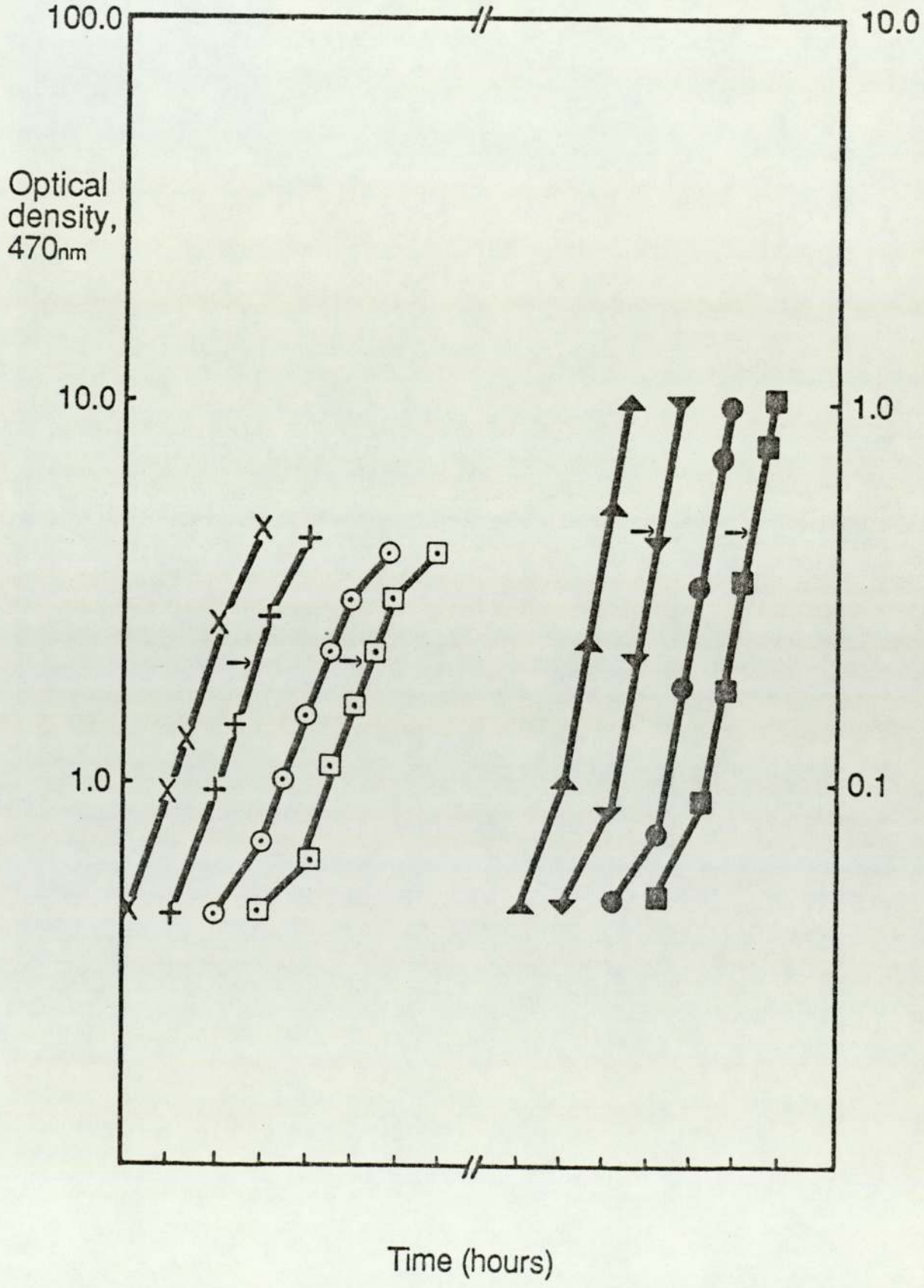


Figure 14

Surface hydrophobicity of S. aureus NCTC 6571 (t_d = 48 and 24 min) in Fe+ TSB and Fe- TSB and in the absence and presence of 1/16 MIC of penicillin G, as determined in the BATH assay.

a) hexadecane

b) octane

1 Fe+ TSB with no antibiotic

2 Fe+ TSB with penicillin G

3 Fe- TSB with no antibiotic

4 Fe- TSB with penicillin G

Fig 14

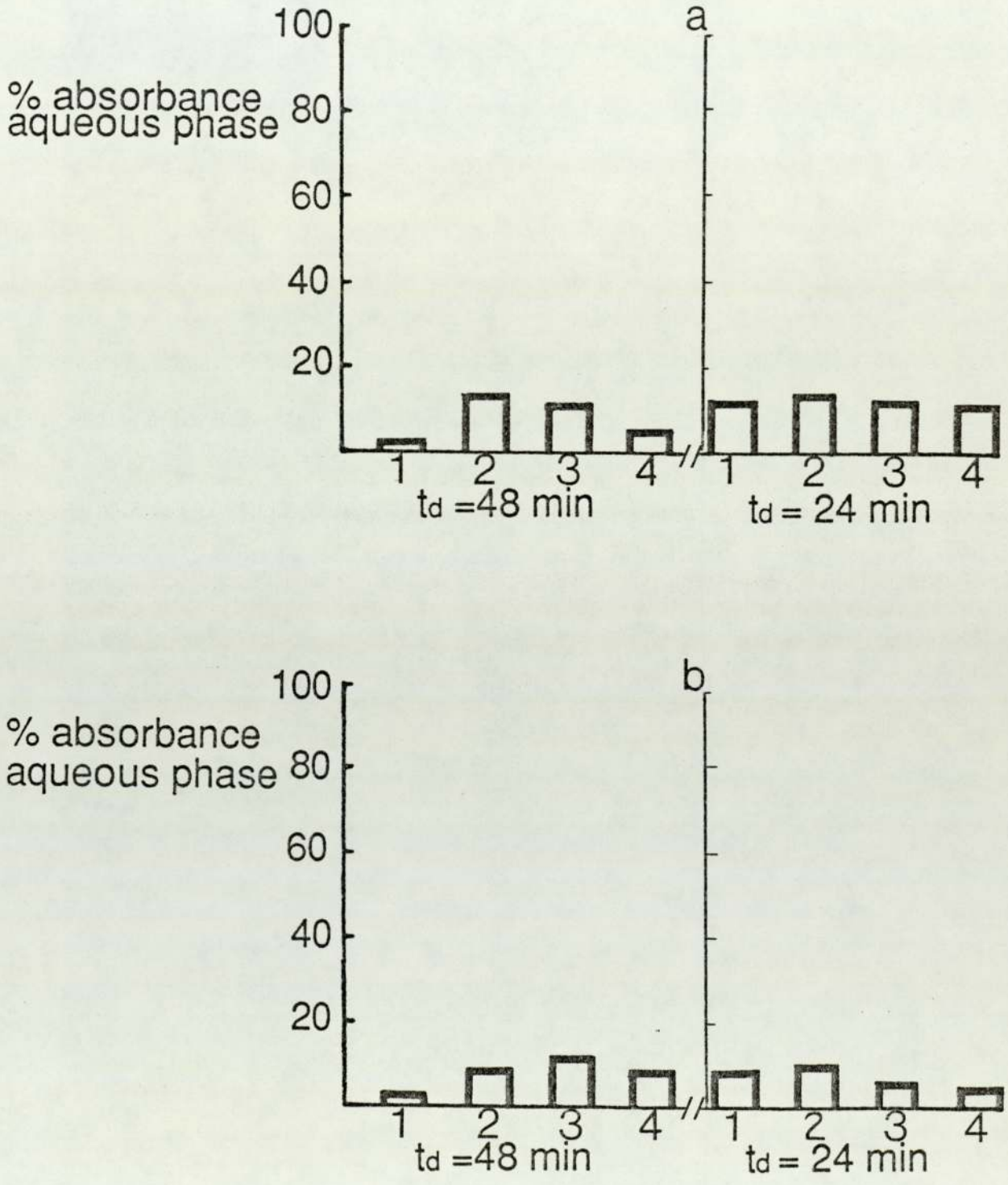

a
100
% absorbance
aqueous phase
80
60
40
20
1
2
3
4
1
2
3
4
td =48 min
td = 24 min
b
100
% absorbance
aqueous phase
80
60
40
20
1
2
3
4
1
2
3
4
td =48 min
td = 24 min

Figure 15

Surface hydrophobicity of S. aureus NCTC 6571 (t_d = 48 and 24 min) in Fe+ TSB and Fe- TSB and in the absence and presence of 1/16 MIC of penicillin G as determined in the HIC assay.

a) 1.0M NaCl in 10mM phosphate buffer, pH 6.8

b) 0.5M NaCl in 10mM phosphate buffer, pH 6.8

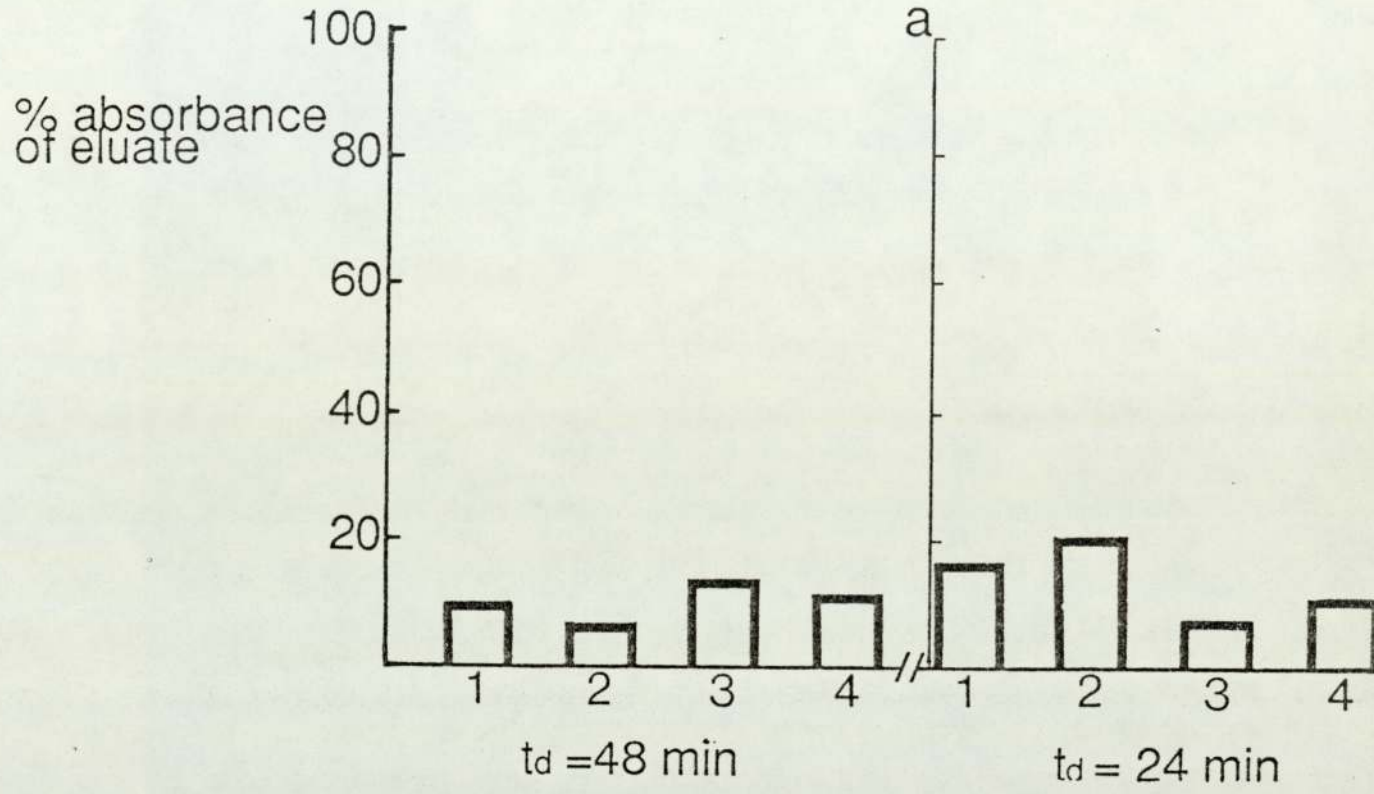
1 Fe+ TSB with no antibiotic

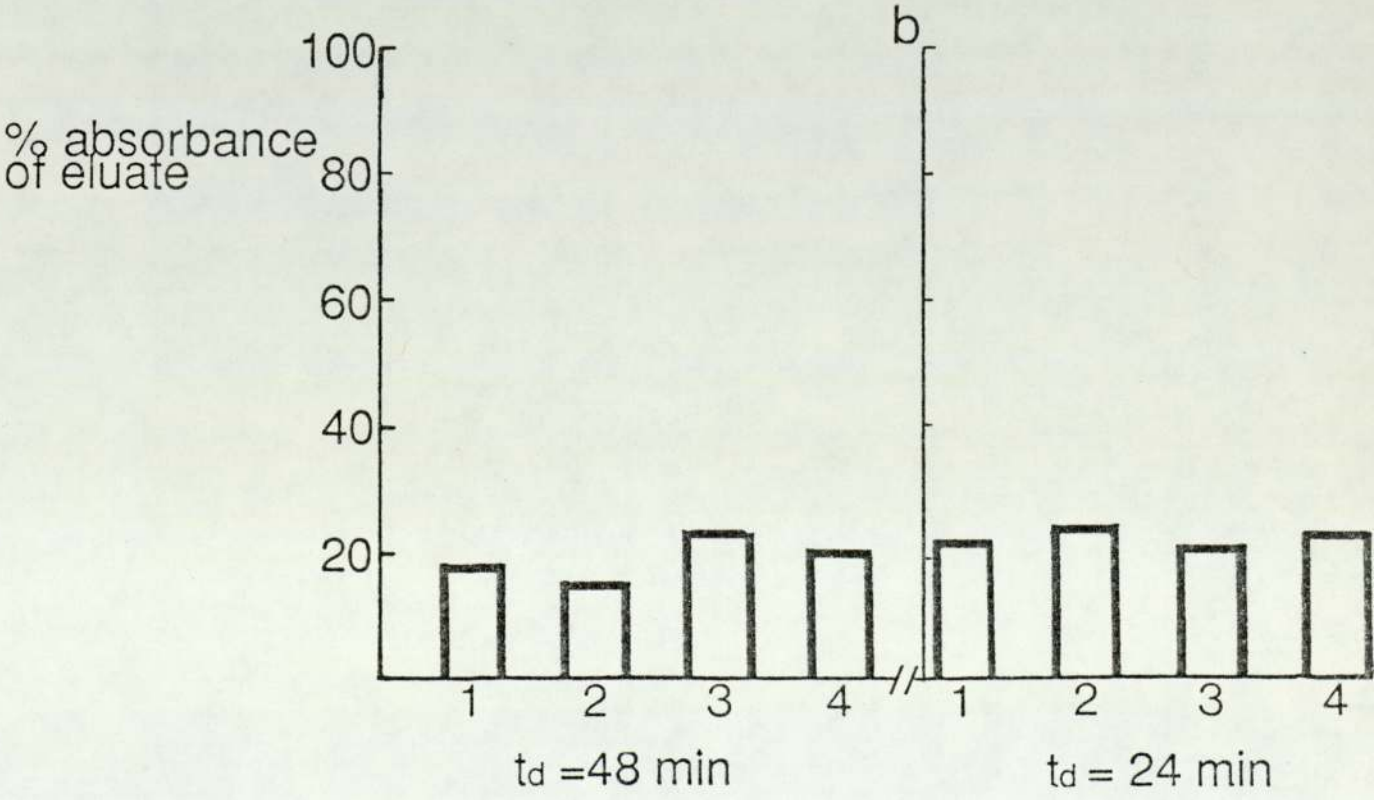
2 Fe+ TSB with penicillin G

3 Fe- TSB with no antibiotic

4 Fe- TSB with penicillin G

Fig 15


a
100
% absorbance of eluate
80
60
40
20
1
2
3
4
1
2
3
4
td =48 min
td = 24 min


b
100
% absorbance of eluate
80
60
40
20
1
2
3
4
1
2
3
4
td =48 min
td = 24 min

c) contact angles

Prior to using Fe+ and Fe- TSB, the method was characterised with respect to drying times of the pads by the use of untreated TSB cultures. It was apparent that a 3 h drying period was optimal (Table 5). Significant differences between the eight coccal types were detected. In the non-opsonised, slow-growing model, iron-poor cocci were markedly less hydrophobic than iron-rich cocci and penicillin G significantly increased the relative hydrophobicity of both despite treatment for only one generation before harvest (Fig 16a).

However, non-opsonised, fast-grown cocci uniformly had a relatively high surface hydrophobicity regardless of iron status or penicillin G treatment (Fig 16c).

Opsonisation of the slow-grown cocci with 20% (V/V) pooled, normal human whole serum (Section 2.2.10) affected the iron-poor cocci treated with or without penicillin G most of all, as their relative hydrophobicity increased to beyond those of the iron-rich equivalents. Opsonisation did not significantly increase the contact angle of iron-rich cocci but decreased that of iron-rich cocci treated with penicillin G (Fig 16b). Evidence in Sections 12 and 13 suggests that the enhanced effect upon iron-poor cocci may be because the antibodies found in normal human serum recognise that antigen better. Opsonisation experiments were not conducted on fast-grown cocci because of insufficient serum due to the need to perform several replicates of the more clinically relevant slow-growing model.

5.3 The surface charge (SC) of S. aureus NCTC 6571

The results obtained were similar to those for HIC in that despite varying the sodium chloride concentration, no significant differences were detected (Fig 17). All coccal types had the same strongly anionic surface character.

Table 5

Influence of drying time upon contact angles of S. aureus NCTC 6571.

Values are means of a minimum of ten separate determinations

± standard deviation (±SD)

	t_d = 48 min		t_d = 24 min	
	Control TSB with no antibiotic	Control TSB with 1/16 MIC	Control TSB with no antibiotic	Control TSB with 1/16 MIC
Drying time, hours				
1	$12.88^{o}\pm2.71$	$18.40^{o}\pm1.60$	$36.50^{o}\pm3.00$	$46.71^{o}\pm2.80$
2	$20.58^{o}\pm1.83$	$32.71^{o}\pm4.57$	$48.25^{o}\pm6.50$	$55.00^{o}\pm2.50$
3	$30.43^{o}\pm1.40$	$48.60^{o}\pm1.60$	$60.00^{o}\pm1.83$	$66.50^{o}\pm4.44$
3.5	$29.28^{o}\pm1.70$	$49.10^{o}\pm2.30$	$51.30^{o}\pm2.14$	65.20 ± 2.80
4	repeated cracking		---	---

Figure 16

Surface hydrophobicity of S. aureus NCTC 6571 (t_d = 48 and 24 min) in Fe+ TSB and Fe- TSB and in the absence and presence of 1/16 MIC of penicillin G as determined by the contact angle method.

a) non-opsonised, t_d = 48 min

b) opsonised, t_d = 48 min

c) non-opsonised, t_d = 24 min

Bars represent standard deviations and values are means of a minimum of ten separate determinations.

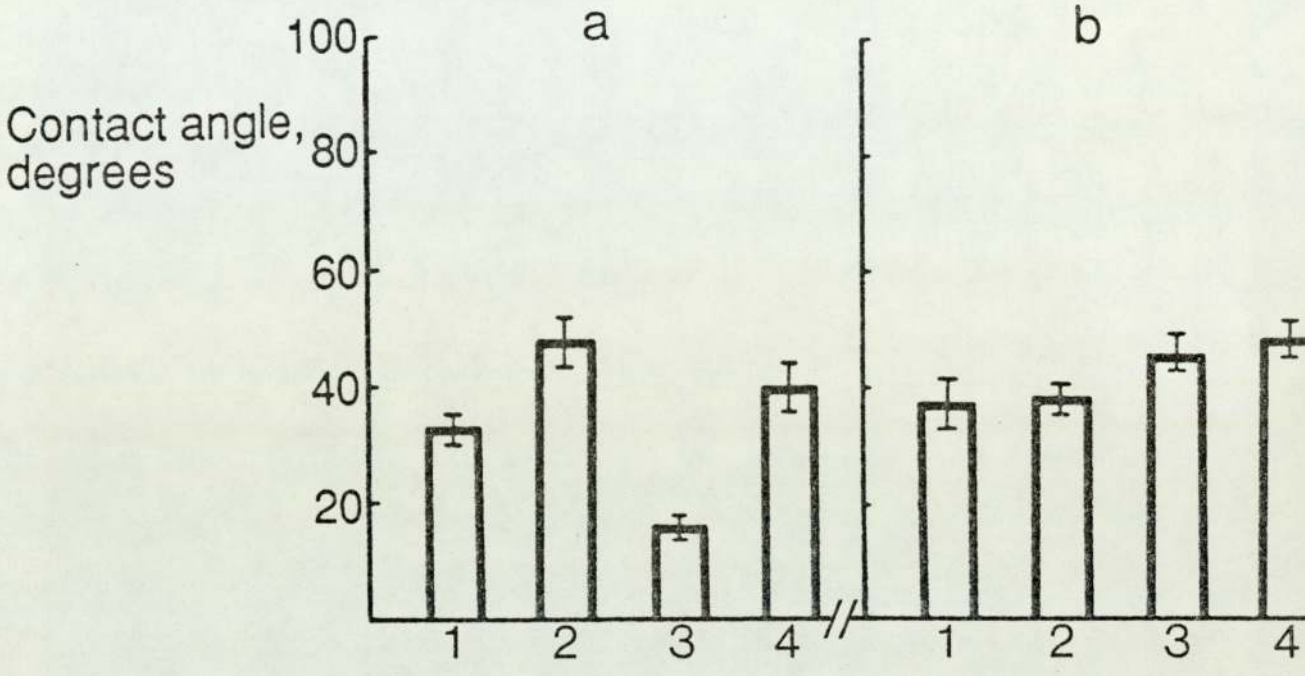
1 Fe+ TSB with no antibiotic

2 Fe+ TSB with penicillin G

3 Fe- TSB with no antibiotic

4 Fe- TSB with penicillin G

Fig 16


a
b
100
80
60
40
20
Contact angle, degrees
1
2
3
4
1
2
3
4

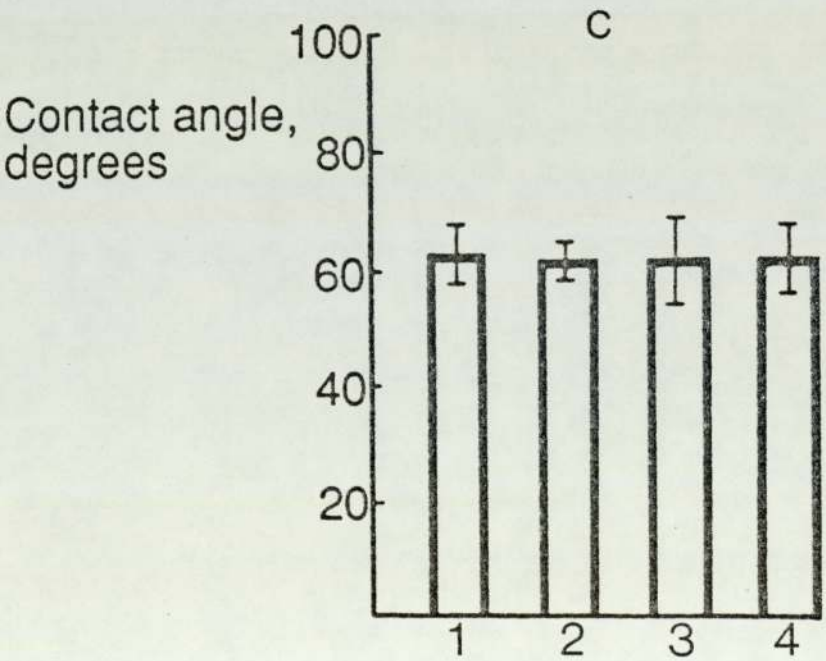

c
100
80
60
40
20
Contact angle, degrees
1
2
3
4

Figure 17

The surface charge of S. aureus NCTC 6571 (t_d = 48 and 24 min) in Fe+ TSB and Fe- TSB and in the absence and presence of 1/16 MIC of penicillin G as determined in the EIC assay.

a) 0.5M NaCl in 10mM phosphate buffer, pH 6.8

b) 0.1M NaCl in 10mM phosphate buffer, pH 6.8

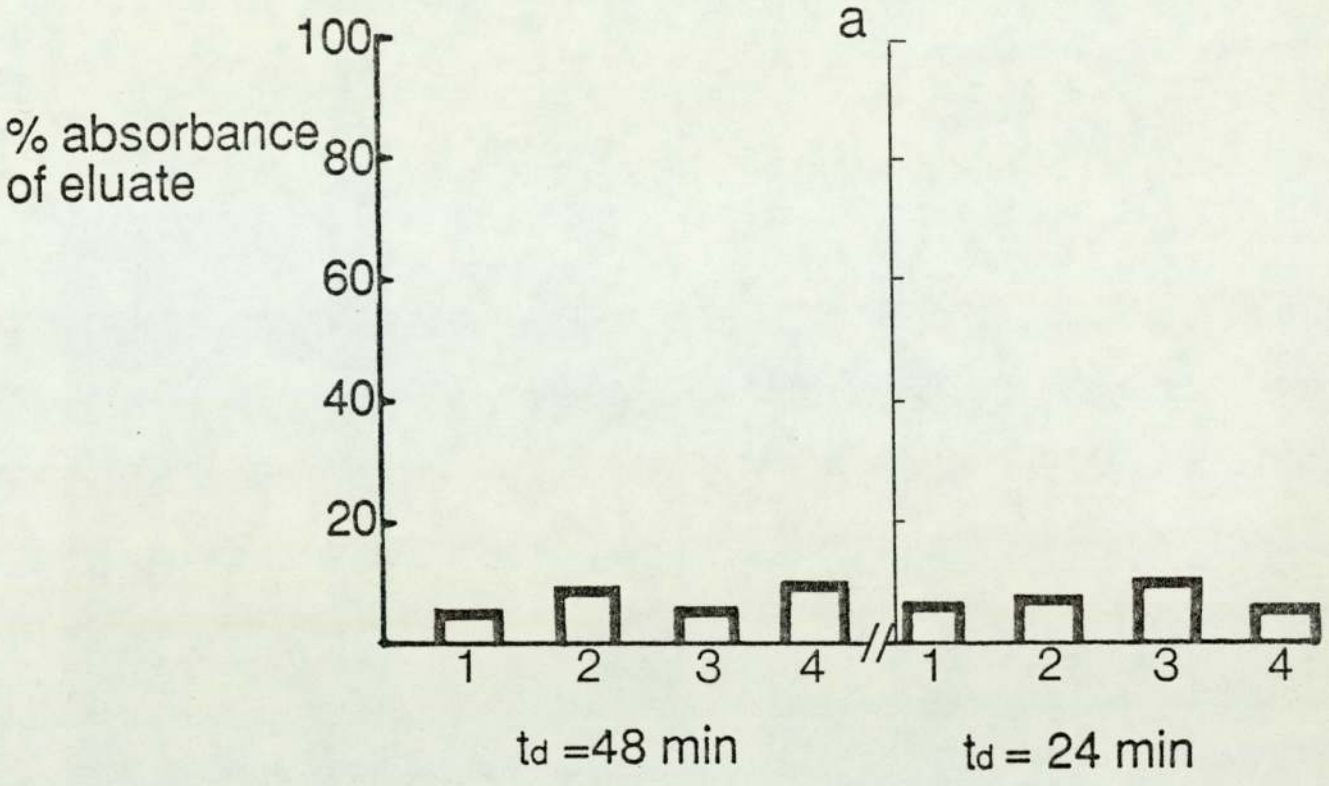
1 Fe+ TSB with no antibiotic

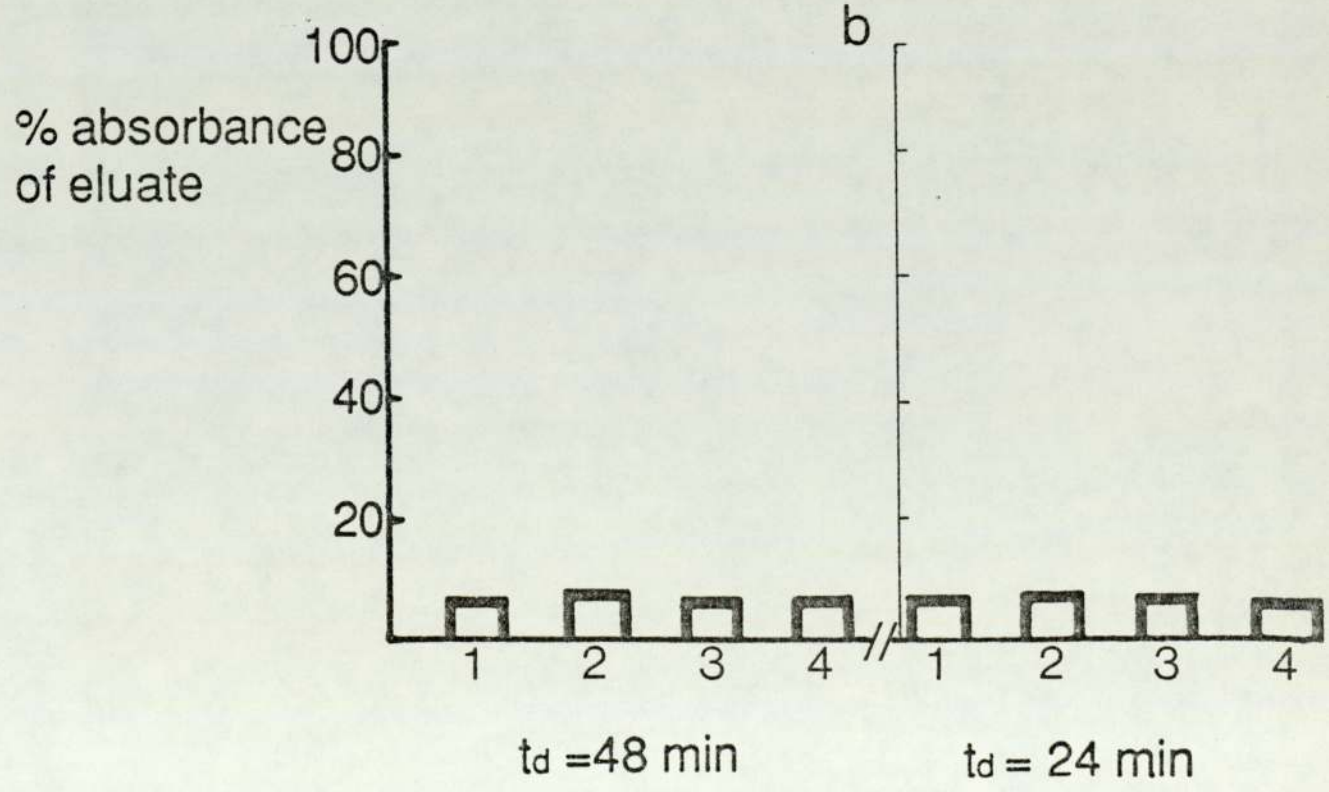
2 Fe+ TSB with penicillin G

3 Fe- TSB with no antibiotic

4 Fe- TSB with penicillin G

Fig 17


a
100
80
60
40
20
% absorbance of eluate
1
2
3
4
1
2
3
4
td =48 min
td = 24 min


b
100
80
60
40
20
% absorbance of eluate
1
2
3
4
1
2
3
4
td =48 min
td = 24 min

5.4 Discussion

No single method adequately investigates SH as the experimental conditions employed influence results and also, may allow non-SH effects to intrude (Sections 1.5.4 and 1.5.5).

Differences detected by the contact angle method were not visible in BATH although it had been adequate in the analysis of stationary phase iron-rich and iron-poor cocci and their opsonic needs (Section 4.1). It is debatable whether increasing the hydrocarbon volume beyond the recommended 0.2 ml (Rosenberg et al., 1980) would be of use. BATH is said to detect varying degrees of affinity to hydrocarbons (Rosenberg, 1984) but most studies have investigated stationary phase cultures and none to date have investigated the effect of sub-MICs of penicillin upon S. aureus. It is therefore difficult to compare the results obtained here and elsewhere. However, BATH using xylene showed very little discrimination between late exponential and stationary phase cultures of S. epidermidis and S. saprophyticus strains (Hogt, Dankert and Feijen, 1985). Xylene was not considered in this study because of its possible membrane disrupting effect.

BATH was found to be a troublesome assay with regards to reproducibility within and between experiments and many repeats and replicates were needed. Results could easily be influenced by surface irregularities within test tubes or their washing. It is now realised that BATH reproducibility is affected by many factors (Rosenberg, 1984). Essentially though, there is very little known about the bacterial surface components that mediate BATH.

HIC correlated with BATH and thus did not detect any significant difference in coccal type. Hydrophobic interactions are increased by high salt concentrations and at 4M and 2M NaCl, all cocci completely resisted desorption from the resin (not shown). As to be expected the

use of 1M and 0.5M NaCl decreased the binding to resin, but this was an overall effect and no significant differences emerged.

Initial contact angle experiments showed that the trend and the drying time needed to reach a plateau of constant angles for S. aureus was in accord with published work (Absolom, Lamberti, Policova, Zingg, van Oss and Neuman, 1983; Busscher, Weerkamp, van der Mei, van Pelt, de Jong and Arends, 1984).

The final contact angles were much higher than published values for S. aureus (van Oss et al., 1975) but the latter were obtained for overnight cultures. Variability was apparent in contact angles obtained on different days which meant that considerable repetition was necessary and this involved heavy consumption of treated TSB and serum. Variability could have been caused by different performances between the vacuum pumps available or, despite precautions, changes in the laboratory environment that may have affected the drying process of the pads. However, trends within an experiment were always the same. The assay has the advantage of not using hydrocarbons or high sodium chloride concentrations that can perturb mammalian membranes and it is thus possible to submit phagocytes to contact angle measurements (Tufano et al., 1985; van Oss et al., 1975).

Opsonisation was acceptable not only because of the evidence from the BATH experiments but also because an increase was noted in the cohesive properties of pellets during the subsequent washing procedure.

If SH measurements are to be of use in understanding in vivo interaction between host and parasite then they must be related to the SH of the human phagocyte which is known to have a contact angle between 18° and 19° (Tufano et al., 1985; van Oss et al., 1975). The results obtained in this study for non-opsonised contact angles predict that in the slow-growing model, iron-poor cocci with their

contact angle of 16.44°, would be less easily phagocytosed than the rest in a non-opsonised system. Penicillin G, even at 1/16 MIC, would enhance the engulfment of iron-poor S. aureus NCTC 6571 by increasing its hydrophobicity which confirms previous findings (Wadström et al., 1981) and may represent a novel action of penicillin G at sub-MIC. Opsonisation of the slow-growing cocci to simulate in vivo, increased the SH of the iron-poor cocci to theoretically render them just as susceptible to engulfment as all the other cocci. The fast-grown cocci had the highest relative SH of all and, in a non-opsonised system, would be apparently very rapidly phagocytosed.

It is unlikely that SC results were interfered with by SH. This is because the sodium chloride concentrations were sufficiently low to enhance SC and diminish SH thus allowing absorption to be due to charge characteristics, (Pedersen, 1980) and the pH of elution was appropriate (Wood, 1980).

Other methods such as particle electrophoresis (Shaw, 1969) might have shown that a difference exists but other work using both HIC and EIC has shown that relatively hydrophobic bacterial surfaces are also relatively anionic (Cockayne, Penn and Bailey, 1986; Stendahl, 1983; Xiu, Magnusson, Stendahl and Edebo, 1983).

6.1 Chemiluminescence (CL) of S. aureus NCTC 6571

Each group of figures shown is from experiments performed on the same day using the same batches of leucocytes and cocci. For consistency, results shown are those obtained with subject one but the same trends were obtained throughout. Analysis of serum from subject one was shown to be normal for titres of antibodies to staphylococcal alpha-haemolysin and DNase (Section 2.1.3).

A high challenge ratio (50 cfu: 1 neutrophil) of non-opsonised cocci showed no distinction between the various slow-grown cocci (Fig

18a) whereas reducing the number of these cocci (10-20 cfu: 1 neutrophil) reduced the chemiluminescence and showed that iron-poor cocci with or without penicillin G treatment produced less chemiluminescence than the rest whether the values produced a sharp (Fig 18b) or a slowly diminishing response (Fig 18c).

When non-opsonised, fast-grown cocci were challenged, all phenotypes and ratios were found to induce high chemiluminescence compared to their slow-grown counterparts (Fig 19 a and b). Even the CL burst produced by the low challenge ratio of fast-grown cocci was higher than that produced by the high challenge ratio of slow-grown cocci (Fig 18a). Fast-grown, iron-rich cocci with or without penicillin G, were the most effective inducers here and at the low challenge ratio, iron-poor cocci with or without penicillin G, produced least CL. Generally, any distinction within the fast-grown cocci was small relative to the overall CL burst (ie. height of the y axis).

S. aureus was then opsonised using the same conditions as for the contact angles (Section 2.2.10). A high challenge ratio of opsonised, slow-grown cocci resulted in a heightened CL response compared to that produced by non-opsonised equivalents, but was still only equal to the burst seen with non-opsonised fast-grown cocci. Differences were not particularly evident with the high ratio and led to apparent reversals. Again the significance of any differences was diminished by the overall magnitude of CL (Fig 20a). When an intermediate challenge ratio of approximately 30 cocci : 1 neutrophil was used it demonstrated the point at which the "blurring" occurred (Fig 20b).

At the low challenge ratio, slow-grown cocci segregated so that the iron-poor cocci were least potent in inducing the burst, and the effect of penicillin G was clearly seen in that it increased the effectiveness of both iron-poor and iron-rich cocci in inducing CL (Fig 20c).

Figure 18

Effect upon chemiluminescence of varying the challenge ratio of NON-OPSONISED S. aureus NCTC 6571 (t_d = 48 min) in Fe+ TSB and Fe- TSB in the absence and presence of 1/16 MIC of penicillin G and neutrophils from subject one.

a) high challenge ratio (50 cfu : 1 neutrophil)

b) low challenge ratio (10-20 cfu : 1 neutrophil)

c) low challenge ratio (10-20 cfu : 1 neutrophil)

× Fe+ TSB with no antibiotic

+ Fe+ TSB with penicillin G

⊙ Fe- TSB with no antibiotic

⊡ Fe- TSB with penicillin G

△ Neutrophil control

The neutrophil control is shown once as all experiments portrayed used the same batch of neutrophils.

N.B. In all chemiluminescent assays the coccal controls failed to elicit a response and are thus not shown in any graphs.

Fig 18

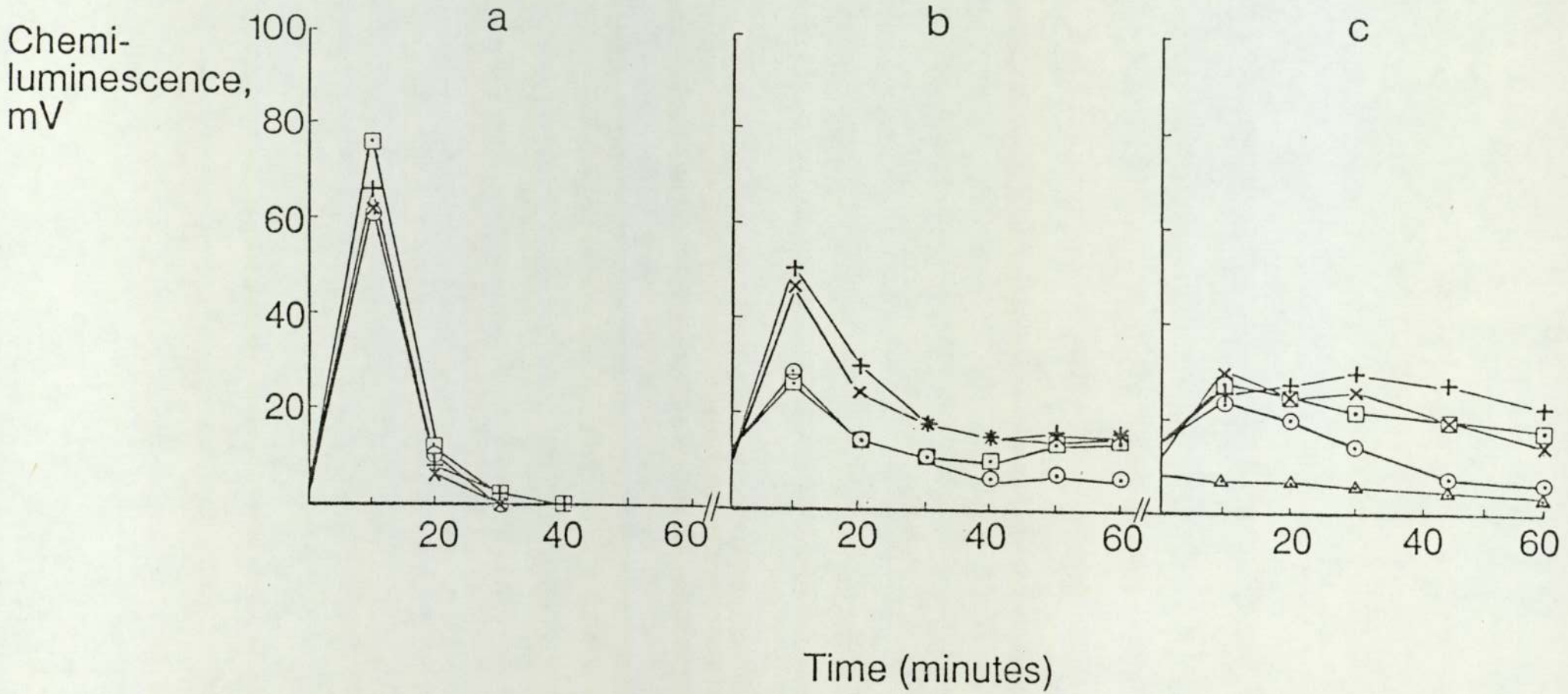

Chemi-
luminescence,
mV
100
80
60
40
20
a
b
c
20
40
60
20
40
60
20
40
60
Time (minutes)

Figure 19

Effect upon chemiluminescence of varying the challenge ratio of NON-OPSONISED S. aureus NCTC 6571 (t_d = 24 min) in Fe+ TSB and Fe- TSB in the absence and presence of 1/16 MIC of penicillin G and neutrophils from subject one.

a) high challenge ratio (50 : 1)

b) low challenge ratio (10-20 : 1)

▲ Fe+ TSB with no antibiotic

▼ Fe+ TSB with penicillin G

● Fe- TSB with no antibiotic

■ Fe- TSB with penicillin G

△ Neutrophil control

The neutrophil control is shown once as all experiments portrayed used the same batch of neutrophils.

Fig 19

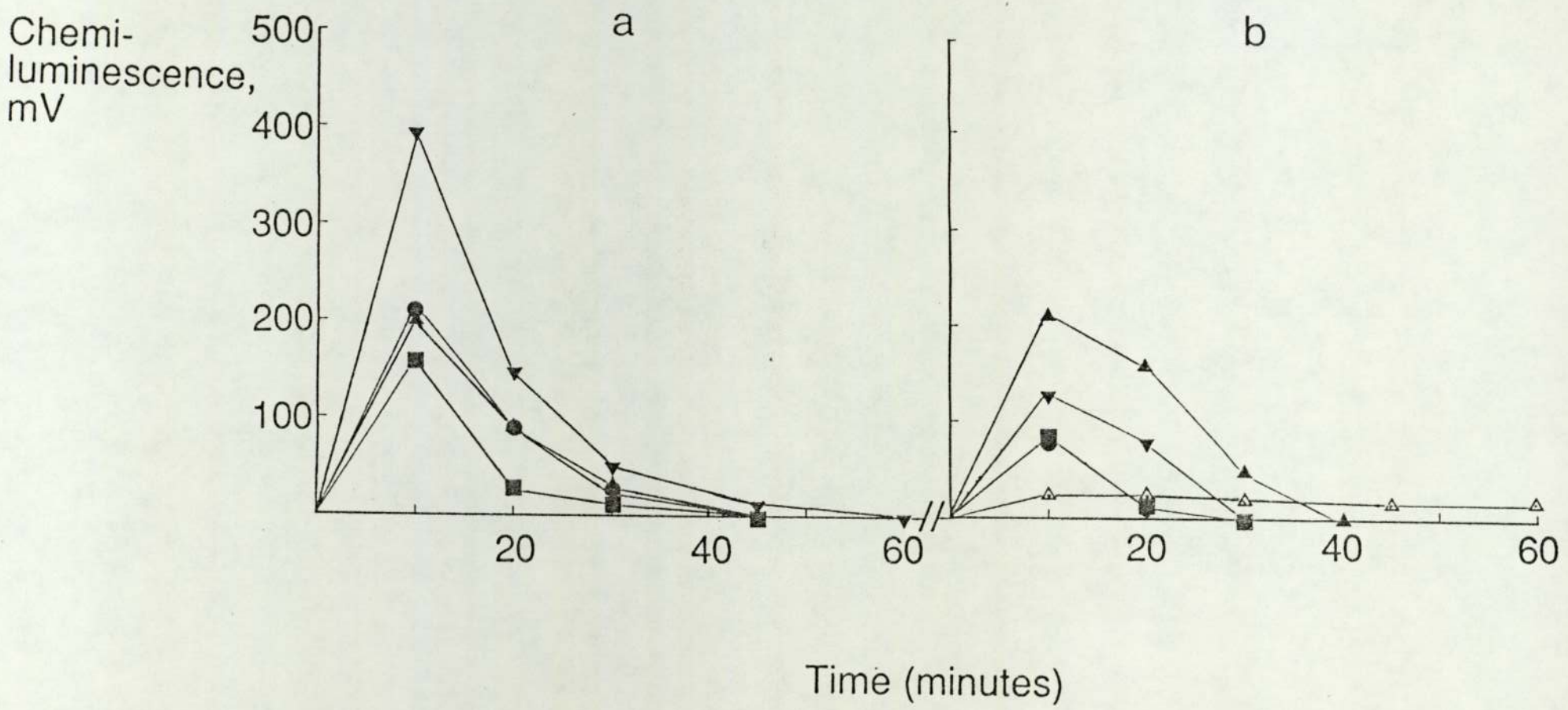

Chemi-
luminescence,
mV
500
400
300
200
100
a
b
20
40
60
20
40
60
Time (minutes)

Figure 20

Effect upon chemiluminescence of varying the challenge ratio of OPSONISED S. aureus NCTC 6571 (t_d = 48 min) in Fe+ TSB and Fe- TSB in the absence and presence of 1/16 MIC of penicillin G and neutrophils from subject one.

a) high challenge ratio (50 : 1)

b) intermediate challenge ratio (30:1)

c) low challenge ratio (10-20:1)

× Fe+ TSB with no antibiotic

+ Fe+ TSB with penicillin G

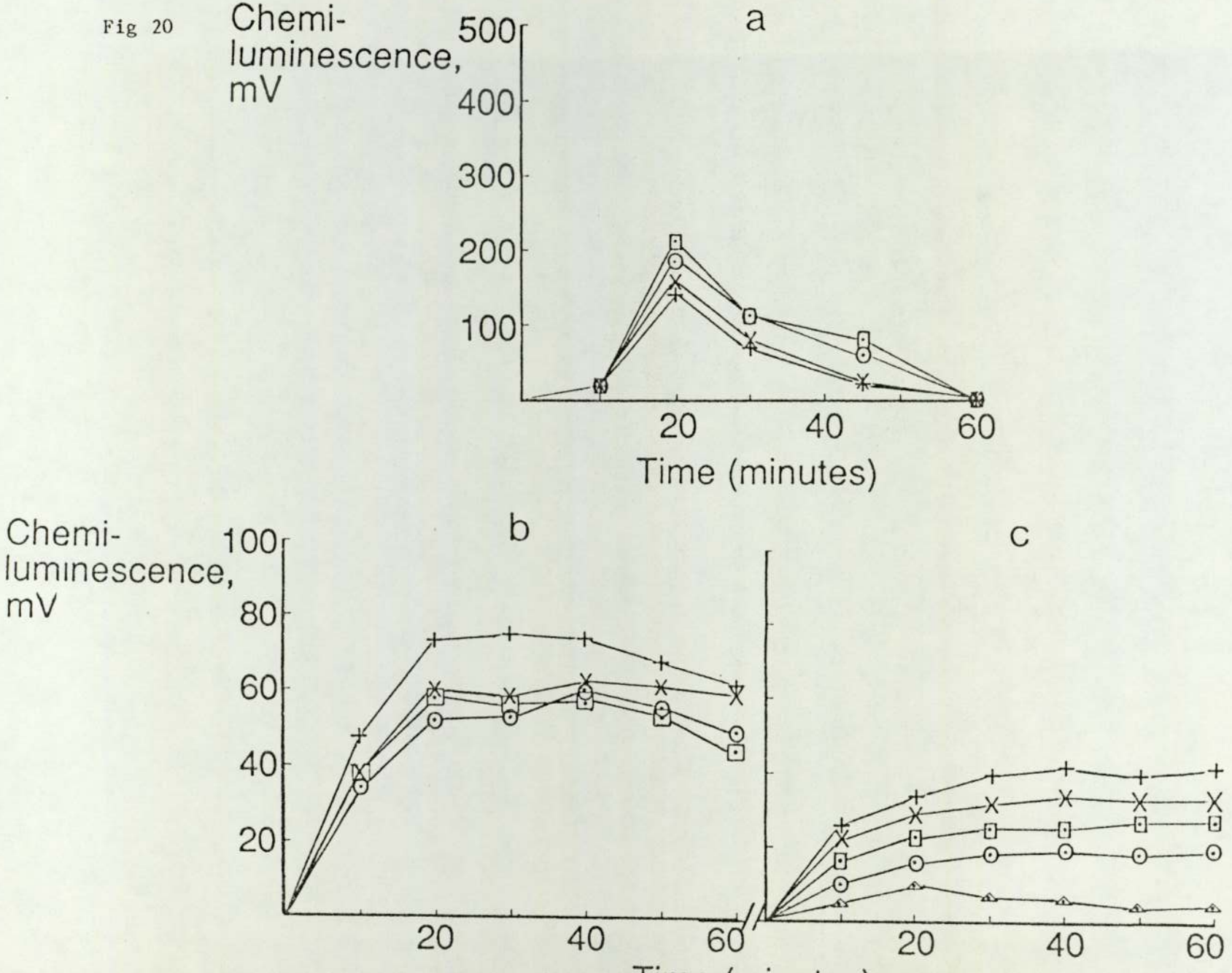
⊙ Fe- TSB with no antibiotic

⊡ Fe- TSB with penicillin G

△ Neutrophil control

The neutrophil control is shown once as all experiments protrayed used the same batch of neutrophils.

Fig 20


Chemi-
luminescence,
mV
500
400
300
200
100
a
20
40
60
Time (minutes)
Chemi-
luminescence,
mV
100
80
60
40
20
b
c
20
40
60
20
40
60

A high challenge ratio of opsonised, fast-grown cocci resulted in the highest CL bursts of all. Essentially there was a "blurred" relationship with some evidence of a faster reduction in CL for neutrophils stimulated by both types of iron-poor cocci (Fig 21a). With a low challenge ratio, iron-poor cocci were least potent, but overall CL bursts were high compared to their slow-grown equivalent (Fig 21b).

6.2 Discussion

Light emission from the phagocyte, ie. the CL burst, is triggered by contact with the target particle and arises from the activation of a membrane NAD(P)H oxidase (Section 1.5.7; Babior, 1982). This catalyses the formation of high energy compounds during phagocytosis and the subsequent bactericidal events (Rosen and Klebanoff, 1976). In this study, it has therefore been used as an index of association to neutrophils in leucocyte preparations. CL has been used synonymously with phagocytosis but more correctly it correlated with phagocytosis (Cockayne, et al., 1986; Gemmell and O'Dowd, 1983; Grebner, Mills, Gray and Quie, 1977; Stevens and Young, 1977; Welch, 1980; Williams, et al., 1980) and is thus an index of phagocyte bactericidal function (Gemmell and O'Dowd, 1983; Stevens and Young, 1977; Stjernholm, Allen, Steele, Waring and Harris, 1973). CL is an indirect method of assessing bactericidal function because it does not distinguish between engulfment and intracellular killing.

CL can be used to measure opsonic activity in serum (Allen, 1977; Grebner, et al.,1977), notably specific opsonisation mediated by immunoglobulins and non-specific opsonisation mediated by complement (Hemming, Hall, Rhodes, Shigeoka and Hill, 1976).

The opsonic activity of the sera used here was presumed normal because of the apparent health of the subjects, their haematological

Figure 21

Effect upon chemiluminescence of varying the challenge ratio of OPSONISED S. aureus NCTC 6571 (t_d = 24 min) in Fe+ TSB and Fe- TSB in the absence and presence of 1/16 MIC of penicillin G and neutrophils from subject one.

a) high challenge ratio (50 : 1)

b) low challenge ratio (10-20 : 1)

▲ Fe+ TSB with no antibiotic

▼ Fe+ TSB with penicillin G

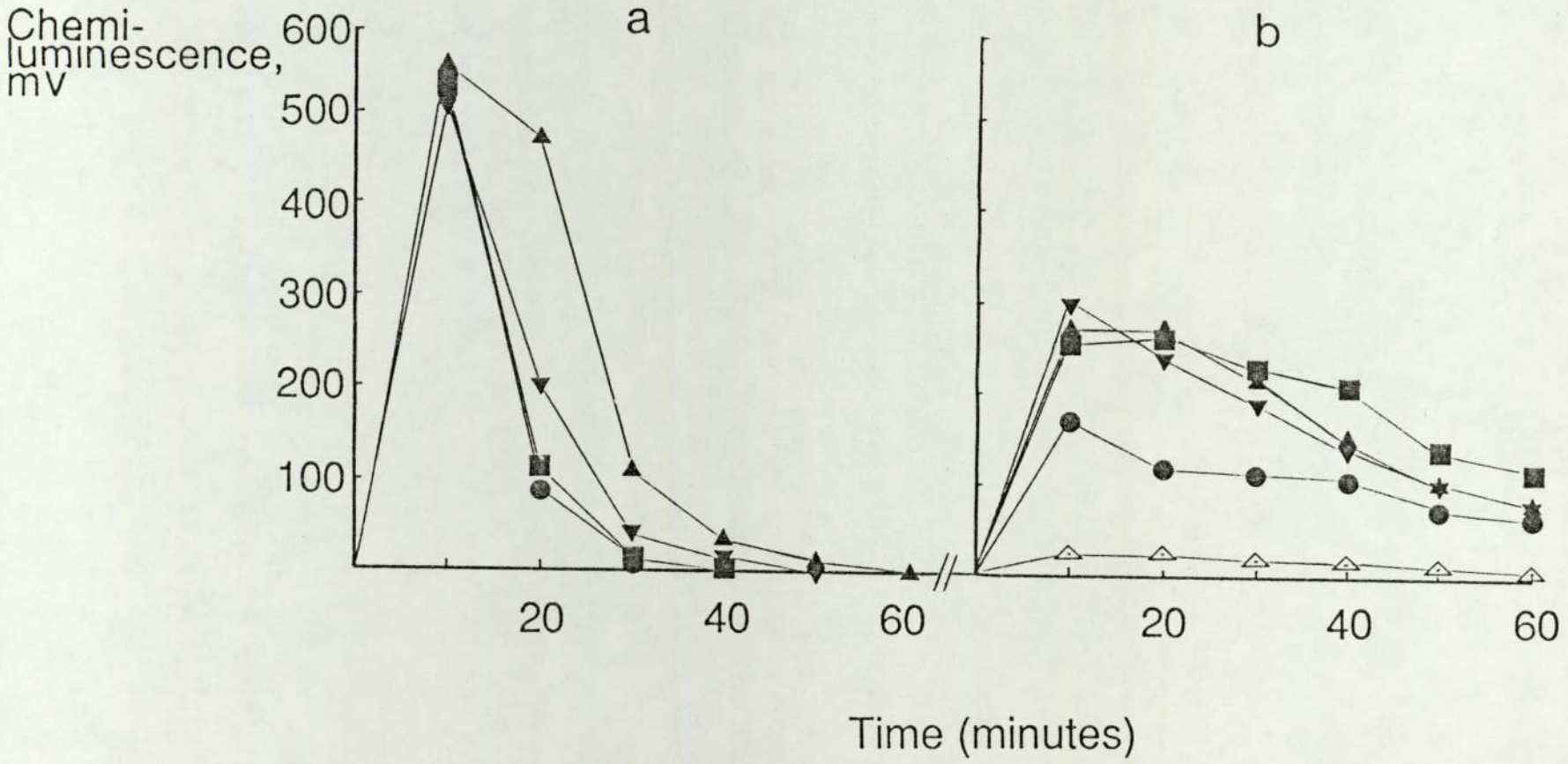
● Fe- TSB with no antibiotic

■ Fe- TSB with penicillin G

△ Neutrophil control

The neutrophil control is shown once as all experiments portrayed used the same batch of neutrophils.

Fig 21


Chemi-
luminescence,
mV
600
500
400
300
200
100
a
b
20
40
60
20
40
60
Time (minutes)

analyses (Section 7.1) and additionally for subject one, the normal titres of anti-staphylococcal antibodies. The same trends were obtained with all subjects which is also confirmatory.

Initially, there appeared to be problems over reproducibility of graph shape within an experiment as either a peak of CL was obtained which varied with time regardless of the experimental conditions, or else a slow rise without a peak and with prolonged decay was observed. This contrasts with CL results obtained in a liquid scintillation counter with isolated neutrophils where S. aureus exhibited only exponential rise and decay (Robinson, Wakefield, Breit, Easter and Penny, 1984). Problems caused by a variable response in CL have been noted elsewhere (Cockayne et al., 1986; Easmon et al., 1980; Maluszynska, Stendahl and Magnusson, 1985).

Variation here may have been due to several factors. The luminometer was a non-heated model and cuvettes were removed from the water bath into the unheated carousel of the machine for reading. It was only possible to do sequential and not simultaneous runs during an experiment. The preparation of leucocytes produced small clumps that did not break up easily and so it is possible that despite precautions, the cells were not distributed evenly between cuvettes. Despite the variation in CL response between replicates, or from day to day with the same subject or from subject to subject, reproducible trends appeared. In the non-opsonised form, CL allowed examination of the non-specific factors, eg. SH, involved in bacteria - neutrophil interactions (Magnusson, et al. 1985). Opsonised CL involved specific interactions caused by antibody and complement. Both forms allowed comparison with the contact angle experiments.

Again, different growth rates, iron deprivation and the exposure for one generation to penicillin G all produced different phenotypes that influenced the CL response of neutrophils in the leucocyte

preparations in different ways.

Non-opsonised CL that used a low number of slow-grown cocci demonstrated that the interaction between neutrophils and coccus was influenced by the relative hydrophobic nature of the bacterial envelope and a parallel with the contact angle relationships was present. As a result, the phenotype that was least efficient at inducing the CL burst was the iron-poor coccus and the sub-MIC of penicillin G improved this efficiency.

This relationship began to disappear in the intermediate challenge and it was lost when a high challenge ratio was used. In this instance the CL peaks were higher and thus it seems that neutrophils were sufficiently excited for cell association to occur to the same approximate extent, regardless of coccal type. CL response has been shown to exhibit saturation kinetics with a plateau at 100 S. aureus:1 neutrophil (Easmon et al., 1980).

An increase in growth rate resulted in a dramatic increase in the ability of cocci to stimulate the neutrophils and appears to be linked to the high relative SH of the fast-grown cocci. Their SH was uniform, as measured by contact angles, and although in CL there was some discrimination between the coccal types, CL was consistently high. Elsewhere, increasing SH in non-opsonised Enterobacteria has been found to correlate with increasing CL response (Maluszynska, et al., 1985).

Opsonised CL with slow-grown cocci gave results that were unlike the opsonised contact angle relationships. Also, conditions of maximum opsonisation were used throughout and so the dependence of the phenotype differences upon serum concentration is not characterised. With a low challenge ratio, the iron-poor cocci stood out as eliciting the least CL and the penicillin G treatment or iron excess or both enhanced this. It seems that here, non-specific interactions such as

SH of the bacterial envelope are more influential than specific ones mediated by serum factors, and ultimately cause lowered cell association. In the same context, the reduced CL of human neutrophils seen with opsonised, virulent Salmonella typhi was possibly due to poor triggering of the neutrophil oxidative metabolism (Kossak, Guerrant, Densen, Schaedlin and Mandel, 1981). When the high challenge ratio was used, the distinction between the various phenotypes was lost and this mirrored the non-opsonised situation.

Opsonised CL of the fast-grown cocci produced the highest CL peaks and although there was no significant difference in coccal type with the high challenge, in the low challenge the iron-poor cocci were again distinguished.

The effect of penicillin G was noted throughout in the low challenge ratios. The contact angle experiments indicated that the drug increased SH and cell association. Elsewhere, a potentiation of CL by sub-MIC treated S. aureus was due to accelerated or enhanced opsonisation as more sites for C3b attachment were available (Gemmell and O´Dowd, 1983; Milatovic et al., 1983). This would enhance cell association.

Overall, low numbers of the slow-grown, iron-poor, opsonised coccus provoked the least CL. This might predict better survival in vivo or in whole blood killing in vitro, compared to the remaining phenotypes.

7. Whole Blood Killing (WBK) and serum stability of S. aureus NCTC 6571

7.1 WBK

The results of each experiment were subject to the variation inherent in the method used. Each subject showed changes from day-to-day which resulted in varying rates of WBK (Boghossian, Wright and Segal, 1983; Gladstone and Walton, 1971). As for CL however, trends did emerge which were reproducible. For continuity and comparison with CL, experiments presented were those obtained with subject one. Coulter counter analysis showed that the subjects conformed to normal haematological criteria and covered the range 4.2×10^9 WBC l^{-1} to 7.6×10^9 WBC l^{-1} with no age or sex bias. Subject one was used most and had a mean WBC count of 4.6×10^9 WBC l^{-1}.

The changes in neutrophil concentration of the subjects' whole blood could have been responsible for only small variations in different rates of killing observed and also there is little difference in the killing capacity of neutrophils from normal subjects, for S. aureus (Verbrugh et al., 1978).

WBK of slow-grown cocci in a high and low challenge ratio showed differences overall. There was no distinction between the slow-grown coccal types when a high challenge ratio was used (Fig 22). In contrast, in the low challenge ratio experiments, iron-poor cocci survived better than all others. Penicillin G treatment reduced this survival but had no effect upon the response of iron-rich cocci as both types were equally sensitive. Iron-poor cocci treated with penicillin G tended to survive better than both types of iron-rich cocci (Fig 23).

The effect of the fast growth rate was again noted. With both high and low challenge ratios, there was no distinction between the coccal types although a faster rate of killing was occasionally noted

Figure 22

Kinetics of killing of a high challenge ratio (50 cfu:1 neutrophil) of S. aureus NCTC 6571 (t_d = 48 min) in Fe+ TSB and Fe- TSB and in the absence and presence of 1/16 MIC of penicillin G, by whole blood from subject one.

× Fe+ TSB with no antibiotic

+ Fe+ TSB with penicillin G

⊙ Fe- TSB with no antibiotic

⊡ Fe- TSB with penicillin G

Figure 23

Kinetics of killing of a low challenge ratio (10-20 cfu:1 neutrophil) of S. aureus NCTC 6571 (t_d = 48 min) in Fe+ TSB and Fe- TSB and in the absence and presence of 1/16 MIC of penicillin G, by whole blood from subject one.

× Fe+ TSB with no antibiotic

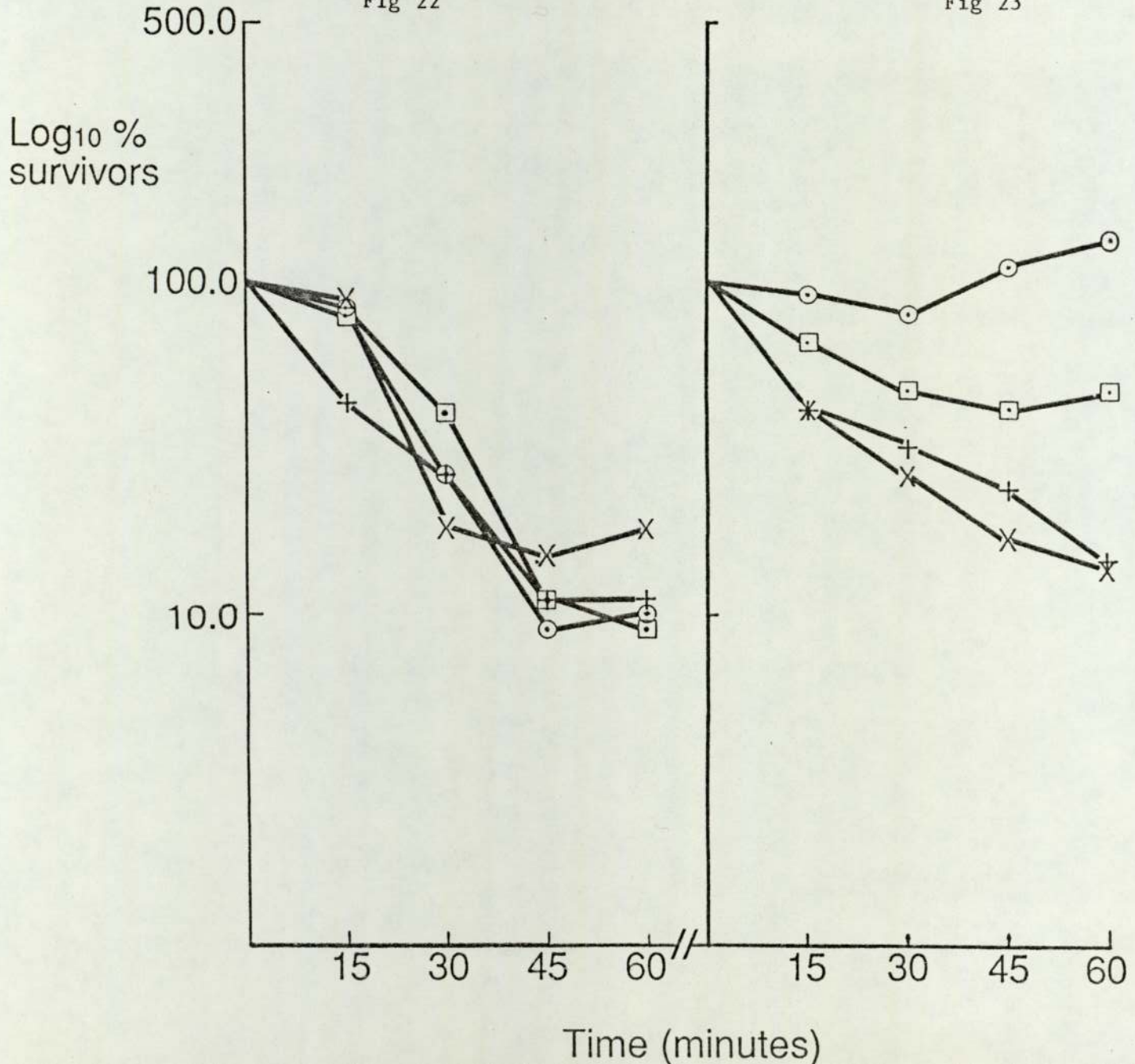
+ Fe+ TSB with penicillin G

⊙ Fe- TSB with no antibiotic

⊡ Fe- TSB with penicillin G

Fig 22

Fig 23


500.0
100.0
10.0
Log10 % survivors
15
30
45
60
15
30
45
60
Time (minutes)

with the high challenge ratio (Fig 24 and 25).

A lag in killing and the outgrowth of S. aureus was independent of experimental conditions and subjects, nor did maximum WBK occur at the same time in each experiment and between replicates (Root et al., 1981). Outgrowth was presumably due to a combination of saturation of uptake of S. aureus and consequent growth outside neutrophils, and disintegration of the neutrophils.

The number of each coccal phenotype associated with each of 30 neutrophils was counted by using light microscopy at 1000x magnification. Sub-populations of neutrophils with different levels of activity exist within the same subject (Bass et al., 1986; Baglin, T., pers. comm.) and many reports have failed to account for skewed distributions in their statistical analyses of cell association (Rosenstein, Grady, Hamilton-Miller and Brumfitt, 1985). The distribution of slow- and fast-grown cocci in the low ratio challenges are shown in Fig 26. Too many bacteria were present in the high challenge ratios to allow accurate counting. Neutrophils challenged with the slow-grown iron-poor cocci had many fewer or no associated cocci. The remaining slow-grown phenotypes and all the fast-grown phenotypes showed approximately Normal distributions and readily associated with neutrophils. The mean number of associated cocci of the slow-grown, iron-poor phenotype was compared to the mean for each remaining slow-grown phenotype by an unpaired t test. The same was done for the fast-grown model. These comparisons were statistically significant for the slow-grown iron-poor coccus but not the fast-grown equivalent (Table 6).

7.2 Serum stability

Alterations in growth rate, availability of iron and treatment with penicillin G did not render any of the phenotypes sensitive to

Figure 24

Kinetics of killing of a high challenge ratio (50:1) of S. aureus NCTC 6571 (t_d = 24 min) in Fe+ TSB and Fe- TSB and in the absence and presence of 1/16 MIC of penicillin G, by whole blood from subject one.

▲ Fe+ TSB with no antibiotic

▼ Fe+ TSB with penicillin G

● Fe- TSB with no antibiotic

■ Fe- TSB with penicillin G

Figure 25

Kinetics of killing of low challenge ratio (10-20:1) of S. aureus NCTC 6571 (t_d = 24 min) in Fe+ TSB and Fe- TSB and in the absence and presence of 1/16 MIC of penicillin G, by whole blood from subject one.

▲ Fe+ TSB with no antibiotic

▼ Fe+ TSB with penicillin G

● Fe- TSB with no antibiotic

■ Fe- TSB with penicillin G

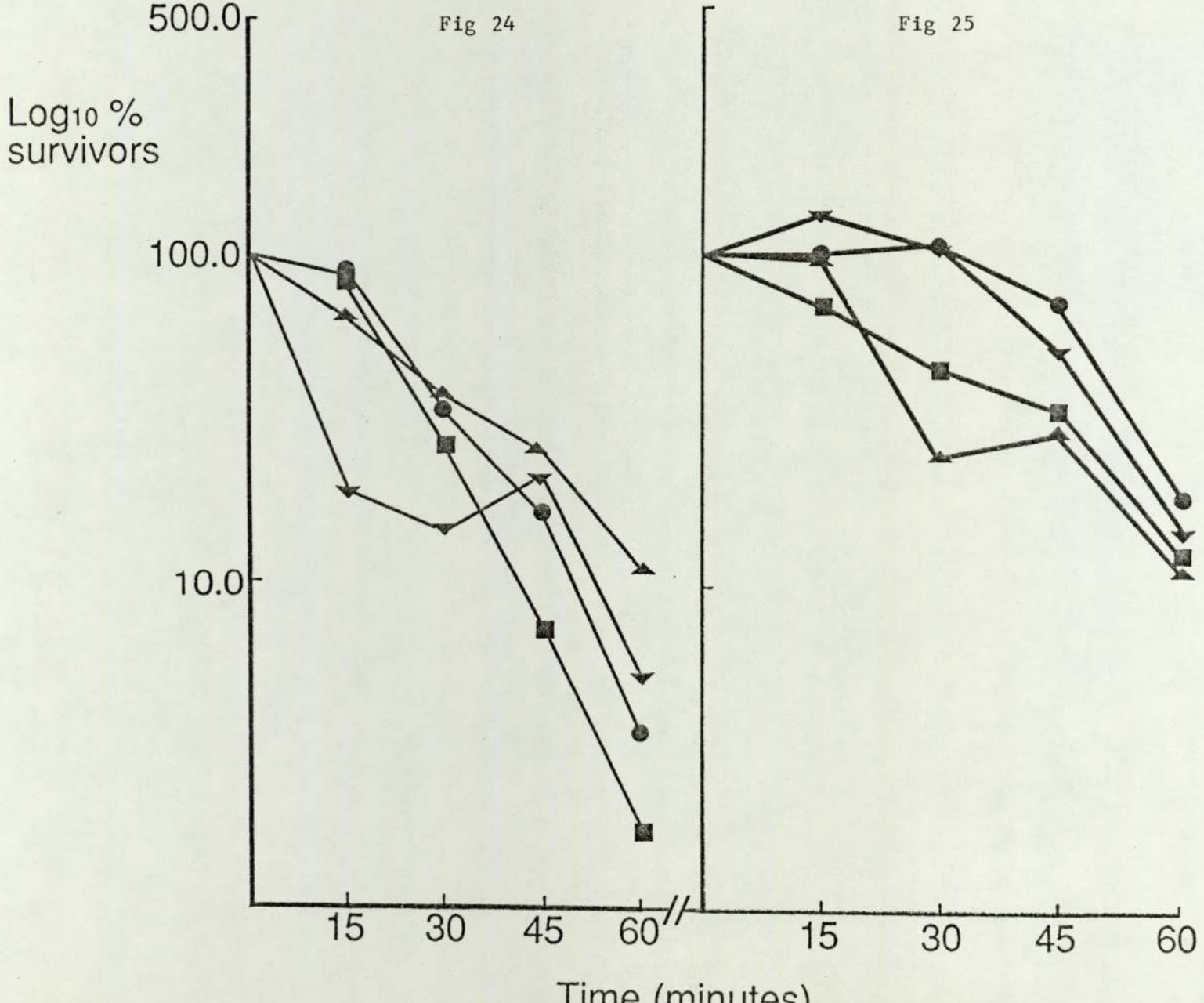


Fig 24
Fig 25
500.0
Log10 % survivors
100.0
10.0
15
30
45
60
15
30
45
60
Time (minutes)

Figure 26

Association to neutrophils of S. aureus NCTC 6571 (t_d = 48 and 24 min) in Fe+ TSB and Fe- TSB in the absence and presence of 1/16 MIC of penicillin G, (low challenge ratio, 10-20:1).

a) Fe+ TSB with no antibiotic
b) Fe+ TSB with penicillin G
c) Fe- TSB with no antibiotic
d) Fe- TSB with penicillin G

t_d = 48 min

e) Fe+ TSB with no antibiotic
f) Fe+ TSB with penicillin G
g) Fe- TSB with no antibiotic
h) Fe- TSB with penicillin G

t_d = 24 min

Fig 26

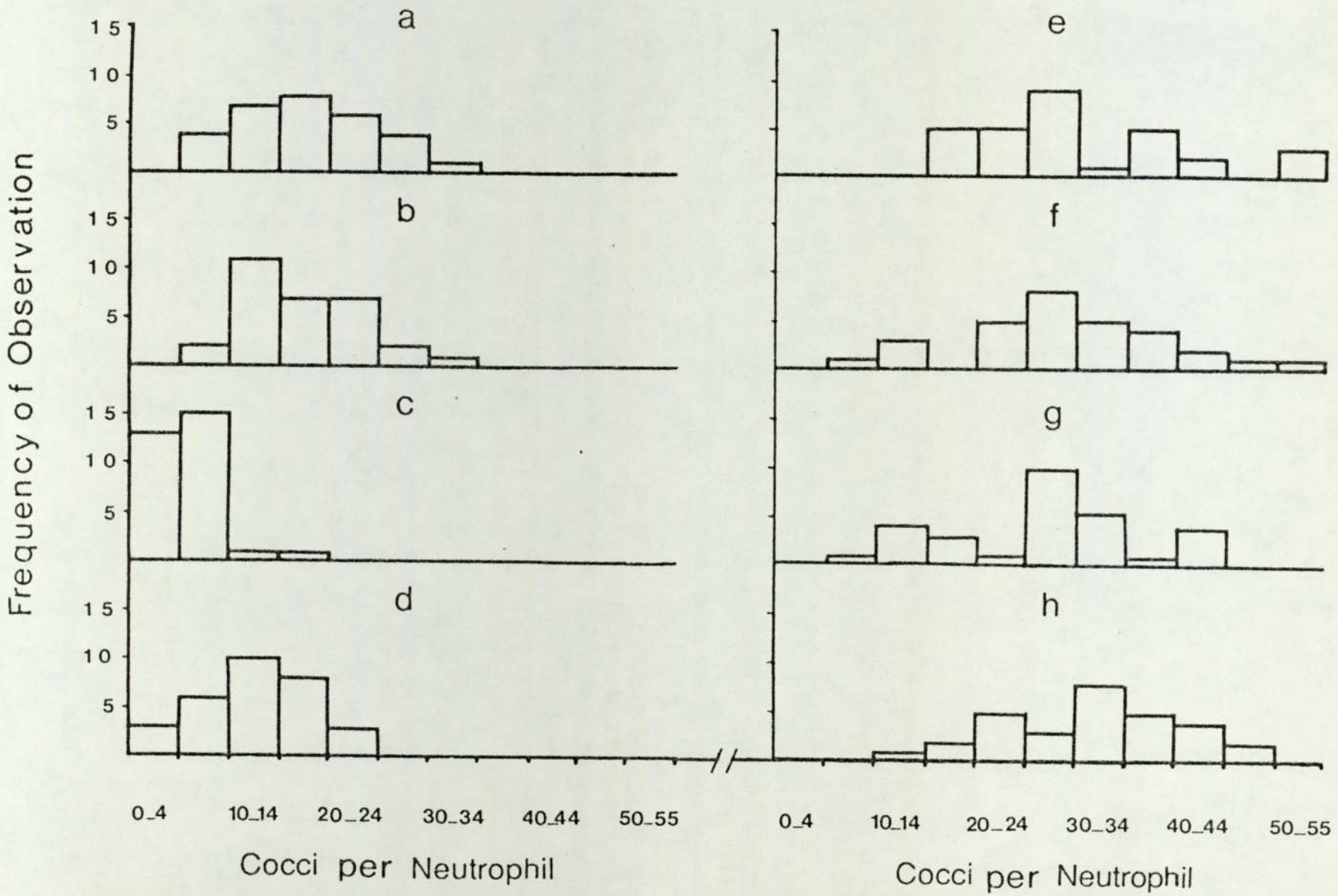

a
b
c
d
e
f
g
h
15
10
5
Frequency of Observation
0_4
10_14
20_24
30_34
40_44
50_55
Cocci per Neutrophil

Table 6

Significance of the differences in mean neutrophil association for S. aureus NCTC 6571 (t_d = 48 and 24 min): comparison of the iron-poor phenotypes with all others.

	Doubling Times					
	t_d = 48 min			t_d = 24 min		
Phenotype	$\bar{x}$	SD	Significance Level*	$\bar{x}$	SD	Significance Level*
Fe+ TSB	17.50	7.92	p<0.001	29.53	10.76	p=0.250
Fe+ TSB with penicillin G	18.90	7.11	p<0.001	28.77	9.93	p=0.400
Fe- TSB	6.10	4.43	-------	26.50	9.32	-------
Fe- TSB with penicillin G	12.10	6.20	p<0.001	30.97	8.70	p=0.100

* Significance level for comparison with Fe- TSB mean.

lysis by serum. Examples are presented in Fig 27 and 28.

7.3 Discussion

The pretreatment of S. aureus before actual challenge with whole blood allows the effects of penicillin G to be distinguished from those of WBK. In this study, penicillin G was not inactivated but washed off thoroughly. It is thus unlikely that there was appreciable carry-over of such a low sub-MIC into the WBK (and CL) assays. Additionally, the reversible nature of the effects of low penicillin concentrations upon cell wall synthesis would also have negated any carry-over (Lorian and Atkinson, 1975; Tomasz, 1979 a and b).

In this study, CL was not performed with whole blood (Tono-Oka, Ueno, Matsumoto, Okhawa and Matsumoto, 1983) nor were phagocytic and bactericidal investigations made using isolated neutrophils. Thus, the extrapolation from the CL results to those obtained in the WBK assays is partly reliant upon published work. However, a survey of the literature pertaining to S. aureus-neutrophil interactions showed that it is difficult to compare closely results obtained here and elsewhere. This is because major methodological differences exist not only between this study and others, but also between published studies.

Here, whole blood and May-Grünwald-Giemsa staining were used to probe the responses of different phenotypes of S. aureus NCTC 6571. The avoidance of a separation step means that killing was tested under more physiological conditions and that interactions between blood components were retained. The method also involves small volumes of blood and is, for example, applicable to infants (Al-Hadithy et al., 1981; Boghossian et al., 1983). The phenotypes were produced by alterations in growth rate, nutrition and penicillin G treatment, and were emphatically unlike the stationary phase, nutritionally replete

Figure 27

Kinetics of killing of a low challenge number of S. aureus NCTC 6571 (t_d = 48 min) in Fe+ TSB and Fe- TSB and in the absence and presence of 1/16 MIC of penicillin G, by whole serum from subject one.

× Fe+ TSB with no antibiotic

+ Fe+ TSB with penicillin G

⊙ Fe- TSB with no antibiotic

⊡ Fe- TSB with penicillin G

Figure 28

Kinetics of killing of a low challenge number of S. aureus NCTC 6571 (t_d = 24 min) in Fe+ TSB and Fe- TSB and in the absence and presence of 1/16 MIC of penicillin G, by whole serum from subject one.

▲ Fe+ TSB with no antibiotic

▼ Fe+ TSB with penicillin G

● Fe- TSB with no antibiotic

■ Fe- TSB with penicillin G

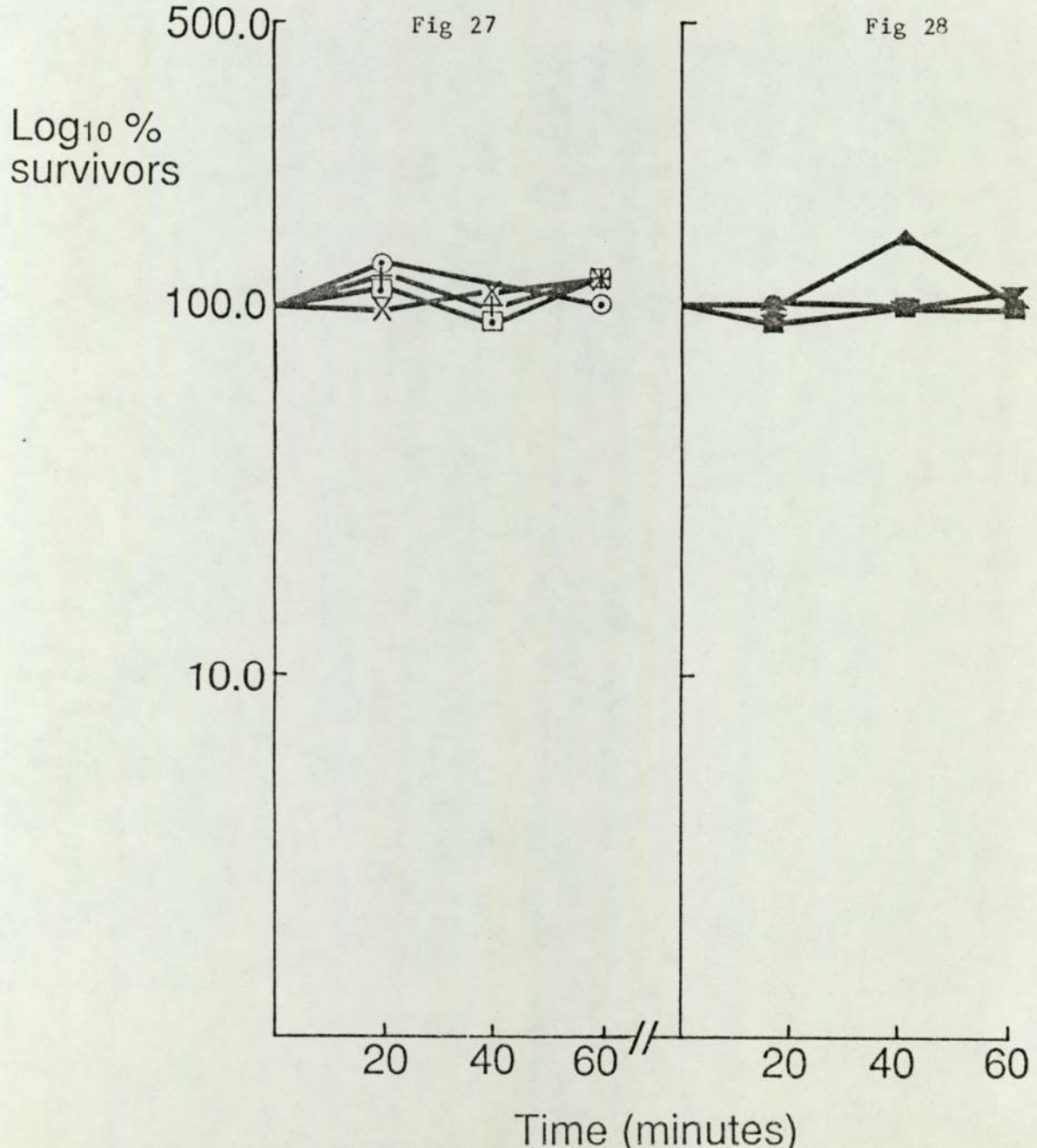


Fig 27
Fig 28
500.0
100.0
10.0
Log10 % survivors
20
40
60
20
40
60
Time (minutes)

bacteria commonly used elsewhere. Prolonged vigorous shaking was used to promote interactions which were then monitored by viable counting.

Current literature centres around the use of pre-opsonised S. aureus and isolated neutrophils to elucidate the steps from cell association to bacterial lysis. Comparison is made difficult because neutrophils from different species differ and also neutrophils recruited by peritoneal injections of glycogen are more active chemotactically and biochemically than their peripheral blood counterparts (Bamberger, Gerding, Bettin, Elson and Forstrom, 1985). Different strains of S. aureus that represent a spectrum of in vivo and in vitro responses have been used and interactions between them and neutrophils have been promoted by slow tumbling and rolling, over relatively short periods. Finally, the situation is compounded by the different analyses used to scrutinise cell association, phagocytosis and bactericidal events which themselves were examined with different techniques.

Results here demonstrated that a concommitant reduction in growth rate and iron availability produced a S. aureus phenotype, which when present in small numbers relative to neutrophils, was better able than the other phenotypes, to survive killing by whole blood. Reduced neutrophil association was implicated, which correlated with CL trends.

Growth rate changes complemented changes in challenge ratios. Thus, the WBK kinetics of high challenges of both slow- and fast-grown cocci did not differ significantly. In contrast, a high and low challenge ratio of fast-grown cocci did produce different results. The neutrophils appeared to be excited to a higher degree by the greater number of cocci and this was reflected in greater killing.

A slow growth rate allowed the iron-poor cocci, and to a lesser extent, their penicillin G-treated counterparts, to survive better in

a low challenge ratio. Exposure to penicillin G for one generation did increase the susceptibility of iron-poor cocci to WBK possibly by increasing neutrophil association. There was no distinction in the killing rates of the iron-rich cocci with or without penicillin G. It seems unlikely that the differences detected here were due to a direct effect of penicillin G upon neutrophil chemotaxis (Yourtree and Root, 1982). It is not known whether iron-poor cocci are less chemoattractive than iron-rich cocci and any differences would probably have been nullified by the rapid shaking, the concentrations of cocci and neutrophils and the relatively enclosed reaction space.

Work with isolated neutrophils (Craven, Williams and Anderson, 1982; Lorian, 1980; Root et al., 1981) and with rabbit peritoneal infections (Lam et al., 1984) confirm an enhancing effect of penicillin G pretreatment upon the response of S. aureus to phagocytosis and killing. It is apparent that in these studies, longer periods of pretreatment than used in this study were employed. The effect could also be small relative to the overall kill (Craven et al., 1982) and masked by inherent variability (Root et al., 1981). Penicillin G seemed to render S. aureus more sensitive to the non-oxidative arm rather than the oxidative arm of intracellular killing (Root and Metcalf, 1979) and this contrasts with results obtained here.

The precise step at which the slow-grown, iron-poor coccus exhibits enhanced survival in the WBK assays is not known for certain and may involve more than one step. The CL and staining evidence was that there was a fundamental difference in cell association caused by non-specific interactions at the lower challenge ratios for the slow-grown, iron-poor S. aureus. This also seems to correlate with the WBK results and certainly, nutrient depletion does effect significant changes in cell association in WBK assays (Anwar, Brown and Lambert,

1983). Presumably, such conditions allow non-specific interactions such as surface hydrophobicity to dominate specific interactions such as opsonisation even when whole blood is used. If so, then the effect of penicillin G would be to increase the surface hydrophobicity of the cocci thus rendering them more susceptible to phagocytosis (van Oss, 1978). Interestingly, pathogenic E. coli serotypes which when non-opsonised had hydrophilic contact angles, were resistant to phagocytosis when opsonised. Opsonised contact angles were not performed (Tufano et al., 1985). Further experiments would be needed to determine whether the decrease in cell association is due to a reduction in actual number of contact events, a reduction in time of each contact, or less firm contact thereby allowing the iron-poor cocci to escape phagocytosis.

Other trends were evident in WBK and some were no doubt caused by the different "normal" immunities of each subject, which are reflections of their previous histories. Others were due to the different challenge ratios. These need discussion because the rate of interaction between bacteria and neutrophils is dependent not only on the ratio of bacteria to neutrophils but also on the actual concentrations of each (Clawson and Repine, 1976; Leijh et al., 1979). Variation in the total number of neutrophils occurred from subject to subject although the cocci to neutrophil ratio was constant.

Large variations in the ratio of cfu to neutrophils were employed in this study even though the majority of reports surveyed used only low to moderate ratios, eg. 1:1 to 20:1. However, the importance of large variations has been stressed. Neutrophils could be stimulated to greater and greater metabolic activity by successive ingestions of S. aureus, so that within limits, the rate of killing increased as the number of cocci ingested increased (Clawson and Repine, 1976; Melly, Thomison and Rogers, 1960). Indirectly, stepwise oxygen consumption

was noted after similar progressive stimulation (Repine, White, Clawson and Holmes, 1974).

The influence of neutrophil and coccal concentrations upon the WBK trends here are best discussed in the light of what is known about the multi-step sequence leading to exposure of bacteria to the bactericidal systems of neutrophils.

Chemotaxis is followed by cell association and no differences in this were reported for moderate alterations in ratios of cocci to neutrophils whether an effect of penicillin G was investigated (Root et al., 1981) or not (Björksten, Peterson, Verhoef and Quie, 1977). The alterations used did not approach the magnitude of those in this study and so it is possible that the trends here would not have been detected.

The phagocytic step is also affected by alterations in total numbers of cocci and neutrophils. Increasing the concentration of neutrophils increased the rate of phagocytosis of S. aureus (Leijh et al., 1979; Vandenbroucke-Grauls, et al., 1985) even when the coccus: neutrophil ratio was kept constant (Verbrugh, Peters, Peterson and Verhoef, 1978).

It is likely that the large differences between similar experimental conditions were influenced more by the changes in numbers of cocci rather than in neutrophils. The phagocytic rate did increase with increasing numbers of S. aureus (Bassøe and Solberg, 1984; Clawson and Repine, 1976; Gladstone and Walton, 1971; Leijh et al, 1979; Verbrugh et al., 1978; Williams, Craven, Field and Bunch, 1985). Maximum ingestion rates occurred sooner with the increasing numbers (Leijh et al., 1979) and the uptake demonstrated saturation kinetics (Bassøe and Solberg, 1984; Leijh et al, 1979; Verbrugh et al., 1978). A minority of workers though have found that phagocytosis remained constant and high despite alterations in input of S. aureus (Björksten

et al., 1977; Clawson and Repine, 1976).

If phagocytosis and killing are positively correlated then alterations in phagocytic rate caused by large alterations in the number of input cocci could explain the differences in rates of killing between the high challenge and low challenge ratios.

The exact relationship between phagocytosis and killing is confused, this is no doubt due to the plethora of methods used. Rate of killing of S. aureus was reportedly enhanced as the rate of ingestion increased (Clawson and Repine, 1976; Melly, Thomison and Rogers, 1960), whilst the rate of kill was said to be constant regardless of the number of cocci ingested (Verbrugh et al., 1978). Bovine neutrophils exhibited a plateau level of killing with increasing ingestion of S. aureus and eventually killing decreased, apparently due to excessive degranulation (Williams et al., 1985). An enhanced kill of penicillin G-treated S. aureus was found not to be due to increased ingestion (Root et al., 1981) but to the non-oxidative arm of the bactericidal system (Root and Metcalf, 1979). But, in a study using S. aureus treated with sub-MICs of clindamycin, ingestion not only correlated with CL, but also with killing (Gemmell and O'Dowd, 1983).

Changes in neutrophil concentration can affect the rate of killing. The speed and magnitude of decline in S. aureus viability was directly related to the number of leucocytes (Rogers and Tompsett, 1952) whilst holding the ratio constant but increasing both coccal and neutrophil concentrations led to enhanced killing (Clawson and Repine, 1976).

In isolated neutrophil systems, both big and small variations in S. aureus input had little effect on the rate of killing (Root et al., 1981) whilst a drop in killing efficiency was reported to be due to overdegranulation caused by excessive input (Clawson and Repine,

1976). This contrasts with the results of this study and is possibly due to extensive differences in methodology.

The clinical significance of the findings presented here can only be tested by further *in vitro* and *in vivo* experimentation. With regard to the latter, a wide range of infection models would be needed. Above all, the nutritional, physiological and immunological status and the clinical significance of low numbers of *S. aureus* *in vivo* is not known. These findings may be helpful in explaining the persistence of *S. aureus* in situations such as abscesses.

There is abundant evidence that for many drug-free *S. aureus* strains, human serum is either bacteriostatic or allows growth under many different conditions (Adlam, Pearce and Smith, 1970a; Clawson and Repine, 1976; Cybulska and Jeljaszewicz, 1966; Grebner *et al.*, 1977; Lam *et al.*, 1984; Leijh *et al.*, 1979; Pruul, Wetherall and MacDonald, 1982; Schade, 1963). This also applies to bovine (Anderson and Wilson, 1985) and rabbit serum (Gladstone and Walton, 1970). However, *S. aureus* was found to have variable sensitivity to human serum after sub-MIC treatment (Lorian and Atkinson, 1978). Also, pretreatment by growth to stationary phase in 1/5 MIC of penicillin G conferred complement-mediated sensitivity to rat serum which also induced morphological and growth rate changes (Perna, Di Lillo, Andreana, Utili and Ruggiero, 1984). In this study serum sensitivity did not significantly influence trends in the WBK assays.

8.1 Protein A (Pr A) biosynthesis

Pr A standards were run simultaneously and results for cell-bound Pr A were expressed per 10^8 cfu ml^{-1} as this took into account the different yields at harvest. Pr A in the supernatant was expressed relative to 10^8 cfu ml^{-1}.

The slow-grown cocci had, overall, far less cell-bound Pr A than the fast-grown ones (Table 7). Higher levels were detected in iron-rich cocci especially the fast-grown ones. Penicillin G treatment and or iron unavailability reduced cell-bound Pr A in both slow and fast models of growth so that these phenotypes were relatively uniform especially amongst the slow-grown cocci. Slight differences were still seen between the fast-grown cocci so that iron-rich cocci treated with penicillin G had more cell-bound Pr A than either of the iron-poor ones.

Supernatant Pr A was only detected in slow-grown iron-rich cultures regardless of penicillin G treatment.

8.2 Discussion

In one sense, these results correlated with those obtained by contact angles in that fast-grown S. aureus had high contact angles, produced no detectable supernatant Pr A and had more cell-bound Pr A, a structure which significantly contributes to surface hydrophobicity (Jonsson and Wadström, 1983); this confirms that Gram-positive surface protein characteristics can be altered by growth rate (Weerkamp and Handley, 1986). However, no significant differences were found due to iron unavailability or the penicillin G treatment that correlated with the contact angles or CL, indeed penicillin G reversed the expected trend by reducing Pr A values. It appears that SH is also decided by factors other than Pr A appearance on the surface of the envelope especially in the penicillin G-treated phenotypes.

A reduction in cell-bound Pr A caused by sub-MICs is noted elsewhere (Gemmell and O'Dowd, 1983) and perhaps impairment of Pr A appearance or stability in the envelope probably increases susceptibility to phagocytosis. Whether topological changes like these occur in vivo remains to be seen.

Table 7

Production of Protein A (Pr A) by S. aureus NCTC 6571 (t_d = 48 min and 24 min) in Fe+ TSB and Fe- TSB and in the absence and presence of 1/16 MIC of penicillin G.

	µg Pr A per 10^8 cfu ml^{-1}	µg Pr A in supernatants*
Phenotype		
t_d = 48 min:		
Fe+ TSB	0.0739	0.0198
Fe+ TSB with penicillin G	0.0330	0.0180
Fe- TSB	0.0313	not detected
Fe- TSB with penicillin G	0.0326	"
t_d = 24 min:		
Fe+ TSB	1.2200	not detected
Fe+ TSB with penicillin G	0.1780	"
Fe- TSB	0.1520	"
Fe- TSB penicillin G	0.1430	"

* values are relative to 10^8 cfu ml^{-1}

9.1 Adhesion to polyvinyl chloride (PVC) catheters

Results were expressed on a percentage binding basis and so are relative. A small less-adherent subpopulation was present for both slow- and fast-grown S. aureus which was displaced by vortexing for merely 30 sec. Removal of the populations was constant at between 2 and 4 min vortexing so a 2 min period was used throughout (Fig 29).

The most adherent phenotype was the slow-grown, iron-rich coccus and penicillin G treatment reduced its adhesive capability (Fig 30). Slow-grown, iron-poor cocci demonstrated even less adhesion and this too was reduced by penicillin G. The fast-grown cocci had fairly uniform adhesion to the catheters regardless of iron or penicillin G status. The lowered result for the iron-rich phenotype here was caused by sampling error in one of the replicate experiments.

9.2 Discussion

Intravascular devices for the administration of fluids, parenteral nutrition and drugs are indispensable but their use may be complicated by infection and thrombophlebitis. The mechanisms by which intravascular device infections develop are not fully understood but studies suggest that potential pathogens adhere to and colonise the external catheter surface, invade via the skin-catheter interface and subsequently colonise the intravascular segment (Maki, 1983).

This adhesive process can be measured in terms of rate or affinity. The latter is important in early pathogenesis (Vosbeck and Mett, 1983) and the conditions chosen here allowed its elucidation. Adhesion is proportional to bacterial concentration rather than total number of cfu and needs careful consideration because if it is too dense, a linear relationship is lost and trends obscured (Vosbeck and

Figure 29

The influence of different vortexing times upon the catheter adhesion of S. aureus NCTC 6571 (t_d = 48 and 24 min) previously grown in Fe+ TSB and Fe- TSB in the absence and presence of 1/16 MIC of penicillin G.

a) t_d = 48 min

b) t_d = 24 min

☐ 30 sec vortex

▤ 2 min vortex

▨ 4 min vortex

1, 2, 3, 4, = see Fig 30 below

Values are from one experiment in duplicate.

Figure 30

The catheter adhesion of S. aureus NCTC 6571 (t_d = 48 and 24 min) previously grown in Fe+ TSB and Fe- TSB in the absence and presence of 1/16 MIC of penicillin G.

1 Fe+ TSB with no antibiotic

2 Fe+ TSB with penicillin G

3 Fe- TSB with no antibiotic

4 Fe- TSB with penicillin G

Values are means of three experiments.

Fig 29

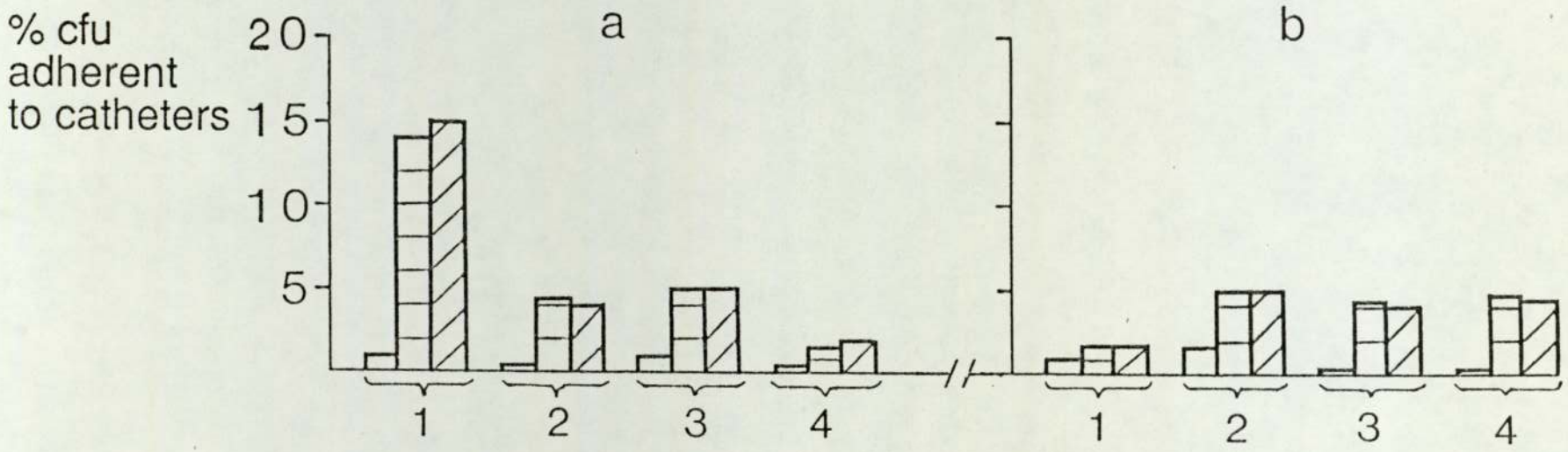
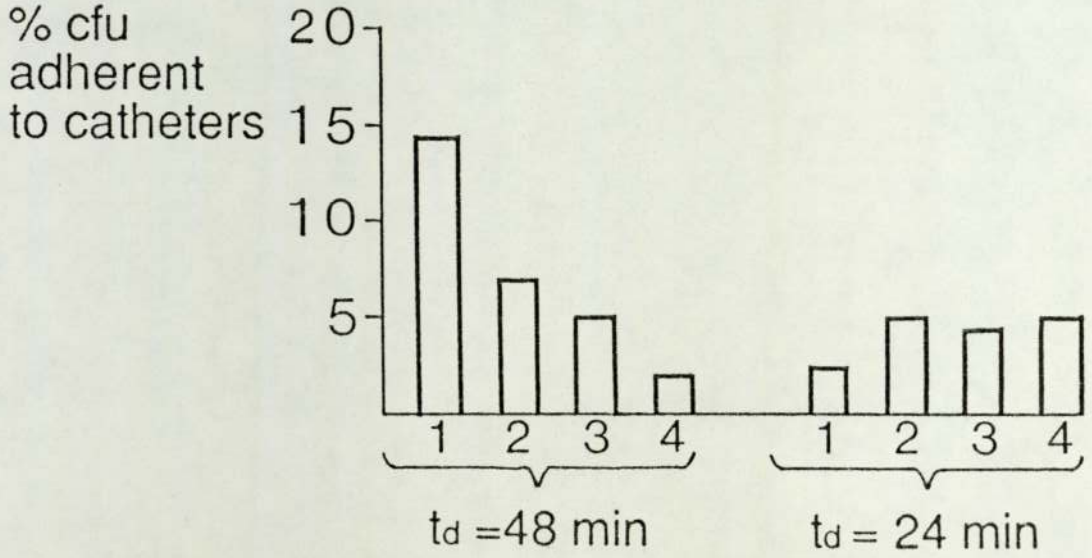

% cfu
adherent
to catheters
20
15
10
5
a
b
1
2
3
4
1
2
3
4

Fig 30


% cfu
adherent
to catheters
20
15
10
5
1 2 3 4
1 2 3 4
td =48 min
td = 24 min

Mett, 1983). This study used approximately 10^8 cfu ml^{-1} which is appropriate (Absolom, et al., 1983). Also, the affinity or extent of adhesion to PVC catheter segments was determined only by the surface properties of the three phases involved, namely, the surface tensions of the cocci, the segments and the suspending saline. Thus, only physico-chemical factors were investigated.

The recent interest in the in vitro colonisation of catheters has revealed the importance of the material of which they are made (Bayston, 1984). Many polymers are now used and adhesion has been shown to be most extensive to the least hydrophobic substrates that also have relatively high surface tensions (Absolom et al., 1983). PVC catheters from administration sets were used in this study because they are very common in British hospitals and especially because Staphylococci are known to adhere to them better than, for example, Gram-negative bacteria (Sheth, et al., 1985). However, their surface properties were not made known despite approaches to the manufacturer.

It was found that S. aureus NCTC 6571 with surface characteristics due to a slow growth rate, were better able to adhere to the catheters. This adhesion was noticeably reduced by the penicillin G treatment and by iron unavailability which are conditions found in vivo. However, none of the trends correlated with either SH or SC so other interactions were involved. Aminosugars have been implicated in staphylcoccal adhesion to PVC catheters (Franson et al., 1984). Elsewhere, a non-specific proteinaceous factor(s) and not SH, seemed to be involved in adhesion of S. aureus to CSF shunts (Barrett, 1985). However, there could also be a role for lipoteichoic acid adhesins (Christensen and Courtney, 1985).

10. Staphylocoagulase and alpha (α)-haemolysin production

10.1 Staphylocoagulase

Supernatant results were expressed relative to 10^8 cfu ml^{-1}. Fast-growing cocci produced no staphylococagulase detectable in this assay. The four slow-growing phenotypes produced similar amounts of enzyme (Table 8).

10.2 α-haemolysin

Results were expressed as above. All phenotypes produced the enzyme and titres are presented in Table 8.

10.3 Discussion

Staphylocoagulase was not produced by any fast-growing phenotypes. This is not surprising in retrospect, as maximum production of the enzyme occurs in stationary phase (Jeljaszewicz et al., 1983) and S. aureus NCTC 6571 is a low producer. No significant differences were induced by iron deprivation or penicillin G treatment. Iron-poor stationary phase cocci had reduced titres of staphylocoagulase (Section 3.1) so it appears that the effects of the deprivation are not manifest until then, when the iron-rich cocci proceed to manufacture more. Penicillin G does not affect protein synthesis hence its lack of activity.

More α-haemolysin was produced by the slow-growing phenotypes than by the fast-growing ones. This again reflects the late onset of extracellular enzyme production in S. aureus, in this instance, α-haemolysin is produced, maximally, in late exponential to early stationary phase.

The effects of the iron and penicillin G treatment were not significant. Possibly more was produced by the slow-growing iron-poor cocci treated with penicillin G, and the fast-growing iron-poor cocci compared to their respective phenotypes.

Table 8

Production of staphylocoagulase and α-haemolysin by S. aureus NCTC 6571 (t_d = 48 min and 24 min) in Fe+ TSB and Fe- TSB in the absence and presence of 1/16 MIC of penicillin G.

(values are means of three estimations).

	Titre in supernatants*	
	staphylocoagulase	α-haemolysin
Phenotype		
t_d = 48 min		
Fe+ TSB	1:0.45	1:2.97
Fe+ TSB with penicillin G	1:0.54	1:3.39
Fe- TSB	1:0.39	1:3.78
Fe- TSB with penicillin G	1:0.54	1:4.73
t_d = 24 min		
Fe+ TSB	not detected	1:0.38
Fe+ TSB with penicillin G	"	1:0.38
Fe- TSB	"	1:1.98
Fe- TSB with penicillin G	"	1:1.75

* Values are relative to 10^8 cfu ml^{-1}

Essentially, S. aureus NCTC 6571 is not renowned for its extracellular enzyme production and a more appropriate strain could be used in future studies.

11. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and lectinblotting of S. aureus NCTC 6571

11.1 Choice of preparative method for samples

Whole cells proved unsatisfactory. Despite solubilisation (Section 2.2.21) most of the sample stayed in the stacking gel. Penicillin G treatment reduced this but generally whole cells did not run very well (Fig 31).

Lysostaphin digests did move onto the running gel satisfactorily but the patterns were relatively uniform. However, some differences were seen, the titres of the antisera to the Fe+ TSB, Fe+ TSB with penicillin G, Fe- TSB and Fe- TSB with penicillin G cocci were known to be satisfactory (1:1,600; 1:3,200; 1:3,200; 1:3,200 respectively) and so immunoblotting was attempted. Only one example is shown as all results were identical. A major antigenic band was visualised at 35.5 Kilodaltons (Kd) (Fig 32). Rabbit-raised anti-rat IgG-peroxidase conjugate was used as a "probe" for Pr A and showed specific reactivity with this band. The pre-immune sera from rabbits subsequently immunised with Fe- TSB grown cocci showed a reaction to the antigen preparation from the Fe+ TSB cultures treated with penicillin G.

Attempts to locate Pr A by simultaneously running a commercial standard (Sigma Ltd.) failed because of diffuse reactions (Fig 33).

11.2 Discussion

When these experiments were first performed, there were no reports about the processing of S. aureus for SDS-PAGE.

Figure 31

SDS-PAGE of different preparations of S. aureus NCTC 6571 (t_d=48 min)

Lane		
1	Fe+ TSB with no antibiotic	Whole cells
2	Fe- TSB with no antibiotic	
3	Fe+ TSB with penicillin G	
4	Fe- TSB with penicillin G	
5	Fe+ TSB with no antibiotic	Lysostaphin digests
6	Fe- TSB with no antibiotic	
7	Fe+ TSB with penicillin G	
8	Fe- TSB with penicillin G	

N.B. The molecular weights of all bands in all figures are expressed in kilodaltons (Kd).

Figure 32

Differential strip immunoblot of lysostaphin digest of S. aureus NCTC 6571 grown in Fe+ TSB in the presence of 1/16 MIC of penicillin G and separated by SDS-PAGE.

Lane		
1	Probed with antisera to Fe+ cocci	Pre-immune
2	" " " " Fe+ penicillin G cocci	
3	" " " " Fe- cocci	
4	" " " " Fe- penicillin G cocci	
5	Probed with Pr A "probe"	
6	" " antisera to Fe+ cocci	Hyper-immune
7	" " " Fe+ penicillin G cocci	
8	" " " Fe- cocci	
9	" " " Fe- penicillin G cocci	
10	Amido black stain for proteins	

Fig 31

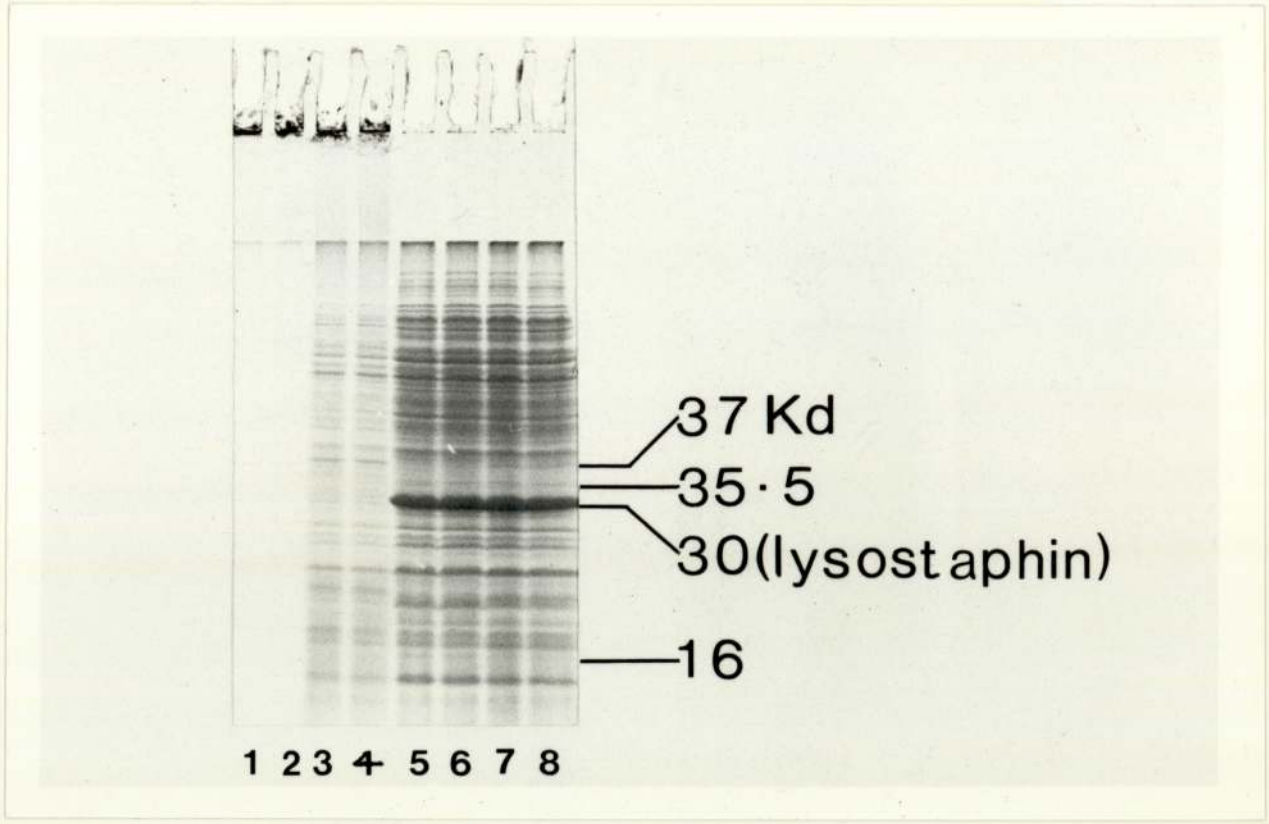


Fig 32

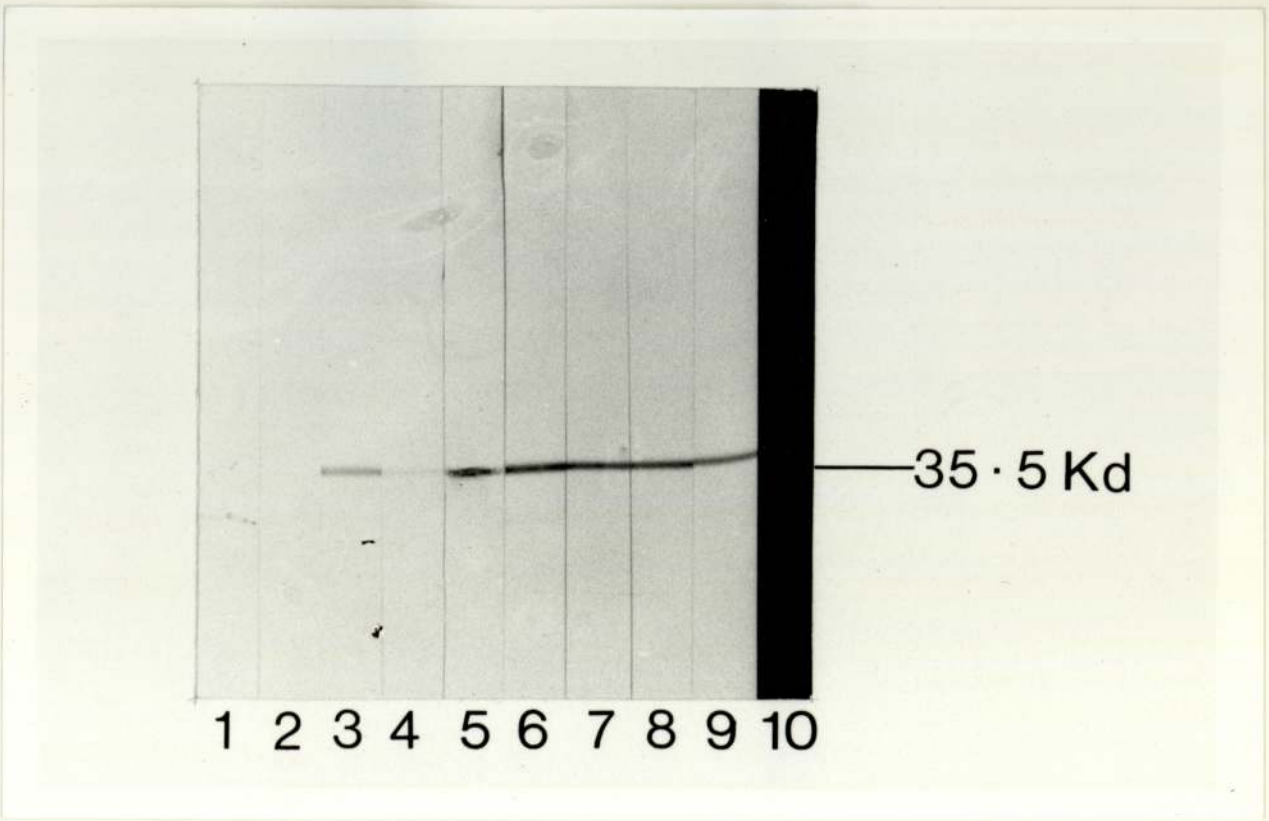
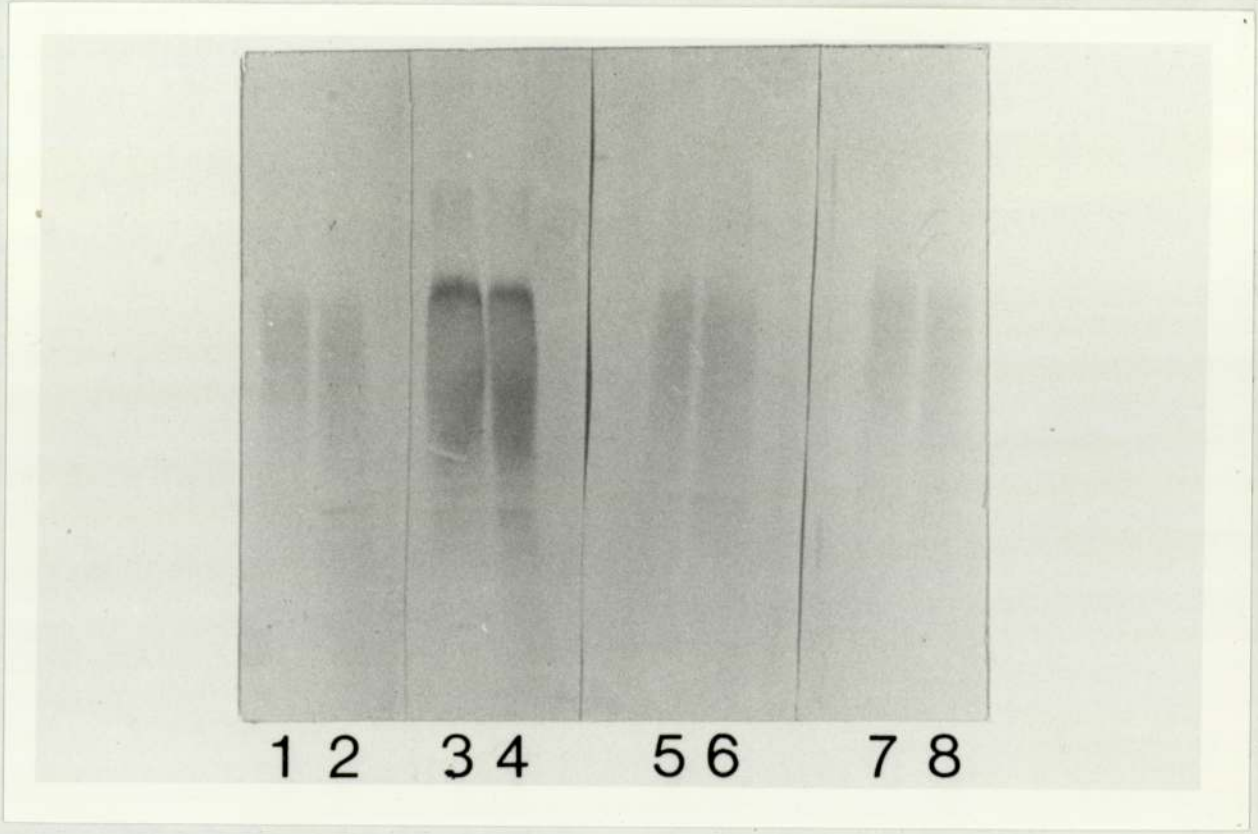


Figure 33

Immunoblot of a commercial preparation of Pr A using four different hyperimmune antisera.

Lane

1 and 2	Probed	with	antisera	to	Fe+ cocci
3 and 4	"	"	"	"	Fe+ penicillin G cocci
5 and 6	"	"	"	"	Fe- cocci
7 and 8	"	"	"	"	Fe- penicillin G cocci



Sonication was rejected because although frequently used, it destroys the clumping factor (Jeljaszewicz et al., 1983).

Whole cocci, cell envelopes and lysostaphin digests had been used in other fields, e.g. ELISA (Jacob et al., 1985; Jarløv et al., 1985) or Pr A characterisation (Björk et al., 1972). Accordingly, these were all used to determine the most suitable preparation.

The slow-grown phenotypes were first investigated because their final yield was greater.

Immunoblotting was disappointing in that the antigenic appearance of the phenotypes was all very similar and very stark in appearance. The SDS-PAGE profiles had abundant bands so it is not clear why lysostaphin should have drastically simplified the profiles. Lysostaphin has three enzymic activities, a glucosaminidase, an endopeptidase and a slow-acting amidase (Sjöquist, Meloun and Hjelm, 1972; Strominger and Ghuysen, 1967) so it is possible to conclude that the antigenicity of the peptidoglycan but not that of Pr A has been destroyed. Other minor proteins that may be surface associated could have been exposed to proteases from the lysed cocci. It is known from immunoblotting that protease-sensitive antigens in S. aureus are derived mainly from the surface of the envelope (Thomas, Sanford, Jones, Triplett and Keogh, 1986) and this might explain the loss of antigens here.

Alternatively, lysostaphin is a basic protein (Schindler and Schuhardt, 1965) and it is possible that it may have interacted non-specifically with minor proteins and pulled them into the 30 Kd range thus simplifying the profile.

The most likely explanation though is that many commercial preparations of lysostaphin are contaminated by traces of various proteases (Wilkinson, B.J.; pers. comm.) and that these hydrolysed the "missing" antigens. It was decided not to subject the fast-grown

cocci to lysostaphin but to proceed to using the cell envelope fractions.

Pr A runs in a diffuse manner on SDS-PAGE if it is not freshly prepared (Björk et al., 1972). It was not possible to prepare fresh Pr A nor use a monoclonal or monospecific antibody. The Sigma preparation ran diffusely whilst the Pharmacia Ltd. preparation is known to produce several bands upon SDS-PAGE even under non-reducing conditions (Langvad, Espersen and Briand, 1984). So the ability of Pr A to bind the F_c portion of IgG was exploited. The IgG-peroxidase conjugate was raised in rabbit and had a specificity for rat IgG (a titre of 1:64 by immunodiffusion assay; manufacturer's instructions). It was used in this study at a 1:300 dilution compared to the 1:50 dilution of the immune antisera. Pr A has a high affinity for the F_c portion of rabbit IgG (Forsgren et al., 1983) and so the interaction between the "probe" and an antigen at 35.5 Kd confirms that the latter is Pr A.

The molecular weight of 35.5 Kd for Pr A contrasts with the often quoted molecular weight of 42 Kd but this is a distillation of the results of several different methods of analysis (Björk et al., 1972; Forsgren et al., 1983). Its molecular weight is not only affected by its freshness but by the method of preparation. Thus Pr A prepared by lysostaphin digests had a molecular weight of 56 Kd in 8.25% (V/V) SDS-PAGE (Björk et al., 1972) whilst when prepared by saline washes it had an SDS-PAGE derived value of 71 Kd (Tewari, Tripathi, Nangia and Gupta, 1984). It is therefore misleading to regard Pr A as an homogenous entity of fixed molecular weight and the use of a "probe" is justified as it enabled the location of Pr A in situ.

12. SDS-PAGE, immunoblotting and lectinblotting of cell envelope fractions of S. aureus NCTC 6571

12.1 SDS-PAGE

When the cell envelope fractions were analysed by SDS-PAGE the differences were more marked than the lysostaphin digest preparations (Fig 34). Laser densitometry (not shown) confirmed this (Ultrascan; LKB Ltd.) The alteration in growth rate did not confer noticeable changes in the SDS-PAGE profiles but the treatment with penicillin G and the level of available iron did.

12.2 Immunoblot with normal human sera

Serum was taken from the four subjects, at the same time as blood for the WBK, and used at a 1:10 dilution, to probe envelope antigens of the four slow-grown phenotypes.

There was heterogeneity between the patterns of recognition. Those for subjects one and four were similar to each other (Fig 35a and b) but differed from those for subjects two and three (Fig 35c and d). Subject three differed from the others in not distinctly visualising a 21 Kd band.

The iron-rich phenotype was notable because it was not recognised by subjects one, two and four and only poorly by subject three. In contrast, the iron-rich penicillin G-treated phenotype was most strongly recognised, especially by subjects one, two and four. The Pr A (35.5 Kd) in this phenotype was most strongly visualised by subjects two and four whereas subjects one and three recognised it only in the iron-poor preparation. The antigen most strongly visualised by all the sera was at 68 Kd.

Figure 34

SDS-PAGE of envelope preparations of S. aureus NCTC 6571 (t_d = 48 and 24 min) in Fe+ TSB and Fe- TSB in the absence and presence of 1/16 MIC of penicillin G.

Lane		
1	Fe+ TSB with no antibiotic	t_d 48 min
2	Fe+ TSB with penicillin G	
3	Fe- TSB with no antibiotic	
4	Fe- TSB with penicillin G	
5	Fe+ TSB with no antibiotic	t_d = 24 min
6	Fe+ TSB with penicillin G	
7	Fe- TSB with no antibiotic	
8	Fe- TSB with penicillin G	

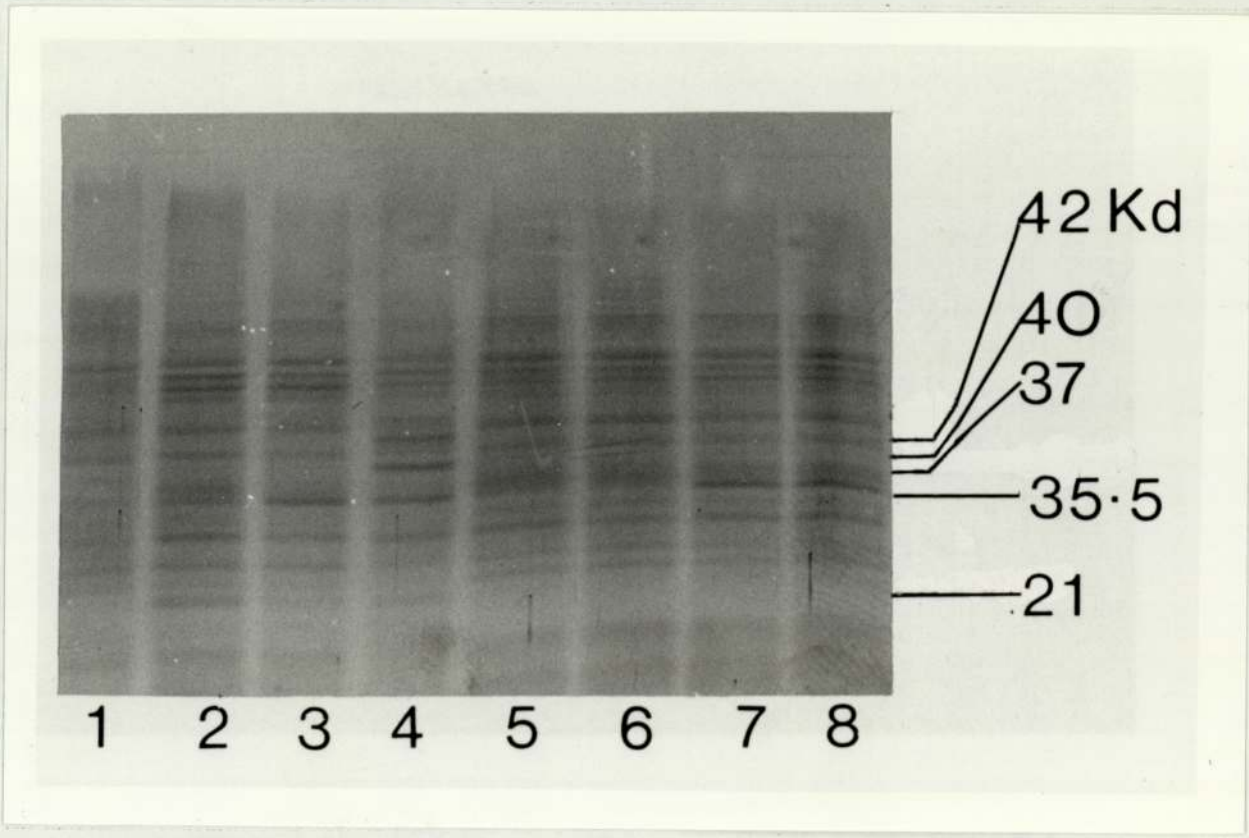


Figure 35

Immunoblot of cell envelope fractions of S aureus NCTC 6571 (t_d = 48 min) grown in Fe+ TSB and Fe- TSB in the absence and presence of 1/16 MIC of penicillin G, and separated by SDS-PAGE.

a = probed with serum from subject 1

b = " " " " " 4

c = " " " " " 2

d = " " " " " 3

Lane

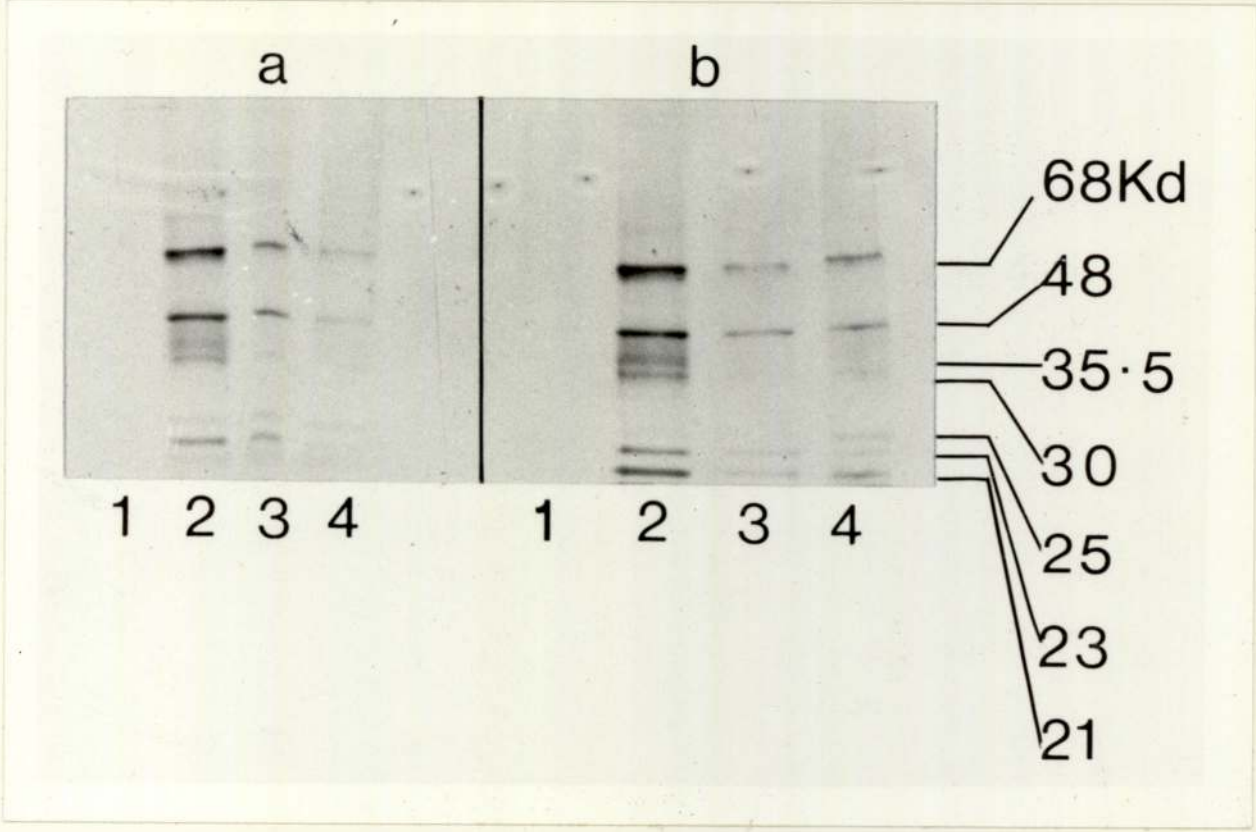
1 Fe+ TSB with no antibiotic

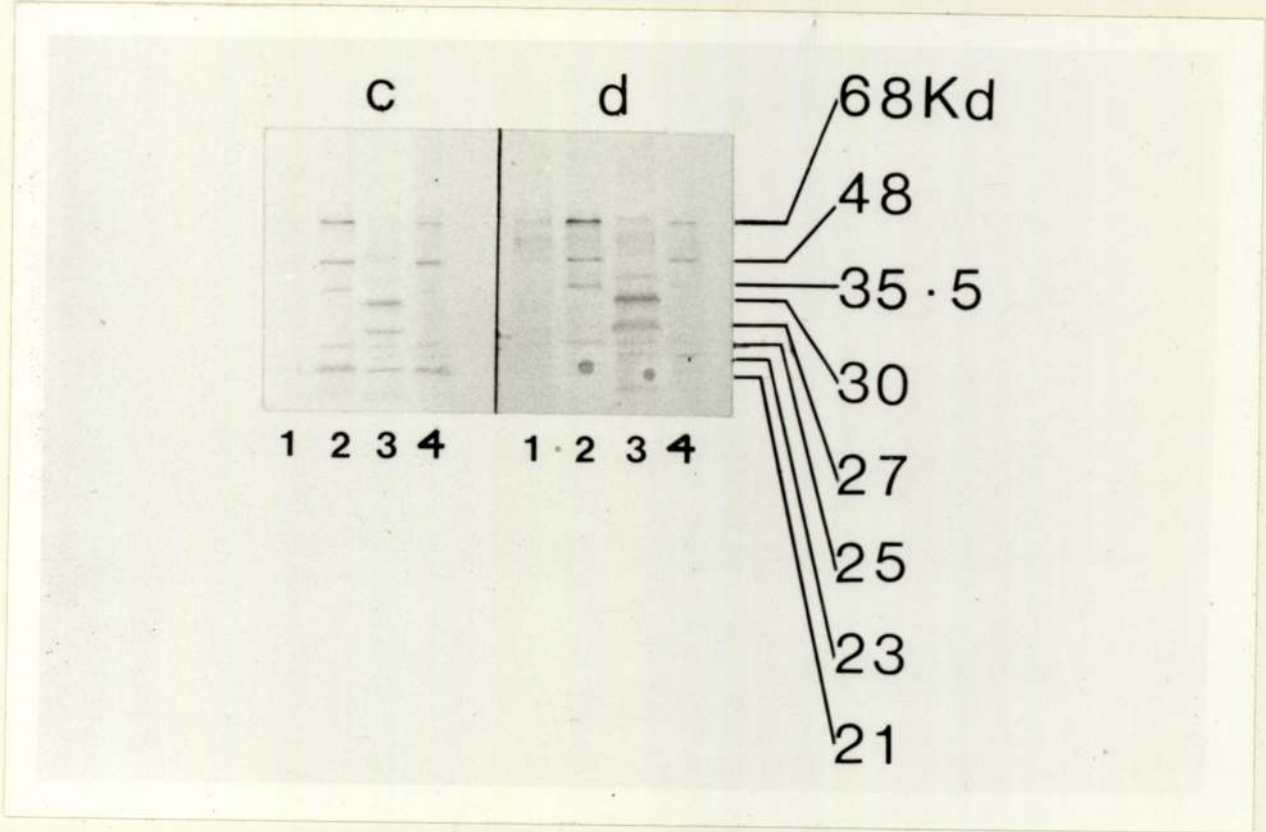
2 Fe+ TSB with penicillin G

3 Fe- TSB with no antibiotic

4 Fe- TSB with penicillin G

Fig 35


a
b
68Kd
48
35·5
30
25
23
21
1 2 3 4
1 2 3 4


c
d
68Kd
48
35·5
30
27
25
23
21
1 2 3 4
1 2 3 4

12.3 Immunoblotting, lectinblotting, avidin binding and surface proteins

Immunoblotting of envelope preparations of S. aureus NCTC 6571 did not show any differences between the fast and slow-grown cocci. Thus to avoid duplication only representative results are shown. All phenotypes were remarkable in that the banding was much simpler and structures were present that were not prominent in stained gels.

The control strips developed with pre-immune sera showed that the anti-Fe+ TSB cocci serum not only failed to recognise the Pr A band in the iron-rich preparation (Fig 36, Lane 1) but also failed to do so in all other preparations (Fig 37 to 39, Lane 1). This was in contrast to the three other types of pre-immune sera (Fig 37 to 39, Lanes 2 to 4). When the strips were developed with the four hyperimmune antisera, the fast and slow-grown iron-rich preparations stood out in that up to 18 immunogenic bands were visualised when both homologous and heterologous antisera were used. More bands were produced however in the homologous challenge particularly at 46 and 48 Kd. However, iron-rich preparations lacked an antigen at 64 Kd (Fig 36).

The fast and slow-grown iron-rich cocci treated with penicillin G, and the fast and slow-grown iron-poor cocci with or without the penicillin G treatment had much simpler profiles so that only nine bands were seen upon challenge with homologous or heterologous antisera. The Pr A band stood out again, at 35.5 Kd (Fig 37 to 39).

Blots of the fast-grown cells had the Pr A "probe" included to bring them into line with the slow-grown cocci (Section 11.1) and the 35.5 Kd band was again confirmed as Pr A. All phenotypes had common bands (Fig 36).

Figure 36

Differential strip immunoblot of envelope preparation of S. aureus NCTC 6571 grown in Fe+ TSB in the absence of 1/16 MIC of penicillin G and separated by SDS-PAGE

Lane

1	probed with antisera to Fe+ cocci	Pre-immune
2	" " " " Fe+ penicillin G cocci	
3	" " " " Fe- cocci	
4	" " " " Fe- penicillin G cocci	
5	" " " " Fe+ cocci	Hyper-immune
6	" " " " Fe+ penicillin G cocci	
7	" " " " Fe- cocci	
8	" " " " Fe- pen G cocci	
9	Amido black stain for proteins	

✳ Denotes antigenic bands common to all eight phenotypes

Figure 37

Differential strip immunoblot of envelope preparation of S. aureus NCTC 6571 grown in Fe+ TSB in the presence of 1/16 MIC of penicillin G and separated by SDS-PAGE

Lane

1	Probed with antisera to Fe+ cocci	Pre-immune
2	" " " " Fe+ penicillin G cocci	
3	" " " " Fe- cocci	
4	" " " " Fe- penicillin G cocci	
5	" " " " Fe+ cocci	Hyper-immune
6	" " " " Fe+ penicillin G cocci	
7	" " " " Fe- cocci	
8	" " " " Fe- penicillin G cocci	
9	Amido black stain for proteins	

❖ Denotes an antigenic band not found in the fast- and slow-grown Fe+ TSB phenotypes

Fig 36

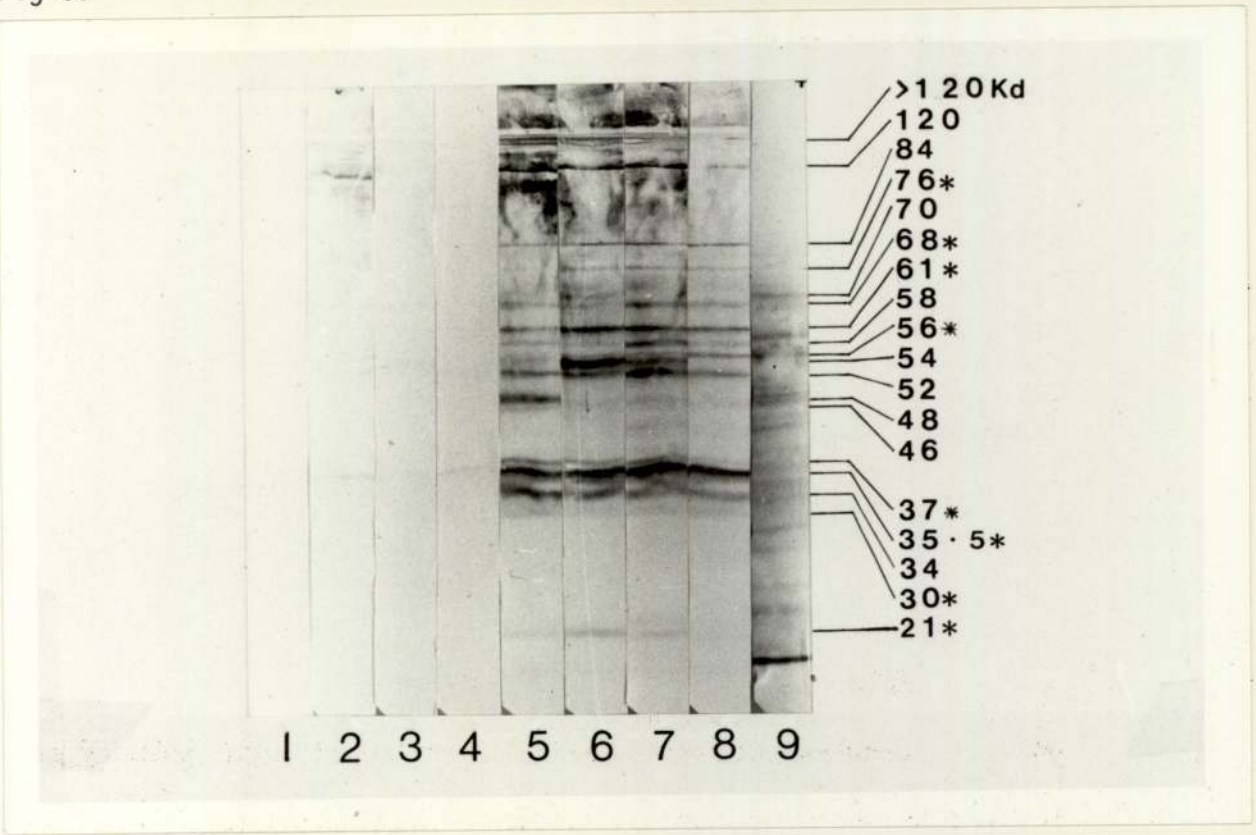

>120Kd
120
84
76*
70
68*
61*
58
56*
54
52
48
46
37*
35·5*
34
30*
21*
1 2 3 4 5 6 7 8 9

Fig 37

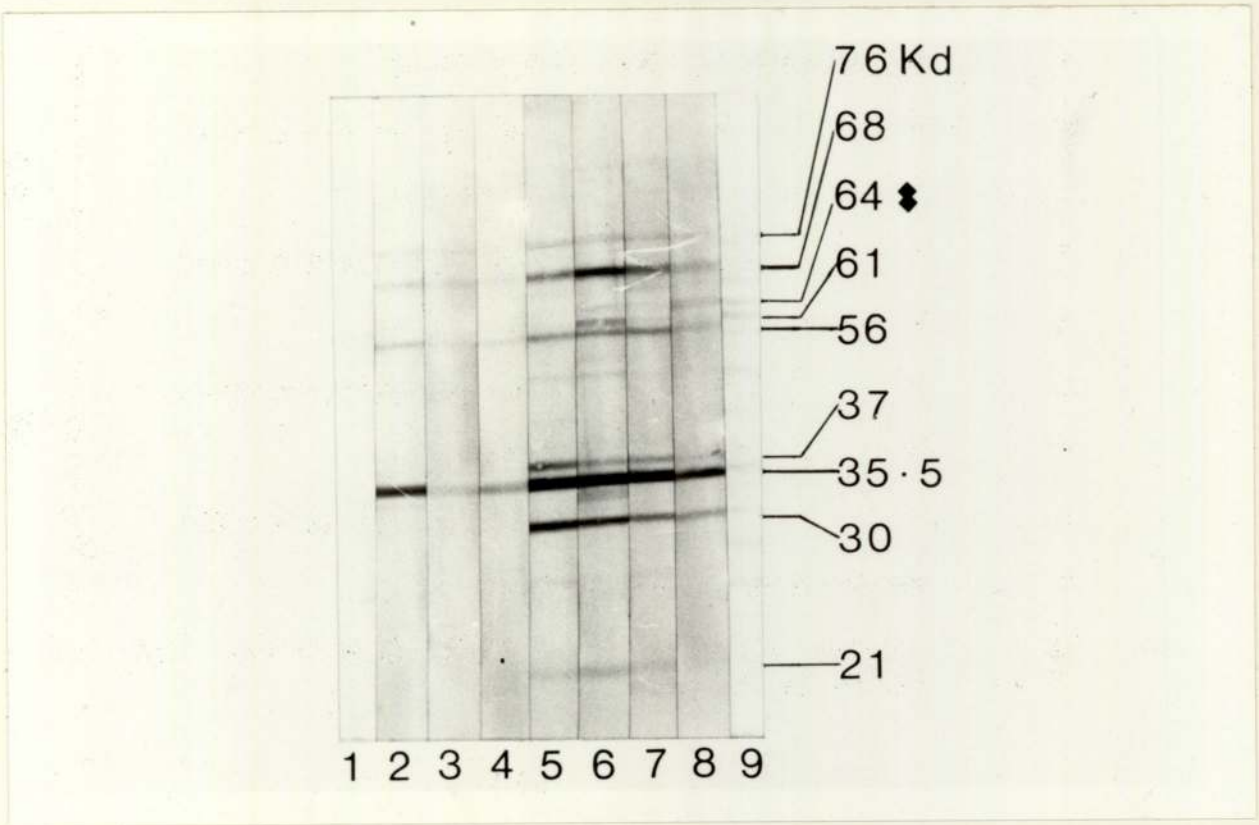

76Kd
68
64
61
56
37
35·5
30
21
1 2 3 4 5 6 7 8 9

Figure 38

Differential strip immunoblot of envelope preparation of S. aureus NCTC 6571 grown in Fe- TSB in the absence of 1/16 MIC of penicillin G and separated by SDS-PAGE.

Lane			
1	Probed with antisera to	Fe+ cocci	Pre-immune
2	" " " "	Fe+ penicillin G cocci	
3	" " " "	Fe- cocci	
4	" " " "	Fe- penicillin G cocci	
5	" " " "	Fe+ cocci	Hyper-immune
6	" " " "	Fe+ penicillin G cocci	
7	" " " "	Fe- cocci	
8	" " " "	Fe- penicillin G cocci	
9	Amido black stain for proteins		

❖ Denotes an antigenic band not found in the fast- and slow-grown Fe+ TSB phenotypes.

Figure 39

Differential strip immunoblot of envelope preparation of S. aureus NCTC 6571 grown in Fe- TSB in the presence of 1/16 MIC of penicillin G and separated by SDS-PAGE.

Lane			
1	Probed with antisera to	Fe+ cocci	Pre-immune
2	" " " "	Fe+ penicillin G cocci	
3	" " " "	Fe- cocci	
4	" " " "	Fe- penicillin G cocci	
5	" " " "	Fe+ cocci	Hyper-immune
6	" " " "	Fe+ penicillin G cocci	
7	" " " "	Fe- cocci	
8	" " " "	Fe- penicillin G cocci	
9	Amido black stain for proteins		

❖ Denotes an antigenic band not found in the fast- and slow-grown Fe+ TSB phenotypes.

Fig 38

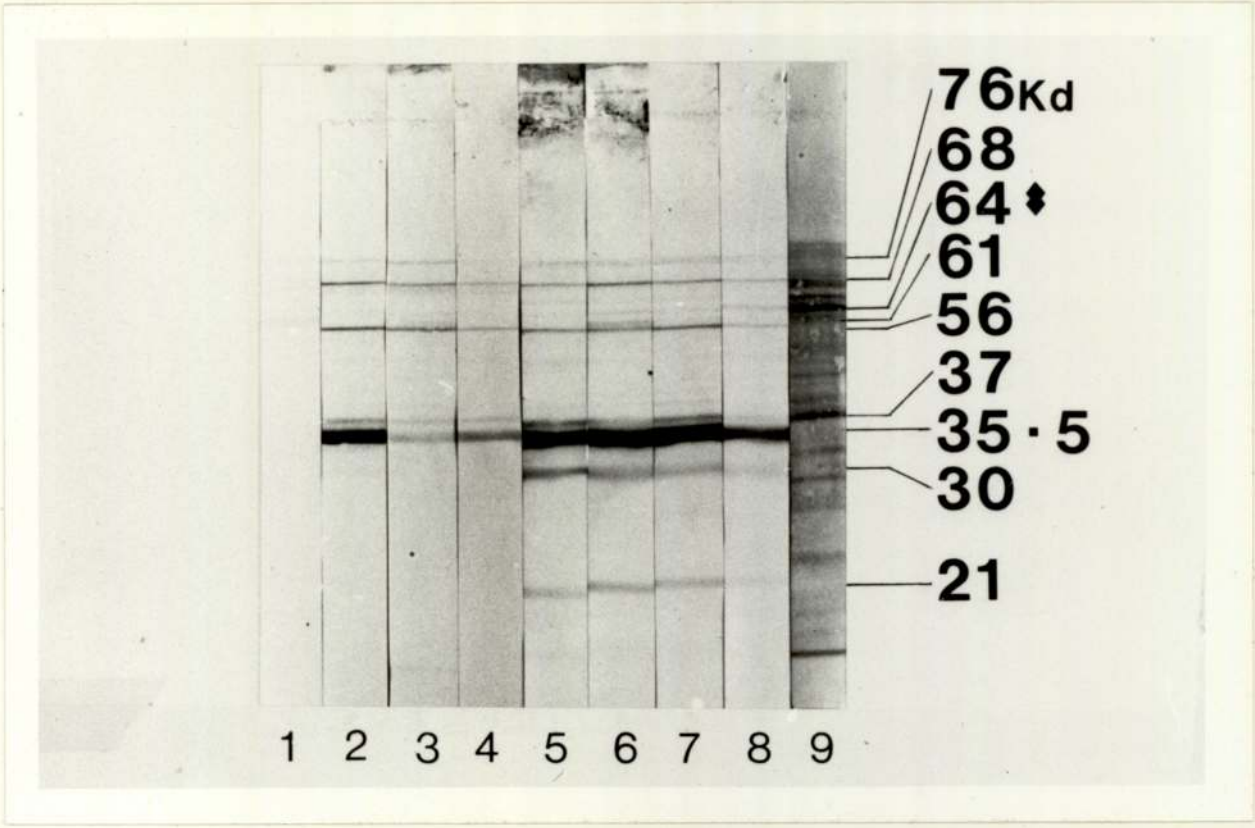
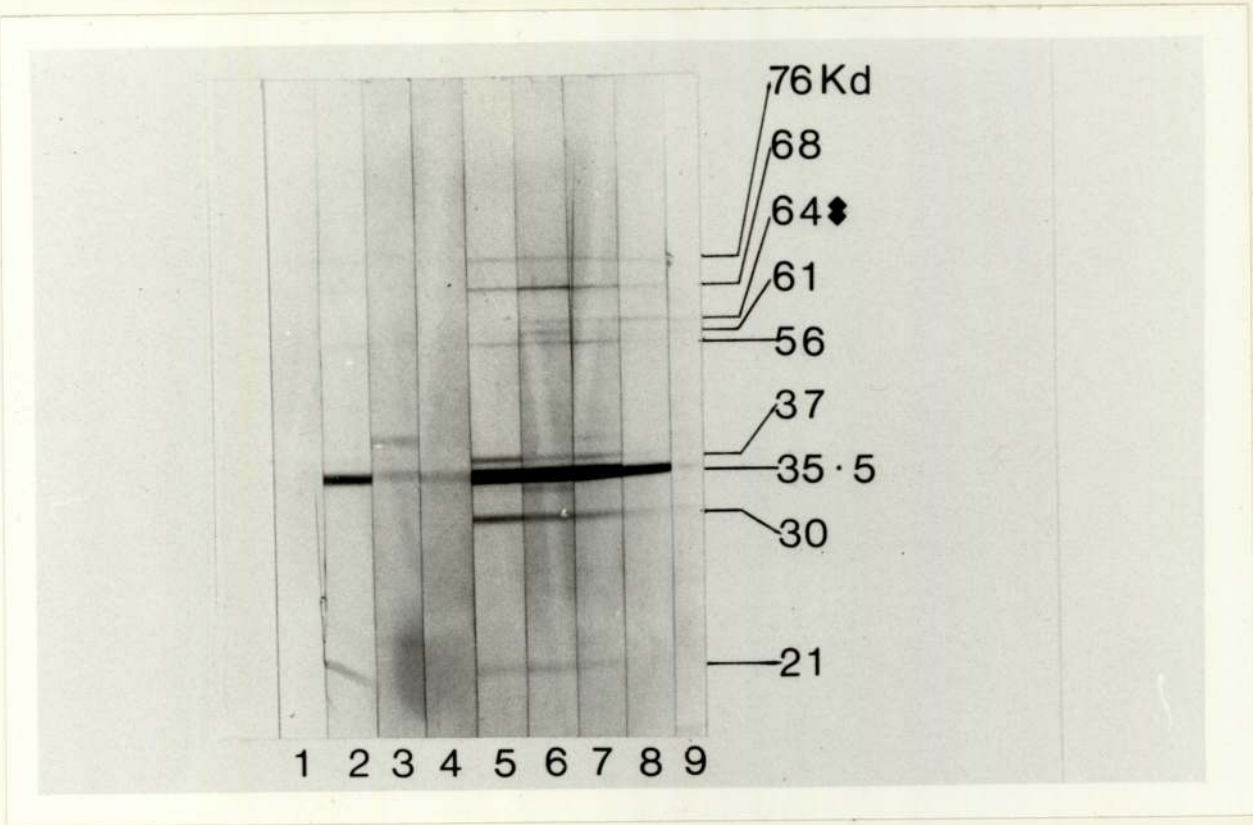

76Kd
68
64
61
56
37
35·5
30
21
1 2 3 4 5 6 7 8 9

Fig 39


76Kd
68
64
61
56
37
35·5
30
21
1 2 3 4 5 6 7 8 9

Subsequent strip blots were also incubated with four lectin-peroxidase conjugates:

Concanavalin A, Con A	binds to glucose and mannose
Soy bean agglutinin, SBA	binds to N-acetylgalactosamine
Asparagus pea lectin, APL	binds to fucose
Wheat germ agglutinin, WGA	binds to N-acetylgalactosamine and N-acetylmuramic acid

Alterations in growth rate, and penicillin G treatment did not influence lectin and avidin binding therefore only representative figures are shown.

Binding of Con A and APL demonstrated that the major antigen, Pr A, was not pure but contained glycosylated residues. Pr A also showed a minor affinity for avidin (Fig 40 and 41). An antigen at 30 Kd in iron-poor preparations with or without penicillin G also bound APL.

Both SBA and WGA showed no affinities although WGA was bound by material left in the stacking gel (Fig 41, Lane 14).

The major avidin binding site in S. aureus NCTC 6571 had a molecular weight of 86 Kd a structure not seen in any immunoblotting experiments, with a minor site in Pr A at 35.5 Kd (Fig 40 and 41, Lane 15).

This last series of immunoblotting used envelopes from cocci grown in treated TSB of a different batch number. No major alterations in antigenicity resulted; the 61 Kd band present most prominently in Fe+ TSB cocci treated with penicillin G and probed with homologous antiserum (eg. Fig 37, Lane 6) was seen now to be most prominent in Fe+ TSB cocci probed with homologous antisera (eg. Fig 40, Lane 6). This change presumably reflects the "environmental" differences between different media (Brown and Williams, 1985a).

Slow-grown whole cocci had their surface proteins radiolabelled

Figure 40

Differential strip immunoblot of envelope preparation of S. aureus NCTC 6571 grown in Fe+ TSB in the absence of 1/16 MIC of penicillin G and separated by SDS-PAGE.

Lane		
1	Amido black stain for proteins	
2	Probed with antisera to Fe+ cocci	Pre-immune
3	" " " " Fe+ penicillin G cocci	Pre-immune
4	" " " " Fe- cocci	Pre-immune
5	" " " " Fe- penicillin G cocci	Pre-immune
6	" " " " Fe+ cocci	Hyper-immune
7	" " " " Fe+ penicillin G cocci	Hyper-immune
8	" " " " Fe- cocci	Hyper-immune
9	" " " " Fe- penicillin G cocci	Hyper-immune
10	Probed with Pr A "probe"	
11	" " Con-A peroxidase	
12	" " SBA-peroxidase	
13	" " APL-peroxidase	
14	" " WGA-peroxidase	
15	" " Avidin-peroxidase	

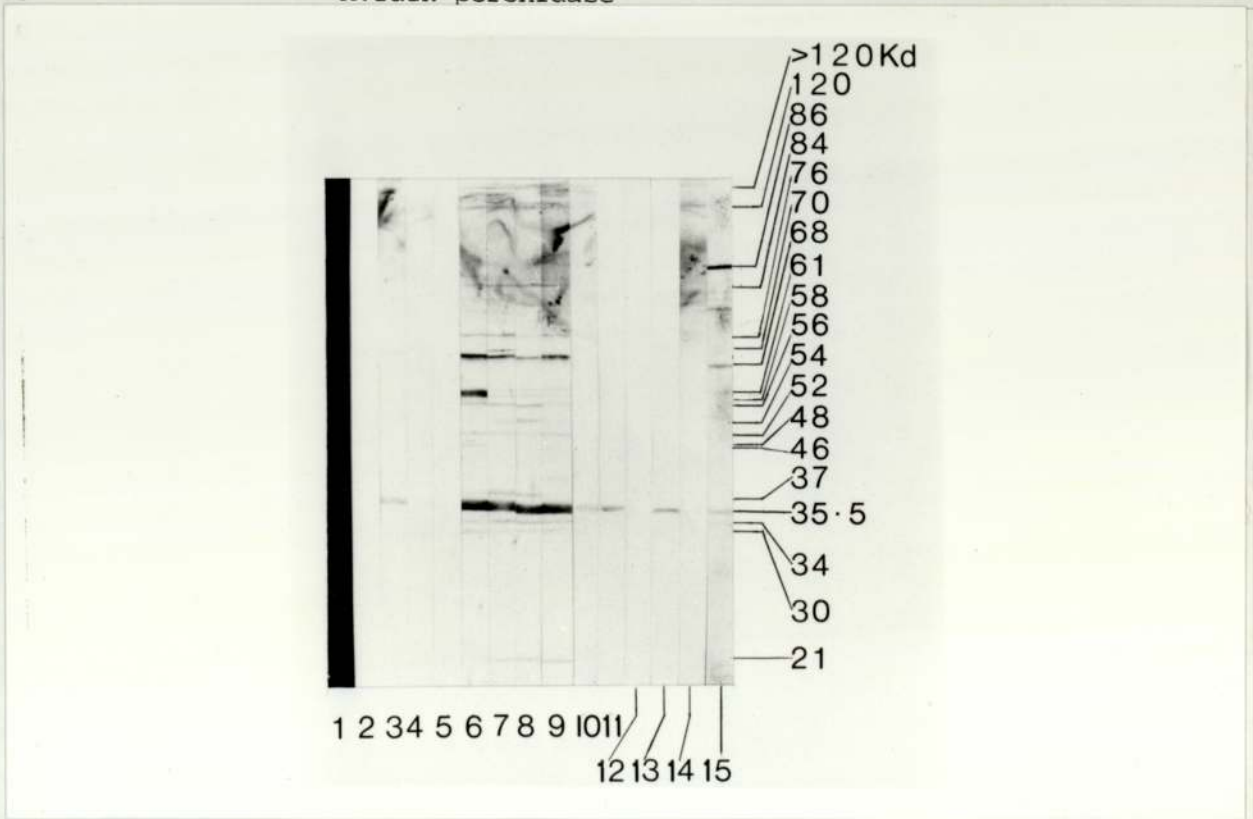
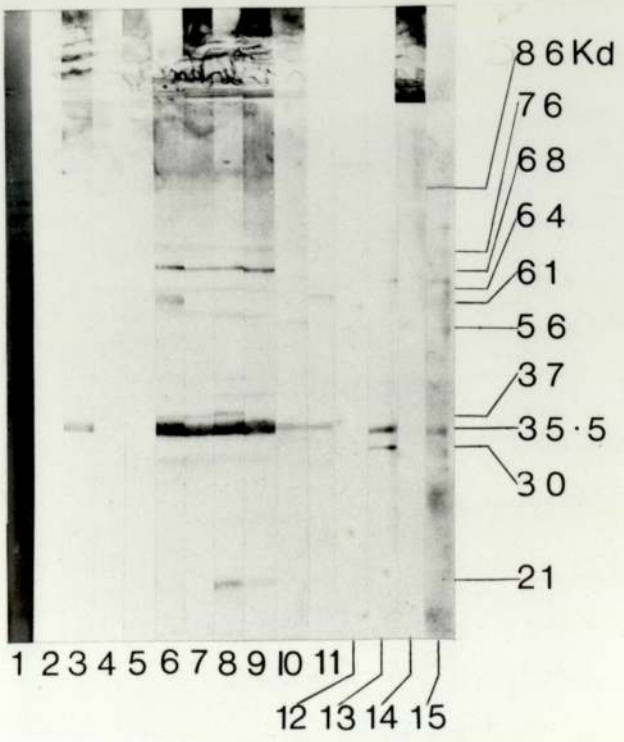


Figure 41

Differential strip immunoblot of envelope preparation of S. aureus NCTC 6571 grown in Fe- TSB in the presence of 1/16 MIC of penicillin G and separated by SDS-PAGE.

Lane		
1	Amido black stain for proteins	
2	Probed with antisera to Fe+ cocci	Pre-immune
3	" " " " Fe+ penicillin G cocci	Pre-immune
4	" " " " Fe- cocci	Pre-immune
5	" " " " Fe- penicillin G cocci	Pre-immune
6	" " " " Fe+ cocci	Hyper-immune
7	" " " " Fe+ penicillin G cocci	Hyper-immune
8	" " " " Fe- cocci	Hyper-immune
9	" " " " Fe- penicillin G cocci	Hyper-immune
10	Probed with Pr A "probe"	
11	" " Con A-peroxidase	
12	" " SBA-peroxidase	
13	" " APL-peroxidase	
14	" " WGA-peroxidase	
15	" " Avidin-peroxidase	



(Fig 42). There was very little difference between the four phenotypes and in contrast to the Coomassie Blue Black-stained gel profiles (Fig 34, Lanes 1-4) only five major bands were noticeable here and were seen in all types. However only three of these, 68, 35.5 and 21 Kd were common to all in immunoblotting and of these the 21 Kd band was labelled most strongly.

12.4 Discussion

S. aureus SDS-PAGE profiles were notable for their similarity, their profusion of bands and poor quality of staining even when the background had been well destained. The lack of obvious heterogeneity of S. aureus profiles is not surprising because profiles for six phage types of S. aureus have been found to be virtually indistinguishable and thus no attempt was made to characterise individual bands by molecular weight, function or chemical nature (Krikler, Pennington and Petrie, 1986). This homogeneity may also explain the extensive cross-reactivity between strains of S. aureus and also the considerable but lesser cross-reactions between different species of Staphylococci that have hampered crossed immuno-electrophoretic analysis of S. aureus antigens (Schiötz, Höiby and Hertz, 1979).

SDS-PAGE has been used to study alterations caused by surface hydrophobicity (SH). Hydrophobic strains of Streptococcus mutans were able to incorporate certain high molecular weight proteins, especially one at 190 Kd, into the cell envelope. The nature of the SH was unlike that investigated in this study because it was retained throughout several years serial subculture on complex, solid media and was always high even in stationary phase cultures (McBride, Song, Krasse and Olsson, 1984). The SH in S. aureus seems to depend mainly upon the amount of cell-bound Pr A (Jonsson and Wadström, 1983) and this would not be detected by SDS-PAGE which is poorly quantitative.

Figure 42

^{125}I-labelling of whole cells of S. aureus NCTC 6571 (t_d = 48 min) grown in Fe+ TSB and Fe- TSB in the absence and presence of 1/16 MIC of penicillin G.

Lane

1 Fe+ cocci with label

2 Fe+ cocci without label

3 Fe+ penicillin G cocci with label

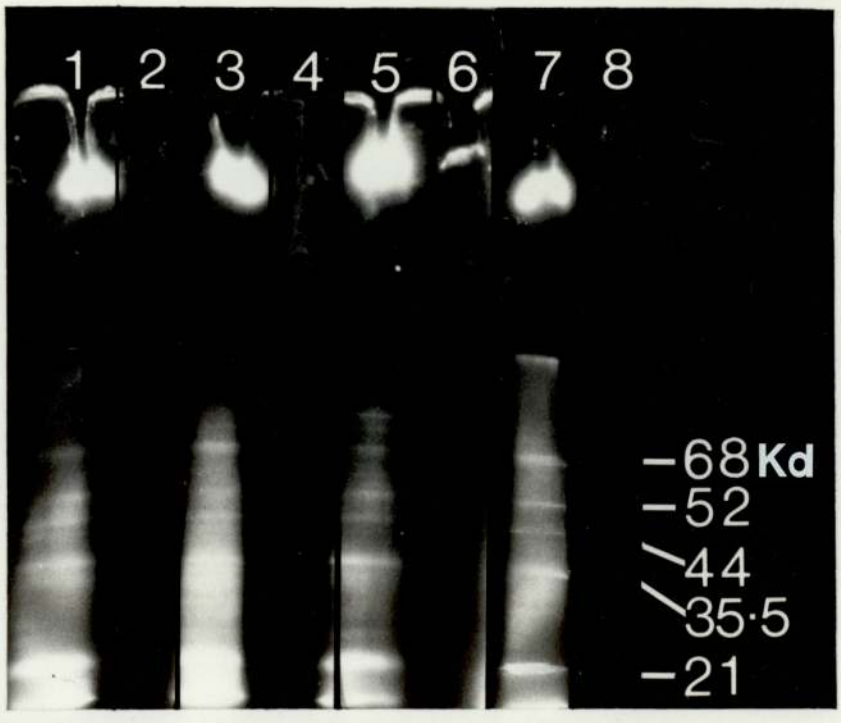
4 Fe+ penicillin G without label

5 Fe- cocci with label

6 Fe- cocci without label

7 Fe- penicillin G cocci with label

8 Fe- penicillin G cocci without label



Besides this, the gel system used did not allow analysis of bands of molecular weight greater than 120 Kd.

SDS-PAGE and immunoblotting have only recently been applied to S. aureus. Currently, counter-immunoelectrophoretic (CIE) techniques are extensively used but to date, little progress has been made in determining the molecular weights, structures and functions of Staphylococcal antigens. This is because of the intrinsic nature of CIE and the multiplicity of antigens which can be described with great precision with regard to position but not identity (Owen, 1981).

Admittedly, SDS-PAGE of Gram-positive bacteria yields a profusion of bands and the processing eventually produces a denatured antigen bound onto nitrocellulose (NC) paper. However there is no doubt from this study and others that antisera raised to whole cells or whole cell extracts recognise antigens treated in such a manner (Krikler et al., 1986; Thomas et al., 1986). It is also known that even after SDS-PAGE resolution and transfer onto NC paper, the enzymic activity of PBPs from S. aureus was still present (Wyke, 1984). The fact remains that a clearer and thus more valuable picture emerges when immunoblotting is applied to S. aureus. This offsets a possible objection that crude envelope preparations have, in other assays, not allowed individual antigens to be monitored.

The reactions seen with the pre-immune sera are presumably a reflection of the rabbits' colonisation with Staphylococci. It is interesting that none of the pre-immune antisera recognised the iron-rich antigenic preparation and this point recurred throughout the study. The recognition patterns obtained with the pre-immune sera were, though, unlike those obtained with the normal, human sera. It is not known why penicillin G or iron unavailability should make the Pr A band more easily recognised in the other preparations by these sera. Evidence has been presented that suggests that the antigens of

the iron-rich coccus are not easily recognised by non-immune human subjects and it may explain these findings.

Immunoblotting with sera from the four subjects confirmed that normal, human serum contains relatively low amounts of antibodies to specific components of the S. aureus envelope.

Subjects one and four, both male, behaved in a very similar fashion, whilst the two female subjects showed differences between themselves. Subject three stood out from the rest as faintly visualising the iron-rich phenotype and not recognising a 21 Kd band. The reason for this is not known but the results presumably reflect the immunological status of the subjects.

Reactions with hyperimmune rabbit antisera revealed that alterations in growth rate did not result in changes detected by SDS-PAGE and immunoblotting. This is probably because antisera was only raised against whole cocci in late logarithmic to early stationary phase (Section 2.2.22). The unavailability of iron and/or the treatment with penicillin G produced changes that although not remarkable in SDS-PAGE dramatically simplified immunoblotting profiles. The effect produced by reduced environmental iron is the reverse of that seen with Gram-negative bacteria both in vitro and in vivo, because they derepress iron-regulated outer membrane proteins to offset iron unavailability and thus produce more antigens (Brown and Williams, 1985a).

Antisera raised against the different phenotypes recognised heterologous antigens. This implies that this method of iron deprivation in TSB and the sub-MIC of penicillin G result in cocci that may have smaller amounts of antigen compared to iron-rich cocci. These amounts are sufficient though to stimulate the production of specific antibodies which are then capable in immunoblotting of recognising the greater amounts of these antigens in iron-rich

preparations. It may be that such binding occurs to the other antigenic preparations at a level not detectable by immunoblotting. Penicillin G and the iron deprivation may loosen cell wall integrity so that antigens are not incorporated properly. Additionally, iron is metabolically so important that synthesis of antigens may have been disrupted.

The majority of iron taken up by a bacterium is located in the cytochromes (Neilands, 1974). The S. aureus cytochromes are a and b types but to date, have been characterised for their absorption spectra and not molecular weights (Schleifer, 1983) so it is not possible to say where they migrated to in SDS-PAGE and immunoblotting.

Immunoblotting of serial serum samples from rabbits revealed that the first S. aureus antigens seen in experimental osteomyelitis were at 128, 76, 65 and 52 Kd. Later, antibodies were detected to antigens between 42 and 17 Kd. The antigens were whole cell sonicates of S. aureus grown on complex media but despite these disparities there are similarities in those findings and the results here (Thomas et al., 1986).

Isolated clumping factor is known to be immunogenic and to give significant protection in mouse infection studies (Espersen and Clemmensen, 1985), it is also known to have a molecular weight of 21 Kd (Jeljaszewicz et al., 1983).

This could be the identity of the 21 Kd band detected here. The immunoblot reaction was weak possibly because S. aureus NCTC 6571 is a relatively weak clumping factor producer and also, although clumping factor resists 100°C (Jeljaszewicz et al., 1983) its stability in sample buffer at this temperature is not known.

The binding of lectins to microbial components (Pistole, 1981) is poorly characterised for S. aureus. In this study, immunoblotting was adapted so that immunogenic structures could also be investigated to

see if they were glycoproteins (Section 2.2.23). The latter may play an important part in host-parasite relationships (Aitchison, Lambert and Farrell, 1986; Scheld, Valone and Sande, 1978).

Pr A reacted with Con A and APL indicating the presence of glycosylated residues. As Pr A is not known to be glycosylated (Forsgren et al., 1983), the material that reacted is presumably glycosylated and co-migrated with Pr A. One possible candidate is MTA.

NAG or NAM residues were detected by WGA but only in high molecular weight material that did not move onto the running gel.

The major avidin receptor was shown, in E. coli to be the outer membrane protein OMP F/OMP C with a molecular weight of 39 to 36 Kd, but no attempt was made to characterise that of S. aureus (Korpela, Salonen, Kuusela, Sarvas and Vaheri, 1984). Thus avidin which is released during inflammatory processes (Korpela, Kulomaa, Tuohimaa and Vaheri, 1983) or from macrophages (Korpela, 1984) can bind to the staphylococcal surface and may effectively trap biotin before it can be assimilated by the bacterium. It thus acts as a non-specific defense mechanism.

It was not feasible, in this study, to investigate a possible fibronectin-binding site on S. aureus but SDS-PAGE followed by immuno- and lectinblotting should be applicable.

Lactoperoxidase cannot pass into the bacterial cell because of its large size (78 Kd). Consequently, only surface-exposed proteins are available and of these, only those with tyrosine or histidine residues (Booth, 1980). The technique has previously been applied to Ps. aeruginosa (Lambert and Booth, 1982) but not S. aureus. The most intensely labelled protein was the 21 Kd band which is presumably located on the surface if it is the clumping factor.

The 52 Kd band was seen in all phenotypes here but was only

visualised in immunoblotting in iron-rich cocci but was recognised by all the heterologous antisera. Thus, it seems that this protein is indeed present as an antigen in the other phenotypes but may not be expressed in sufficient amounts for it to be visualised by immunoblotting. The 48 Kd band was also present in all the phenotypes here but was only visualised in immunoblotting when iron-rich envelope fractions were challenged with homologous antisera. Presumably, it did not stimulate antibody formation due to being present in insufficient amounts in those phenotypes. These findings may also be explained by the differences in size of lactoperoxidase (78 Kd) and IgG (150 Kd). The latter may not be able to aproach and recognise small amounts of antigens. Again, radiolabelling used whole cells whilst immunoblotting was applied to cell envelope fractions which therefore may have a reduced protein content compared to whole cells.

Pr A was not heavily labelled and thus was not as obvious as in the immunoblotting. This is because it is not particularly rich in tyrosine or histidine (Forsgren et al., 1983).

13. The serological response of an endocarditis patient to the causative S. aureus

13.1 SDS-PAGE and immunoblotting of S. aureus Hopewell

SDS-PAGE (Fig 43) and a subsequent laser densitometer scan (not shown) (Ultrascan; LKB Ltd.) showed that there were only small differences eg. at 32 Kd between the iron-rich and iron-poor cocci. Again though, immunoblotting analysis revealed major differences which were not reflected by the profusion of bands seen on the gels.

Although the patient's anti-α-haemolysin titre was not known the serum was likely to have a high antibody content because it was taken

Figure 43

SDS-PAGE of envelope preparations of S. aureus Hopewell (t_d = 48 min) in Fe+ TSB and Fe- TSB.

Lane

1 Fe+ TSB

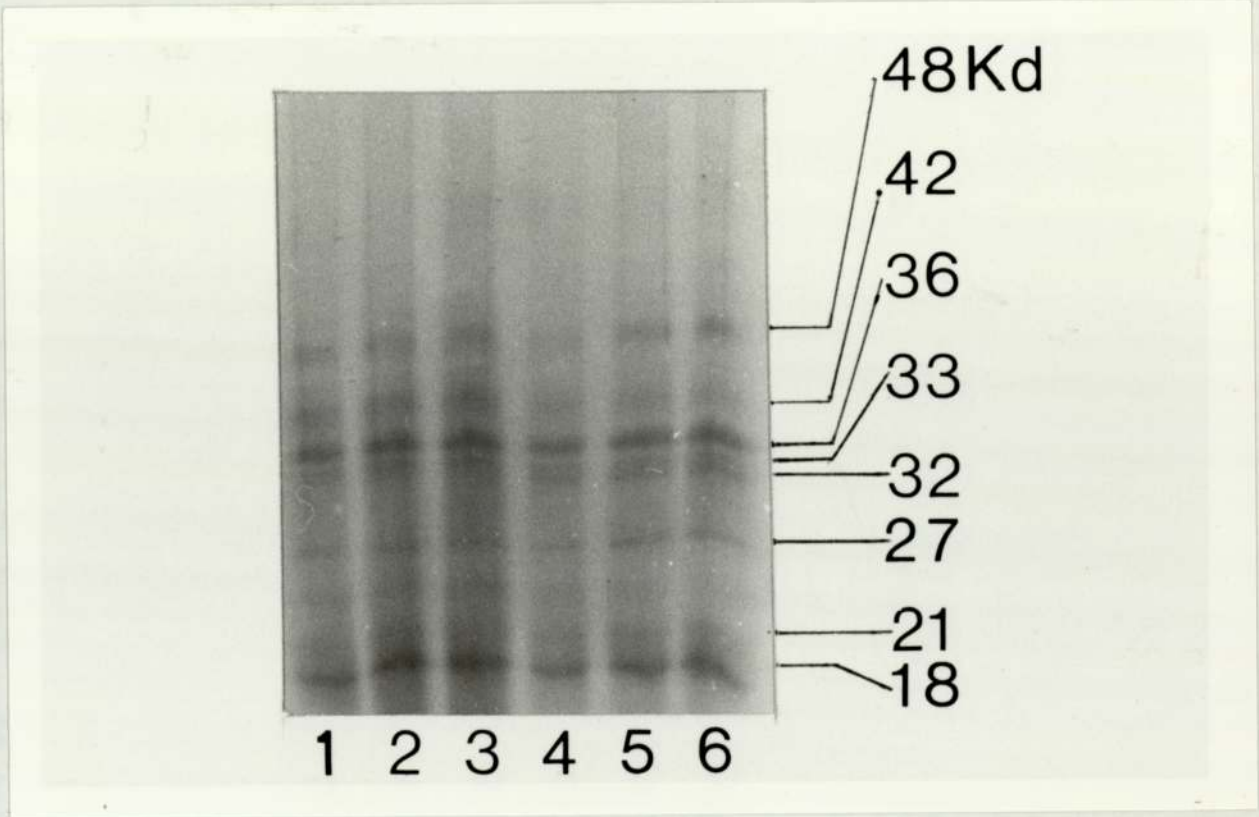
2 Fe+ TSB

3 Fe+ TSB

4 Fe- TSB

5 Fe- TSB

6 Fe- TSB



approximately 3 to 4 weeks into a second episode of S. aureus endocarditis. There were differences in the way this serum recognised the iron-rich (Fig 44 and 45) and iron-poor (Fig 46 and 47) antigens of the infecting strain. The highest and lowest molecular weight bands visualised in the iron-rich cocci were 61 and 18 Kd, whilst they were 76 and 16 Kd in the iron-poor cocci. The 29 and 33 Kd bands were also not visualised in the iron-rich envelope fractions.

The Pr A "probe" bound mainly to two antigens, at 27 and 32 Kd thus emphasising the changeability of the protein's character even between strains. The reaction with the iron-poor preparation showed extra bands associated here at 29 and 33 Kd.

The antiserum raised against iron-rich S. aureus NCTC 6571 recognised a band at 54 Kd in the equivalent S. aureus Hopewell preparation whilst the antiserum raised against iron-poor S. aureus NCTC 6571 recognised this band and an associated band (a doublet) at 55 Kd in the iron-poor S. aureus Hopewell preparation.

Overall, the Hopewell antiserum had an increased immunoblotting reactivity for the iron-poor preparations. This was most noticeable with major antigens present as a doublet at 54 and 55 Kd, one at 42 Kd and a group at between 27 and 33 Kd. The endpoint of this reactivity was not reached on initial blotting due to the serum dilutions used. However, the reactivity against the iron-rich antigen diminished at between 1:4,000 and 1:6,000.

Repeat immunoblotting used a different, overlapping dilution range and incorporated lectin probes and iron-rich and iron-poor S. aureus NCTC 6571 envelope preparations (Fig 45 and 47). The Hopewell antiserum ceased reactivity against the iron-poor preparation of the homologous strain at a 1:12,000 dilution and when reacted at a 1:50 dilution bands developed at 32 Kd in the iron-rich and the iron-poor preparations of S. aureus NCTC 6571.

Figure 44

Differential strip immunoblot of envelope preparation of S. aureus Hopewell (t_d = 48 min) grown in Fe+ TSB.

Lane			
1	Amido black strain for proteins		
2	Probed with Pr A "probe"		
3	"	"	1:50 rabbit-raised antiserum to Fe+ TSB S. aureus NCTC 6571
4	"	"	1:50
5	"	"	1:100
6	"	"	1:200
7	"	"	1:400
8	"	"	1:600
9	"	"	1:800
10	"	"	1:1,000
11	"	"	1:2,000
12	"	"	1:4,000
13	"	"	1:6,000
14	"	"	1:8,000
15	"	"	1:10,000

Hopewell antiserum

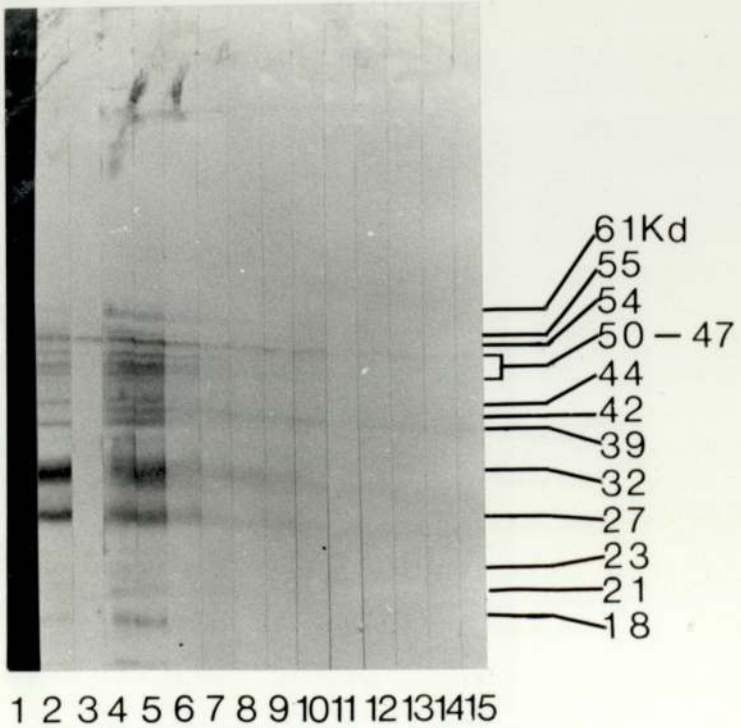


Figure 45

Differential strip immunoblot of envelope preparation of S. aureus Hopewell (t_d = 48 min) grown in Fe+ TSB

Lane		
1	Probed with	1:500
2	" "	1:1,000
3	" "	1:2,000
4	" "	1:3,000
5	" "	1:4,000
6	" "	1:6,000
7	" "	1:8,000
8	" "	1:12,000
9	" "	1:16,000
10	" "	1:32,000

Hopewell antiserum

11	Probed with	Con A-peroxidase
12	" "	SBA-peroxidase
13	" "	APL-peroxidase
14	" "	WGA-peroxidase
15	" "	Avidin-peroxidase
16	Envelope preparation of S. aureus NCTC 6571 grown in Fe+ TSB and probed with 1:50 dilution of Hopewell antiserum	

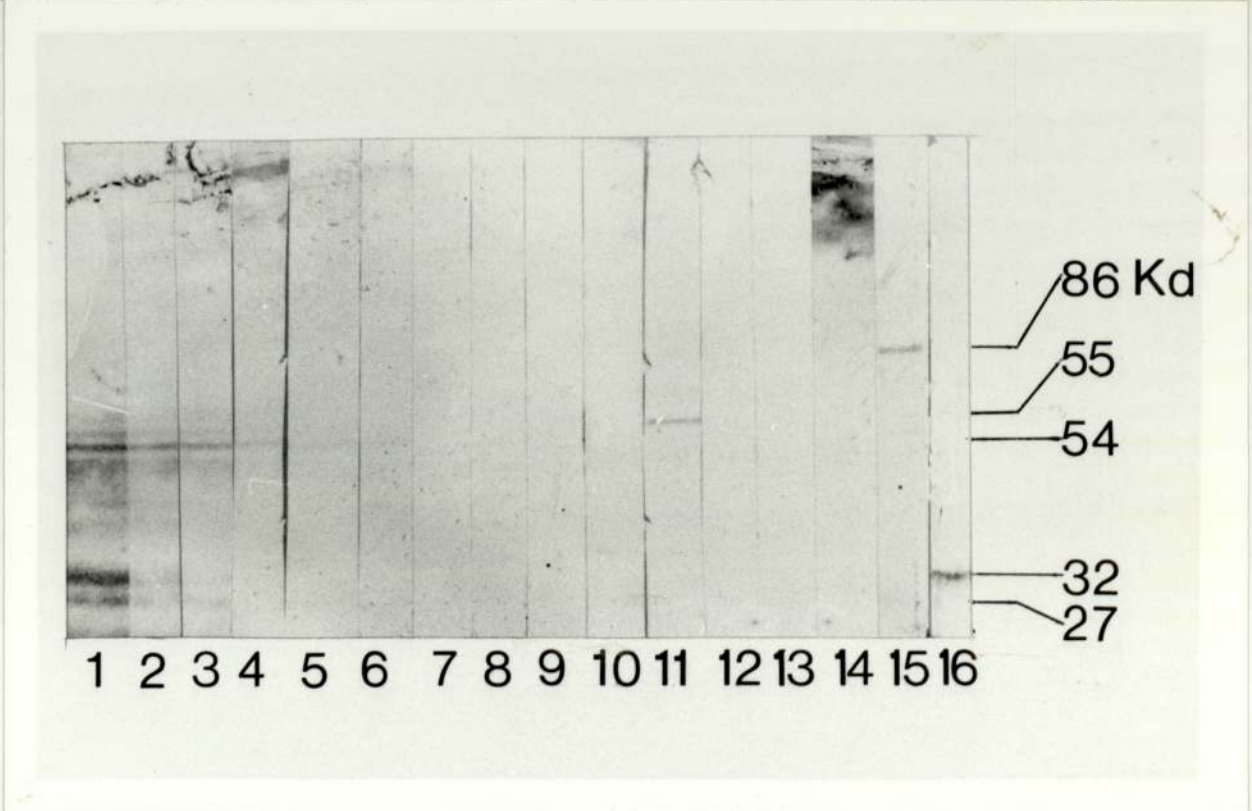


Figure 46

Differential strip immunoblot of envelope preparation of S. aureus Hopewell (t_d = 48 min) grown in Fe- TSB

Lane			
1	Amido black stain for proteins		
2	Probed with Pr A "probe"		
3	"	"	1:50 rabbit raised antiserum to Fe- TSB S. aureus NCTC 6571
4	"	"	1:50
5	"	"	1:100
6	"	"	1:200
7	"	"	1:400
8	"	"	1:600
9	"	"	1:800
10	"	"	1:1,000
11	"	"	1:2,000
12	"	"	1:4,000
13	"	"	1:6,000
14	"	"	1:8,000
15	"	"	1:10,000

Hopewell antiserum

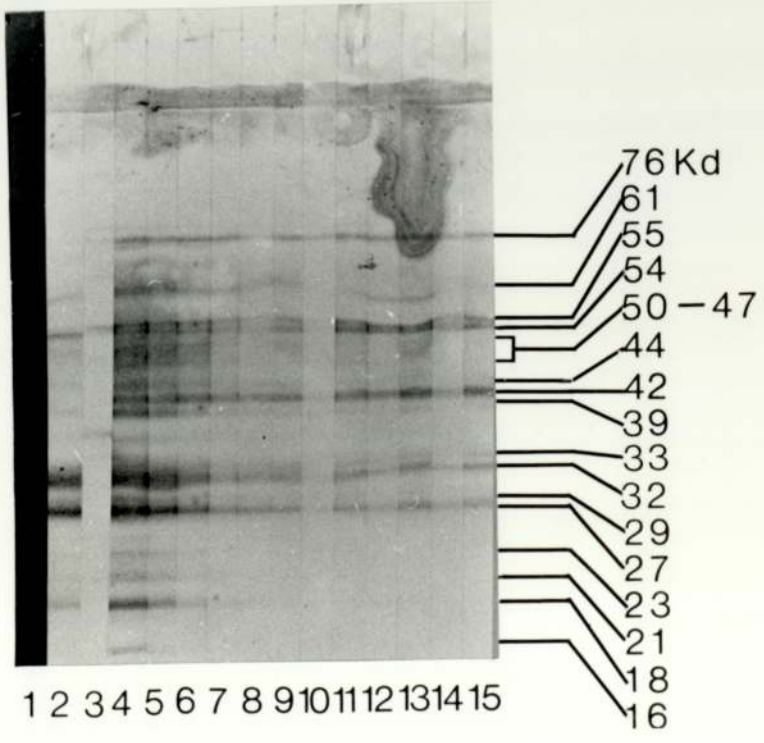


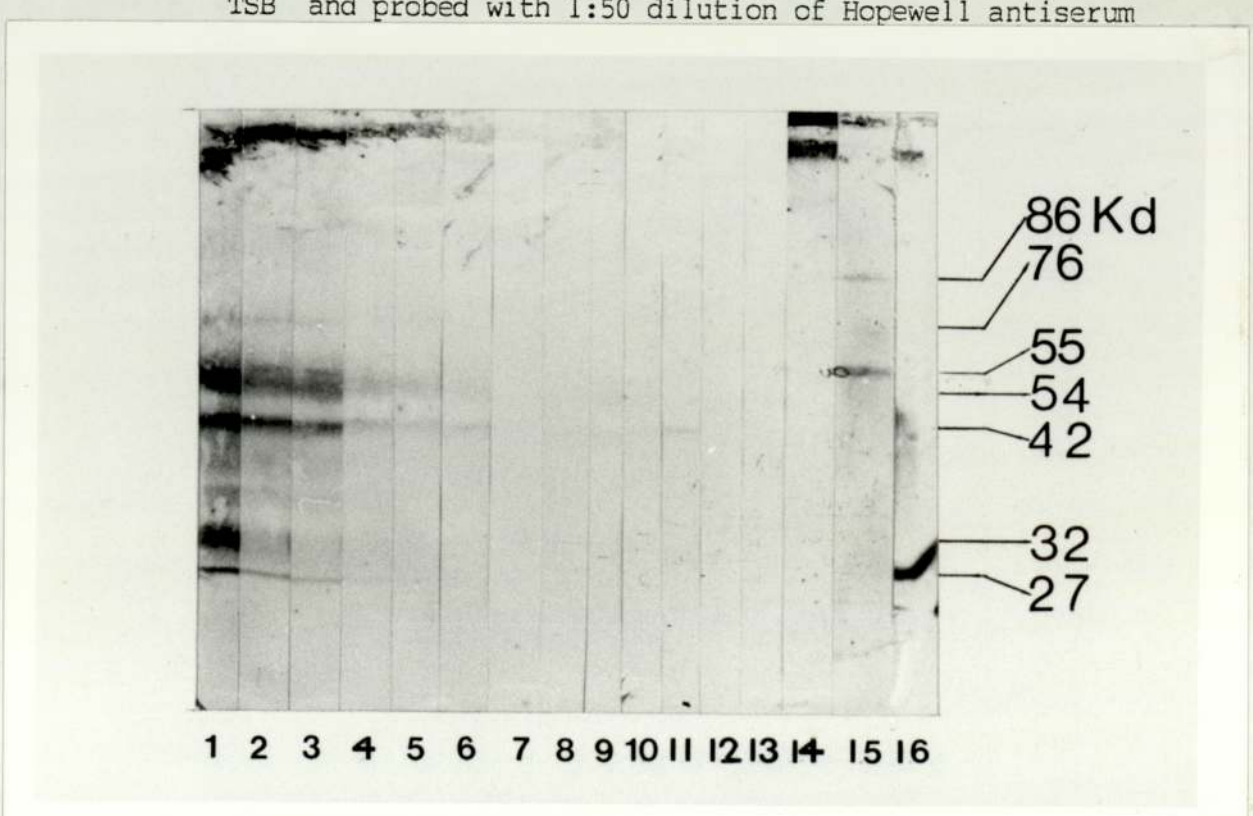
Figure 47

Differential strip immunoblot of envelope preparation of S. aureus Hopewell (t_d = 48 min) grown in Fe- TSB

Lane		
1	Probed with	1:500
2	" "	1:1,000
3	" "	1:2,000
4	" "	1:4,000
5	" "	1:8,000
6	" "	1:12,000
7	" "	1:16,000
8	" "	1:25,000
9	" "	1:32,000
10	" "	1:64,000

Hopewell antiserum

11	Probed with	Con A-peroxidase
12	" "	SBA-peroxidase
13	" "	APL-peroxidase
14	" "	WGA-peroxidase
15	" "	Avidin-peroxidase
16	Envelope preparation of S. aureus NCTC 6571 grown in Fe- TSB and probed with 1:50 dilution of Hopewell antiserum	



Differences between S. aureus Hopewell and S. aureus NCTC 6571 were found. S. aureus Hopewell did not react with APL whilst Con A was bound at two different sites. In the iron-rich and iron-poor preparations it bound at 55 and 42 Kd respectively. Avidin was also bound differently at 86 Kd, and 86 and 55 Kd in iron-rich and iron-poor preparations respectively. Like S. aureus NCTC 6571, S. aureus Hopewell did not react with SBA.

13.2 ELISA for antibody detection to S. aureus Hopewell

The patient's pre-immune serum was not available and so the negative serum control was the pooled, normal human serum (Section 2.2.11). There was thus a big difference in antibody content of the two sera (Fig 48).

Interpretation of ELISA results varies from study to study. Here, the values plotted were the means of duplicate determinations after the mean absorption values for each control well had been deducted. Three points are commonly used to determine the titre of sera. Studies with Gram-positive bacteria have used E_{450} 0.1 (van de Rijn, George, Bouvet and Roberts, 1986) and E_{450} 0.2 (Jacob et al., 1985) whilst elsewhere a 50% decrease in maximum absorption has been used (Shand et al., manuscript submitted). The results derived using all three criteria are presented in Table 9.

13.3 Discussion

This study probably represents the first serological analysis by immunoblotting of an endocarditis-associated S. aureus strain and so it is not known how typical the results are. Unfortunately, it was not possible to work with the organism isolated from in vivo without subculture and so growth in vitro with conditions that might

Figure 48

ELISA of antibody response to envelope preparations of S. aureus Hopewell (t_d = 48 min) grown in Fe+ TSB and Fe- TSB.

X= Fe+ TSB

⊙= Fe- TSB

Solid line = patient's serum; broken line = pooled, non-immune sera

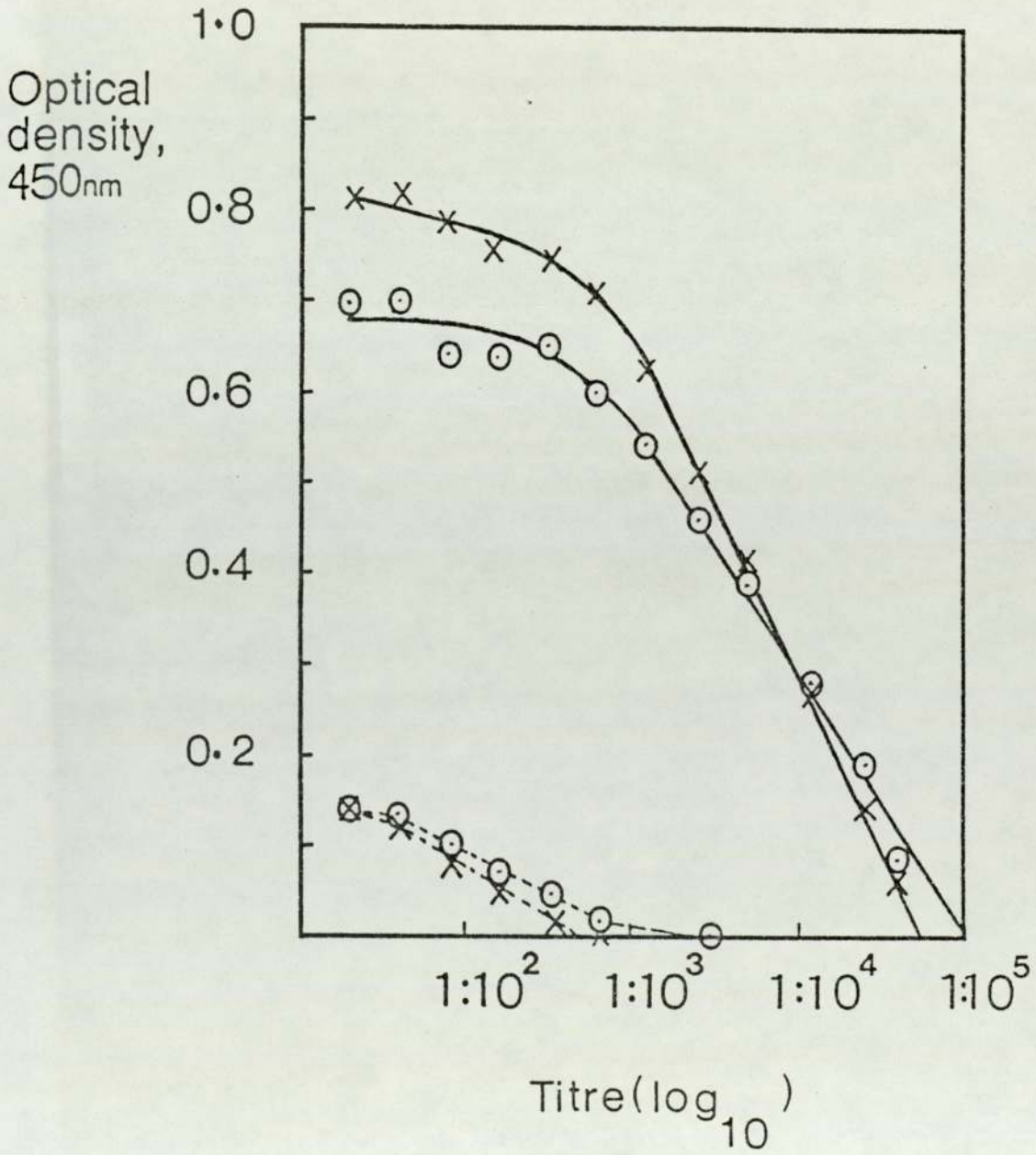


Table 9

ELISA of antibody response to envelope preparations of S. aureus Hopewell (t_d = 48 min) grown in Fe+ TSB and Fe- TSB.

	Titre of serum* against:	
	iron-rich envelope	iron-poor envelope
Criteria:		
E_{450} 0.1	1:30,000 (1:60)	1:50,000 (1:80)
E_{450} 0.2	1:18,000 (----)	1:20,000 (----)
50% of maximum E_{450}	1:4,000 (1:140)	1:5,000 (1:200)

* pooled, normal human serum in brackets

approximate to *in vivo* (Brown and Williams, 1985a) were used. The patient's antibodies reacted best with the iron-poor envelope preparations suggesting, indirectly, that *S. aureus* Hopewell grows *in vivo* under iron restriction.

Common Staphylococcal antigens of molecular weights 55, 54 and 32 Kd were detected when antisera to *S. aureus* NCTC 6571 was reacted with *S. aureus* Hopewell preparations and patient's serum was reacted with *S. aureus* NCTC 6571 preparations.

Two of these antigens appeared to be Pr A but its molecular weights were very different from that of *S. aureus* NCTC 6571 (Section 12.2). An antigen also occurred at 21 Kd in *S. aureus* Hopewell as in *S. aureus* NCTC 6571 but reactions were weak probably for the reasons discussed earlier (Section 12.4). Neither of the antisera to *S. aureus* NCTC 6571 recognised the 21 Kd band in *S. aureus* Hopewell. If this antigen is clumping factor, then it implies a strain-specific immune response would be elicited in protective studies.

Phage lysates of *S. aureus* serotypes I and II yielded type specific antigens with molecular weights between 40 and 50 Kd. Apparently, antigens between 6 and 20 Kd had neither major serological specificity nor mitogenic activity. The major part of the latter was exhibited by the 40 to 50 Kd and 25 to 30 Kd fractions (Ganfield, Gray and Esber, 1985).

Immunoblotting does not feature as a routine assay especially in British microbiology. Its potential has been emphasised here and it could be that modifications, eg. dot immunoblotting (Towbin and Gordon, 1974) will allow its widespread use in staphylococcal serology.

The advantage of immunoblotting is its ability to show which bacterial antigens are most strongly recognised by the host. This may explain the phenomenon of elevated titres against one antigen but not

another in the same serum (Oeding et al., 1983). It partly reflects that S. aureus strains contain the same major antigens whose quantities may be strain-variable, as well as their type-specific agglutinogens. The experiment emphasises the value of preserving clinical isolates for antigenic analysis with patient's serum.

The ELISA method here differed from published studies in that Pr A-peroxidase rather than a second antibody was used to locate patient's bound antibody. Two advantages were apparent. There was no interference caused by a second antibody binding to the antigen as well as to the first antibody. Also, it allowed a direct comparison with the immunoblot.

Envelope preparations were used, again to provide more direct comparisons with immunoblotting, with satisfactory results. But it is realised that at least for S. aureus, purified antigens no longer offer an advantage over whole cell preparations (Christensson et al., 1985; Espersen and Hedström, 1983) or whole cells (Jarløv et al., 1985). For S. epidermidis, whole cell sonicates give better discrimination between negative and positive sera than whole cells (Espersen, Wheat, Bemis and White, 1986).

The antigen concentrations used satisfied criteria for the best differentiation (Christensson, Espersen, Hedström and Kronvall, 1985; Jacob et al., 1985; Jarløv et al., 1985) although small fluctuations in antigen concentration are apparently acceptable (Christensson et al., 1985). Reproducibility between the cobalt-irradiated plates used was good here as elsewhere (Christensson et al., 1985).

ELISA is obviously more sensitive than immunoblotting as the former detects changes in optical density. However, the same trends emerged, and once again evidence was obtained that implied that S. aureus in vivo could be growing under iron restraint, because the patient's serum recognised the antigen from iron-poor S. aureus

Hopewell more efficiently. Such recognition may explain the more effective opsonisation of Fe- TSB cultures (Sections 4.1 and 5.2).

The development of sensitive and specific assays to detect antibodies to S. aureus has been pursued for years. Until very recently, ELISA did suffer from lack of standardisation especially over the choice of antigen. Whole cells can now be used but if the synthetic pentaglycine promoted as the most specific antigen in serology (Oeding et al., 1983) is made commercially available the test should become even easier. It is also encouraging that the cobalt-irradiated plates have been loaded with antigen and stored for up to 3 months at $4^{o}C$ without affecting the assay (Jarløv et al., 1985).

CONCLUSION

Despite high host immunity to Staphylococcus aureus due to sub-clinical exposure, for unknown reasons there is a low host resistance to re-infection and S. aureus can persist in vivo although it is not a classic, intracellular pathogen. It must somehow circumvent host defences (Elek and Conen, 1957) and its surface properties may feature here. However, the Staphylococcal surface in vivo is not well characterised and in vitro simulation of in vivo conditions must be rigorous to ensure bacteria that are physiologically appropriate (Brown and Williams, 1985a and b; Dalhoff, 1985). In vivo, S. aureus often replicates relatively slowly and may encounter drugs at sub-optimal concentrations. Some bacteria also grow in humans under iron-restricted conditions (Anwar et al., 1984 and 1985; Brown et al., 1984; Lam et al., 1984; Shand et al., 1985) however, the nutritional status of S. aureus in vivo is unknown and it is not appreciated how this affects surface properties. One such, surface hydrophobicity (SH), is implicated in the adherence of bacteria to host tissues including phagocytes, and this interaction increases as SH increases (Magnusson et al., 1985; Stendahl, 1983; van Oss, 1978). Sub-MIC's of penicillin G (Wadström et al., 1981) and rapid growth (Malmqvist, 1983; Miörner et al., 1982; Wadström et al., 1985) increase SH whereas it can be repressed or enhanced by different media (Ljungh et al., 1985; Wadström et al., 1985).

Here relatively slow growth (t_d = 48 min) in Fe- TSB of batch cultures of S. aureus NCTC 6571 produced a phenotype that at a ratio of 10-20 cocci: 1 neutrophil resisted human whole blood killing (WBK).

This was directly related to trends in neutrophil association assessed by May-Grünwald-Giemsa staining and non-opsonised and opsonised chemiluminescence (CL), and SH measured by non-opsonised contact angles. This phenotype had a low SH and crucially, low

neutrophil association values. This indicated that under the above conditions, SH interactions can override opsonic interactions and could partly explain the persistence of S. aureus in vivo. 1/16 MIC of penicillin G and/or iron excess increased SH, neutrophil-cocci interaction and subsequent WBK. A fast growth rate (t_d = 24 min) greatly increased SH, neutrophil association and WBK so that neither penicillin G nor iron status were significant.

Further studies are needed with the slow-growing, iron-poor coccus to determine whether antibody or complement binding sites, C3b deposition rate or chemotaxis (Gemmell and O'Dowd, 1983; Milatovic et al., 1983) are altered and whether the contact event between coccus and neutrophil is more scarce, weaker or shorter. Surface phagocytosis (Lee et al., 1983; Vandenbroucke-Grauls et al., 1985) would bring the in vitro events more into line with in vivo. The dose dependence of the phenomenon in relation to amount of penicillin G, and in CL to the serum concentration, and potential differences such as susceptibility to neutrophil killing (Root et al., 1981) and degradation could then be investigated.

Protein A (Pr A) contributes to SH (Jonsson and Wadström, 1983) and the effect of the fast growth rate was explained as it drastically increased cell-associated Pr A. Iron deprivation decreased Pr A in all phenotypes especially the fast-grown ones, however it significantly decreased SH in only the slow-grown phenotypes. There must therefore be a critical concentration of Pr A above which contact angles cannot discriminate between the fast-grown phenotypes. The same applies to the penicillin G-induced decrease in Pr A but as penicillin G increased SH other structures, possibly MTA, are involved.

Slow-grown cocci, i.e. those with a more physiological growth rate, adhered better to PVC catheters. Overall, penicillin G and iron-deprivation reduced this and further studies are needed to determine the extent of the hydrophobic and non-hydrophobic interactions and the structures involved.

SDS-PAGE, immunoblotting and ELISA with normal and hyperimmune sera showed better recognition of iron-poor envelope preparations implying that S. aureus is iron-deprived in vivo. Immunisation of rabbits with killed cells may give a different picture to the infected state as the physiological stress is not the same and so more clinically orientated work is needed. Penicillin G and iron-deprivation simplified the antigenic profiles but it is not known if such changes occur in vivo. There was some evidence for strain-specific immunogenicity which could also partly explain the re-infection phenomenon. Immunoblotting could if developed, become a rapid serodiagnostic test.

S. aureus has not to date, been cultured and tested elsewhere under the above conditions and many of the results currently stand alone. Also, the in vivo status of S. aureus, particularly its surface properties such as Pr A production and antigenicity, is unclear. Finally, the actual concentrations of sub-optimal doses of drugs at sites of infection and their effects upon S. aureus need more investigation.

REFERENCES

Aasjord, P. and Haaheim, C.R. (1985). Antibodies to lipoteichoic acid from Staphylococcus aureus: specificity of murine monoclonal and human antibodies. Acta Pathologica Microbiologica Immunologica Scandinavica. Section C. 93, 245-250.

Abramson, C. (1983). The serolodiagnostic aspects of Staphylococcus aureus infections. Laboratory Management, 21, 48-55.

Absolom, D.R., Lamberti, F.V., Policova, Z., Zingg, W., van Oss, C.J. and Neumann, A.W. (1983). Surface thermodynamics of bacterial adhesion. Applied and Environmental Microbiology, 46, 90-97.

Adlam, C. and Easmon, C.S.F. (1983). Immunity and hypersensitivity to Staphylococcal infection. In: Staphylococci and Staphylococcal Infections, Vol 1. Clinical and Epidemiological Aspects. Eds. Easmon, C.S.F. and Adlam, C. Pub. Academic Press, London, 275-323.

Adlam, C., Pearce, J.H., and Smith, M. (1970a). Virulence mechanisms of Staphylococci grown in vivo and in vitro. Journal of Medical Microbiology, 3, 147-155.

Adlam, C., Pearce, J.H. and Smith, H. (1970b). The interaction of Staphylococci grown in vivo and in vitro with polymorphonuclear leucocytes. Journal of Medical Microbiology, 3, 157-163.

Adlam, C., Ward, P.D., McCartney, A.C., Arbuthnott, J.P. and Thornley, C.M. (1977). Effect of immunisation with highly purified alpha and beta toxins on Staphylococcus aureus mastitis in rabbits. Infection and Immunity, 17, 250-256.

Agarwal, D.S. (1967). Subcutaneous Staphylococcal infection in mice II. The inflammatory response to different strains of Staphylococci and Micrococci. British Journal of Experimental Pathology, 48, 468-482

Aisen, P. (1977). Some physiochemical aspects of iron metabolism. In: Iron Metabolism. Ciba Foundation Symposium. Pub. Elsevier/Excerpta Medica/North Holland. Amsterdam, 1-17.

Aisen, P. and Listowski, I. (1980). Iron transport and storage proteins. Annual Reviews of Biochemistry, 49, 357-393.

Aitchison, E.J., Lambert, P.A. and Farrell, I.D. (1986). Antigenic composition of an endocarditis - associated isolate of Streptococcus faecalis and identification of its glycoprotein antigens by ligand blotting with lectins. Journal of Medical Microbiology, 21, 161-167.

Al-Hadithy, H., Addison, I.E. and Goldstone, A.H. (1981). A rapid whole blood technique for assessment of neutrophil phagocytosis and killing. Journal of Clinical and Laboratory Haematology, 3, 85-88

Alkan, M.L. and Beachey, E.H. (1978). Excretion of lipoteichoic acid by Group A Streptococci. Journal of Clinical Investigation, 61, 671-677.

Allen, R.C. (1977). Evaluation of serum opsonic capacity by quantitating the initial chemiluminescent response from phagocytosing polymorphonuclear leukocytes. Infection and Immunity, 15, 828-833.

Aly, R., Shinefield, H.R., and Maibach, H.I. (1980). Adherence of Staphylococcus aureus to infant nasal mucosal cells. American Journal of Diseases in Childhood, 134, 522-523.

Ambruso, D.R., Sasada, M., Nishiyama, H., Kubo, A., Komiyama, A. and Allen, R.H. (1984). Defective bactericidal activity and absence of specific granules in neutrophils from a patient with recurrent bacterial infections. Journal of Clinical Immunology, 4, 23-30.

Anderson, J.C. (1983). Veterinary aspects of Staphylococci. In: Staphylococci and Staphylococcal Infections, Vol 1. Eds. Easmon, C.S.F. and Adlam, C. Pub. Academic Press, London, 194-241.

Anderson, J.C. and Wilson, C.D. (1981). Encapsulated, coagulase-negative strain of Staphylococcus simulans. Infection and Immunity, 33, 304-308.

Anderson, J.C. and Williams, M.R. (1985). The contribution of a capsule to survival of Staphylococci within bovine neutrophils. Journal of Medical Microbiology, 20, 317-323.

Anwar, H., Brown, M.R.W., Day, A. and Weller, P.H. (1984). Outer membrane antigens of mucoid Pseudomonas aeruginosa isolated directly from the sputum of a cystic fibrosis patient. FEMS Microbiology Letters, 24, 235-239.

Anwar, H., Brown, M.R.W. and Lambert, P.A. (1983). Effect of nutrient depletion on sensitivity of Pseudomonas cepacia to phagocytosis and serum bactericidal activity at different temperatures. Journal of General Microbiology, 129, 2021-2027.

Anwar, H., Shand, G.H., Ward, K.H., Brown, M.R.W., Alpar, K.E. and Gowar, J. (1985). Antibody response to acute Pseudomonas aeruginosa infection in a burn wound. FEMS Microbiology Letters, 29, 225-230.

Arbeit, R.D., Karakawa, W.W., Vann, W.F. and Robbins, J.B. (1984). Predominance of two newly described capsular polysaccharide types among clinical isolates of Staphylococcus aureus. Diagnostic Microbiology and Infectious Disease, 2, 85-91.

Arbuthnott, J.P. (1981). Membrane-damaging toxins in relation to interference with host defence. In: Microbial Perturbation of Host Defences. Eds. O'Grady, F. and Smith, H. Pub. Academic Press. London, 97-120.

Archibald, A.R. (1974). The structure, biosynthesis and function of teichoic acids. Advances in Microbial Physiology, 10, 53-95.

Archibald, A.R. and Heckels, J.E. (1975). Alterations in the composition and bacteriophage-binding properties of walls of Staphylococcus aureus H grown in continuous culture in simple

defined media. Biochemica et Biophysica Acta, 406, 60-67.

Arvidson, S.O. (1983). Extracellular enzymes from Staphylococcus aureus. In: Staphylococci and Staphylococcal Infections, Vol 1. Eds. Easmon, C.S.F. and Adlam, C. Pub. Academic Press. London. 745-808.

Asheshov, E.H. (1966). Chromosomal location of the genetic elements controlling penicillinase production in a strain of Staphylococcus aureus. Nature (London), 210, 804-806.

Atkinson, B.A. and Amaral, L. (1982). Sublethal concentrations of antibiotics, effects on bacteria and the immune system. CRC Critical Reviews in Microbiology, 9, 101-138.

Ausobsky, J.R., Scuitto, M., Trachtenberg, L.S. and Polk, Jr., H.C. (1984). The role of muramyl dipeptide in the therapy of established experimental bacterial infection. British Journal of Experimental Pathology, 65, 1-9.

Babior, B.M. (1982). The Role of active oxygen in microbial killing by phagocytes. In: Pathology of Oxygen. Ed. Autor, A.P. Pub. Academic Press, London. 117-143.

Babior, B.M. (1984). The respiratory burst of phagocytes. Journal of Clinical Investigation, 73, 599-601.

Babior, B.M. and Cohen, M.C. (1981). Leucocyte function. In: Methods in Haematology, Vol 3. Ed. Cline, M.J. Pub. Churchill Livingstone. Edinburgh.

Babu, U.M. and Zeiger, A.R. (1983). Soluble peptidoglycan from Staphylococcus aureus is a murine B-lymphocyte mitogen. Infection and Immunity, 42, 1013-1016.

Baddiley, J. (1972). Teichoic acids in cell walls and membranes of bacteria. Essays in Biochemistry, 8, 35-77.

Bamberger, D.M., Gerding, D.N., Bettin, K.M., Elson, M.K. and Forstrom, L.A. (1985). Relation between in vivo abscess localisation and in vitro migration and adherence of neutrophils. Journal of Infectious Diseases, 152, 903-912.

Barber, M. (1962). Coagulase-positive Staphylococci resistant to benzylpenicillin, methicillin and other penicillins. In: Resistance of Bacteria to the Penicillins. Eds: de Renck, A.V.S. and Cameron, M.P. Pub: Little, Brown, Boston, USA.

Barrett, S.P. (1985). Protein-mediated adhesion of Staphylococcus aureus to silicone implant polymer. Journal of Medical Microbiology, 20, 249-253.

Bass, D.A., Olbrantz, P., Szejda, P., Seeds, M.C. and McCall, C. (1986). Subpopulations of neutrophils with increased oxidative product formation in blood of patients with infection. Journal of Immunology, 136, 860-866.

Bassøe, C.-F. and Solberg, C.O. (1984). Phagocytosis of Staphylococcus aureus by human leukocytes: quantitation by a flow cytometric and a microbiological method. Acta Pathologica Microbiologica Immunologica Scandinavica. Section C, 92, 43-50.

Batteiger, B., Newhall, W.J. and Jones, R.B. (1982). The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. Journal of Immunological Methods, 55, 397-407.

Batish, V.K., Chander, H., Zumdegni, K.C., Singh, R.S. and Bhatia, K.L. (1984). Inhibition activity of lactoferrin against toxigenic Staphylococcus aureus. Archives Lebensmitt Hygiene, 35, 9-11.

Bayston, R. (1984). A model of catheter colonisation in vitro and its relationship to clinical catheter infections. Journal of Infection, 9, 271-276.

Beachey, E.H. (1981). Bacterial adherence: Adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. Journal of Infectious Diseases, 143, 325-345.

Beachey, E.H., Nealon, T.J., Courtney, H.S. and Simpson, W.A. (1985). Effects of antibiotics on Streptococcal adherence: release of fibronectin-lipoteichoic acid complexes from Group A Streptococci by sublethal concentrations of penicillin. In: The Influence of Antibiotics on the Host-Parasite Relationship. II. Eds. Adam, D., Hahn, H. and Opferkuch, W. Pub: Springer-Verlag. Berlin. 203-208.

Beaman, L. and Beaman, B.L. (1984). The role of oxygen and its derivatives in microbial pathogenesis and host defense. Annual Reviews of Microbiology, 38, 27-48.

Beining, P.R. and Kennedy, E.R. (1963). Characteristics of a strain of Staphylococcus aureus grown in vivo and in vitro. Journal of Bacteriology, 85, 732-741.

Bergeron, R.J., Elliott, G.T., Kline, S.J., Ramphal, R. and St. James III, L. (1983). Bacteriostatic and fungostatic action of catecholamide iron chelators. Antimicrobial Agents and Chemotherapy, 24, 725-730.

Beumier, D.L. Caldwell, M.A. and Holbrin, B.E. (1984). Inflammation triggers hypoferremia and de novo synthesis of serum transferrin and ceruloplasmin in mice. Infection and Immunity, 46, 489-494.

Beveridge, T.J. (1980). Bacterial structure and its implications in the mechanisms of infection: a short review. Canadian Journal of Microbiology, 26, 643-653.

Bhakdi, S. and Muhly, M. (1985a). Decomplementation antigen, a possible determination of Staphylococcal pathogenicity. Infection and Immunity, 47, 41-46.

Bhakdi, S. and Muhly, M. (1985b). Isolation and partial characterisation of Staphylococcal decomplementation antigen. Infection and Immunity, 47, 47-51.

Bibel, D.J., Aly, R., Shinefield, H.R. and Maibach, H.I. (1983). The Staphylococcus aureus receptor for fibronectin. Journal of Investigative Dermatology, 80, 494-496.

Bibel, D.J., Aly, R., Shinefield, H.R., Maibach, H.I. and Strauss, W.G. (1982). Importance of the keratinised epithelial cell in bacterial adherence. Journal of Investigative Dermatology, 79, 250-253.

Bierbaum, G. and Sahl, H.G. (1985). Induction of autolysis of Staphylococci by the basic peptide antibiotics Pep 5 and nisin and their influence on the activity of autolytic enzymes. Archives of Microbioloby, 141, 249-254.

Biorad Laboratories Product Information Sheet 2020 (1981).

Björk, I., Petersson, B.-A. and Sjöquist, J. (1972). Some physiochemical properties of Protein A from Staphylococcus aureus. European Journal of Biochemistry, 29, 579-584.

Björksten, B., Peterson, P.K., Verhoef, J. and Quie, P.G. (1977). Limiting factors in bacterial phagocytosis by human polymorphonuclear leucocytes. Acta Pathologica Microbiologica Scandinavica. Section C, 85, 345-349.

Blobel, H. and Brückler, J. (1984). Staphylococcal diseases in animals. In: Staphylokokken und Staphylokokken-Erkrankungen. Ed: Myer, W. Pub: Gustav Fischer Verlag. Jena. 405-418.

Blumberg, P.M. and Strominger, J.L. (1974). Interaction of penicillin with the bacterial cell: penicillin-binding proteins and penicillin-sensitive enzymes. Bacteriological Reviews, 38, 291-335.

Blümel, P., Uecker, W. and Giesbrecht, P. (1978). Turnover of cell wall in Staphylococcus aureus. Abstracts of the XIIth International Congress of Microbiology, Munich, Abstract A19.

Blümel, P., Reinicke, B., Lahav, M. and Giesbrecht, P. (1983). Cell wall degradation of Staphylococcus aureus by autolysins and lysozyme. In: The Target of Penicillin. Eds. Hakenbek, R., Holje, J.V. and Labischinski, H. Pub. Walter de Gruyter and Co. Berlin, 323-328.

Boghossian, S.H., Wright, G. and Segal, A.W. (1983). The kinetic measurement of phagocyte function in whole blood. Journal of Immunological Methods, 60, 125-140.

Booth, B.R. (1980). Cell surface proteins of Escherichia coli. Biochemical and Biophysical Research Communications, 94, 1029-1036.

Boylan, R.J., Mendelson, N.H., Brookes, D. and Young, F.E. (1972). Regulation of the bacterial cell wall: analysis of a mutant of Bacillus subtilis defective in biosynthesis of teichoic acid. Journal of Bacteriology, 110, 281-290.

Brown, D.F.J. and Reynolds, P.E. (1980). Intrinsic resistance to β-lactam antibiotics in Staphylococcus aureus. FEBS Letters, 122, 275-278.

Brown, M.R.W., Anwar, H. and Lambert, P.A. (1984). Evidence that mucoid Pseudomonas aeruginosa in the cystic fibrosis lung grows under iron- restricted conditions. FEMS Microbiological Letters, 21, 113-117.

Brown, M.R.W. and Williams, P. (1985a). The influence of environment on envelope properties affecting survival of bacteria in infections. Annual Reviews of Microbiology, 39, 527-556.

Brown, M.R.W. and Williams, P. (1985b). Influence of substrate limitation and growth phase on sensitivity to antimicrobial agents. Journal of Antimicrobial Chemotherapy, 15, Supplement A, 7-14.

Brown, W.C., Fraser, D.K. and Young, F.E. (1970). Problems in purification of a Bacillus subtilis autolytic enzyme caused by association with teichoic acid. Biochemica et Biophysica Acta, 198, 308-315.

Brückler, J. and Blobel, H. (1984). Vaccination against Staphylococcal infections of animals. In: Staphylokokken und Staphylokokken-Erkrankungen. Ed. Meyer, W. Pub. Gustav Fischer Verlag. Jena, 503-508.

Bullen, J.J. (1981) The significance of iron in infection. Review of Infectious Diseases, 3, 1127-1138.

Bullen, J.J. and Armstrong, J.A. (1979). The role of lactoferrin in the bactericidal function of polymorphonuclear leucocytes. Immunology, 36, 781-789.

Burghaus, P., Johannsen, L., Naumann, D., Labischinski, H., Bradaczek, H. and Giesbrecht, P. (1983). The influence of different antibiotics on the degree of O-acetylation of Staphylococcal cells walls. In: The Target of Penicillin. Eds. Hakenbek, R., Holje, J.V., and Labischinski, H. Pub. Walter de Gruyter and Co. Berlin, 319-322.

Busscher, H.J., Weerkamp, A.H., van der Mei, H.C., van Pelt, A.W.J., de Jong, H.P. and Arends, J. (1984). Measurement of the surface free energy of bacterial cell surfaces and its relevance for adhesion. Applied and Environmental Microbiology, 48, 980-983.

Canepari, P., Del Mar Lleò, M., Cornaglia, C. Fontana, R. and Satta, G. (1986). In Streptococcus faecium penicillin-binding protein 5 alone is sufficient for growth at sub-maximal but not at maximal rate. Journal of General Microbiology, 132, 625-631.

Canepari, P., Varaldo, P.E., Fontana, R. and Satta, G. (1985). Different staphylococcal species contain various numbers of penicillin-binding proteins ranging from four (Staphylococcus aureus) to only one (Staphylococcus hyicus). Journal of Bacteriology, 163, 796-798.

Carruthers, M.M. and Kabat, W.J., (1983). Mediation of Staphylococcal adherence to mucosal cells by lipoteichoic acid. Infection and Immunity, 40, 444-446.

Chandra, R.K. (1973). Reduced bactericidal capacity of polymorphs in iron deficiency. Archives of Diseases in Childhood, 48, 864-866.

Christensen, G.D. and Courtney, V.A. (1985). Lipoteichoic acid inhibits adherence of coagulase-negative Staphylococci to smooth surfaces. Abstracts of the Annual Meeting of the American Society for Microbiology (1985). Abstract B43, 25.

Christensson, B., Espersen, F., Hedström, S.A. and Kronvall, G. (1985). Serological assays against Staphylococcus aureus peptidoglycan, crude Staphylococcal antigen and Staphylolysin in the diagnosis of serious Staphylococcus aureus infections. Scandinavian Journal of Infectious Diseases, 17, 47-53.

Clawson, C.C. and Repine, J.E. (1976). Quantitation of maximal bactericidal capability in human neutrophils. Journal of Laboratory and Clinical Medicine, 88, 316-327.

Cleveland, R.F., Wicken, A.J., Daneo-Moore, L. and Shockman, G.D. (1976). Inhibition of wall autolysis in Streptococcus faecalis by lipoteichoic acids and lipids. Journal of Bacteriology, 126, 192-197.

Cluff, L.E. (1965). Cellular reactions in pathogenesis of Staphylococcal infection. Annals of the New York Academy of Sciences, 128, 214-230.

Cockayne, A., Penn, C.W. and Bailey, M.J. (1986). Surface properties of Treponema pallidum in relation to phagocytosis by human polymorphonuclear leucocytes in vitro. Journal of General Microbiology, 132, 133-141.

Cole, G.W. and Silverberg, N.L. (1986). The adherence of Staphylococcus aureus to human corneocytes. Archives of Dermatology, 122, 166-169.

Coley, J., Duckworth, M. and Baddiley, J. (1975). Extraction and purification of lipoteichoic acids from Gram-positive bacteria. Carbohydrate Research, 40, 41-52.

Coley, J., Tarelli, E., Archibald, A.R. and Baddiley, J. (1978). The linkage between teichoic acid and peptidoglycan in bacterial cell walls. FEBS Letters, 88, 1-9.

Cooke, E.M. and Marples, R.R. (1985a). Outbreaks of nosocomial infection due to Staphylococcus aureus. Journal of Hospital Infection, 6, 241-244.

Cooke, E.M. and Marples, R.R. (1985b). Outbreaks of Staphylococcal infection. PHLS Microbiology Digest, 2, 62-64.

Costerton, J.W. and Marrie, T.J. (1983). The role of the bacterial glycocalyx in resistance to antimicrobial agents. In: Medical Microbiology, Vol 3. Eds. Easmon, C.S.F., Jeljaszewicz, J., Brown, M.R.W., Lambert, P.A. Pub. Academic Press, London, 63-85.

Costerton, J.W., Irvin, R.T. and Cheng, K.-J. (1981). The bacterial glycocalyx in nature and disease. Annual Reviews of Microbiology, 35, 299-324.

Cowan, S.T. and Steel, K.J. (1974). Identification of Medical Bacteria, 2nd Edition. Pub. Cambridge University Press.

Cowart, R.E. and Foster, B.G. (1985). Differential effects of iron on the growth of Listeria monocytogenes: minimum requirements and mechanism of acquisition. Journal of Infectious Diseases, 151, 721-730.

Cowart, R.E., Marquadt, M.P. and Foster, B.C. (1980). The removal of iron and other trace elements from a complex bacteriological medium. Microbios Letters, 13, 117-122.

Craven, N. and Anderson, J.C. (1982). pH-dependent penicillin tolerance may protect intraleukocytic Staphylococcus aureus from killing by cloxacillin. Antimicrobial Agents and Chemotherapy, 21, 618-621.

Craven, N., Williams, M.R. and Anderson, J.C. (1982). Enhanced killing of penicillin-treated Staphylococcus aureus by host defences: effects of amoxycillin, cloxacillin and nafcillin in vitro and in experimental mastitis. Comparative Immunology and Microbiology of Infectious Disease, 5, 447-456.

Craido, M.T., Ferreirós, C.M. and Sainz, V. (1985). Adherence and hydrophobicity in Neissera meningitidis and their relationship with surface charge. Medical Microbiology and Immunology, 174, 151-156.

Crone, P.B. (1948). The counting of surface colonies of bacteria. Journal of Hygiene, Cambridge, 46, 426-430.

Cybulska, A.J. and Jeljaszewicz, J. (1966). Bacteriostatic activity of serum against Staphylococci. Journal of Bacteriology, 91, 953-962.

Dacie, J.V. (1950). Practical Haematology. Pub. Churchill Livingstone. Edinburgh.

Dalhoff, A. (1985). Differences between bacteria grown in vitro and in vivo. Journal of Antimicrobial Chemotherapy, 15, Suppl. A., 175-195.

Diem, K. (1962). (Editor) Documenta Geigy Scientific Tables, 6th Edition. Pub. Geigy Pharmaceutical Co. Ltd., Manchester, UK.

Dobson, B.C. and Archibald, A.R. (1978). Effect of specific growth limitations on cell wall composition of Staphylococcus aureus H. Archives of Microbiology, 119, 285-301.

Domingue, G.J. and Schlegel, J.U. (1977). Novel bacterial structures in human blood: cultural isolation. Infection and Immunity, 15, 621-627.

Donnelly, T.M. and Stark, D.M. (1985). Susceptibility of laboratory rats, hamsters and mice to wound infection with Staphylococcus aureus. American Journal of Veterinary Research, 46, 2634-2638.

Dossett, J.H., Kronvall, G., Williams Jnr., R.C. and Quie, P.G. (1969). Antiphagocytic effects of Staphylococcal Protein A. Journal of Immunology, 103, 1405-1410.

Downie, P.A. (1979). Cash's Textbook of Medical Conditions for Physiotherapists. 6th Edition. Pub: Faber and Faber. London.

Duckworth, M., Archibald, A.R. and Baddiley, J. (1975). Lipoteichoic acid and lipoteichoic acid carrier in Staphylococcus aureus H. FEBS Letters, 53, 176-179.

Duncan, J.L. and Cho, G.J. (1971). Production of Staphylococcal alpha-toxin. 1. Relationship between cell growth and toxin formation. Infection and Immunity, 4, 456-461.

Dunkle, L.M., Blair, L.L. and Fortune, K.P. (1986). Transformation of a plasmid encoding an adhesin of Staphylococcus aureus into a non-adherent Staphylococcal strain. Journal of Infectious Diseases, 153, 670-675.

Dunkle, L.M., Blair, L.L. and Fortune, K.P. (1985). Effect of subinhibitory (subMIC) concentrations of antibiotics on expression of a plasmid-mediated adhesin of Staphylococcus aureus. Abstract of the Annual Meeting of the American Society for Microbiology, 20. Abstract B12.

Dziarski, R. (1985). Antibody responses to Staphylococcus aureus cell wall antigens. Clinical and Immunological Newsletter, 6, 58-62.

Eagle, H. and Musselman, A.D. (1948). The rate of bactericidal action of penicillin in vitro as a function of its concentration, and its paradoxically reduced activity at high concentrations against certain organisms. Journal of Experimental Medicine, 88, 99-131.

Easmon, C.S.F. (1981). Host parasite relationships in experimental Staphylococcal infection. In: The Staphylococci. Eds. Macdonald, A. and Smith, G. Pub. Aberdeen University Press. Aberdeen, 63-72.

Easmon, C.S.F. and Glynn, A.A. (1975a). The role of humoral immunity and acute inflammation in protection against Staphylococcal dermonecrosis. Immunology, 29, 67-85.

Easmon, C.S.F. and Glynn, A.A. (1975b). Cell-mediated immune responses in Staphylococcus aureus infections in mice. Immunology, 29, 75-85.

Easmon, C.S.F., Cole, P.J., Williams, A.J. and Hastings, S.M. (1980). The measurement of opsonic and phagocytic function by luminol-dependent chemiluminescence. Immunology, 41, 67-74.

Easmon, C.S.F. and Glynn, A.A. (1978). Role of Staphylococcus aureus cell wall antigens in the stimulation of delayed hypersensitivity after Staphylococcal infection. Infection and Immunity, 19, 341-342.

Easmon, C.S.F., Hamilton, I. and Glynn, A.A. (1973). Mode of action of a Staphylococcal anti-inflammatory factor. British Journal of Experimental Pathology, 54, 638-645.

Eickenberg, H.-U., Hahn, H. and Opferkuch, W. (1982). Eds. The influence of antibiotics on the host-parasite relationship. Pub. Springer-Verlag. Berlin.

Elek, S.D. and Conen, P.E. (1957). The virulence of Staphylococcus pyogenes for man. A study of the problems of wound infection. British Journal of Experimental Pathology, 38, 573-586.

Ellwood, D.C. and Tempest, D.W. (1972). Effects of environment on bacterial wall content and composition. Advances in Microbial Physiology, 7, 83-117.

Elsbach, P. (1980). Degradation of micro-organisms by phagocytic cells. Review of Infectious Diseases, 2, 106-128.

Elsbach, P. and Weiss, J. (1983). A re-evaluation of the roles of the oxygen-dependent and oxygen-independent microbicidal systems of phagocytes. Reviews of Infectious Diseases, 5, 843-853.

Endl, J., Seidl, H.P., Fiedler, F. and Schleifer, K.H. (1983). Chemical composition and structure of cell wall teichoic acids of Staphylococci. Archives of Microbiology, 135, 215-223.

England, E. (1986). Hydrophobic interaction chromatography. Laboratory Practice, January, 64-67.

Engvall, E., Johnson, K. and Perlman, P. (1971). Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody coated tubes. Biochemica et Biophysica Acta, 251, 427-434.

Eriksen, H.O., Espersen, F. and Clemmensen, I. (1984). Opsonic activity of fibronectin in the phagocytosis of Staphylococcus aureus by polymorphonuclear leukocytes. European Journal of Clinical Microbiology, 3, 108-112.

Espersen, F. (1985). Complement activation by clumping factor and Protein A from Staphylococcus aureus strain E2371. Acta Pathologica Microbiologica Immunologica Scandinavica. Section C, 93, 59-64.

Espersen, F. and Clemmensen, I. (1985). Immunisation of mice with the fibronectin-binding protein and clumping factor from Staphylococcus aureus: antibody response and resistance against intraperitoneal infection. Acta Pathologica Microbiologica Immunologica Scandinavica. Section C, 93, 53-58.

Espersen, F., Clemmensen, I. and Barkholt, V. (1985). Isolation of Staphylococcus aureus clumping factor. Infection and Immunity, 49, 700-708.

Espersen, F., Clemmensen, I., Frimodt-Møller, N. and Jensen, K. (1984). Effect of human IgG and fibrinogen on Staphylococcus aureus intraperitoneal infection in mice. Acta Pathologica Microbiologica Immunologica Scandinavica. Section B, 92, 311-317.

Espersen, F. and Hedström, S.Å. (1983). Serological diagnosis of Staphylococcus aureus septicaemia and endocarditis by means of crossed immuno-electrophoresis. Scandinavian Journal of Infectious Diseases, Suppl. 41, 97-104.

Espersen, F., Jarløv, J.O., Jensen, C., Skov, S. and Norn, S. (1984). Staphylococcus aureus peptidoglycan induces histamine release from basophil human leucocytes in vitro. Infection and Immunity, 46, 710-714.

Espersen, F., Wheat, L.J., Bemis, A.T. and White, A. (1986). Enzyme-linked immunosorbent assay for detection of Staphylococcus epidermidis antibody in experimental Staphylococcus epidermidis endocarditis. Journal of Clinical Microbiology, 23, 339-342.

Evans, S., Arcenaux, M.M., Aranha, H. and Byers, R. (1985). Iron uptake in Streptococcus mutans. 1st International Symposium on iron transport-storage-metabolism. July 10th-12th. University of Tubingen. Federal Republic of Germany.

Fainstein, V., Musher, D.M. and Cate, T.R. (1980). Bacterial adherence to pharyngeal cells during viral infection. Journal of Infectious Diseases, 141, 172-176.

Fattom, A. and Shilo, M. (1984). Hydrophobicity as an adhesion mechanism of benthic cyanobacteria. Applied and Environmental Microbiology, 47, 135-143.

Feingold, D.S. (1986). Bacterial adherence, colonisation and pathogenicity. Archives of Dermatology, 122, 161-163.

Ferreirós, C.M. and Criado, M.T. (1984). Expression of surface hydrophobicity encoded by R-plasmids in Escherichia coli laboratory strains. Archives of Microbiology, 138, 191-194.

Finkelstein, R.A., Sciortino, C.V. and McIntosh, M.A. (1983). Role of iron in microbe-host interactions. Reviews of Infectious Diseases, 5, Suppl. 4, S759-S777.

Fischer, H. and Tomasz, A. (1984). Production and release of peptidoglycan and wall teichoic acid polymers in pneumococci treated with β-lactam antibiotics. Journal of Bacteriology, 157, 507-513.

Fontana, R. (1985). Penicillin-binding proteins and the intrinsic resistance to β-lactams in Gram-positive cocci. Journal of Antimicrobial Chemotherapy, 16, 412-416.

Fontana, R., Canepari, P., Satta, G. and Coyette, J. (1983). Streptococccus faecium ATCC 9790 penicillin-binding proteins and penicillin sensitivity are heavily influenced by growth conditions: proposal for an indirect mechanism of growth inhibition by β-lactams. Journal of Bacteriology, 154, 916-923.

Forsgren, A. (1970). Significance of Protein A production by Staphylococci. Infection and Immunity, 2, 672-673.

Forsgren, A., Ghetie, V., Lindmark, R. and Sjöquist, J. (1983). Protein A and its exploitation. In: Staphylococci and Staphylococcal Infections, Vol 2. Eds. Easmon, C.S.F. and Adlam, C. Pub: Academic Press, London. 429-480.

Franson, T.R., Sheth, N.K., Rose, H.D. and Sohnle, P.G. (1984). Quantitative adherence in vitro of coagulase-negative Staphylococci to intra-vascular catheters: inhibition with D-mannosamine. Journal of Infectious Diseases, 149, 116.

Frazer, J.G. (1935). The Golden Bough: the magic art and the evolution of kings. Pub: MacMillan Co., New York. 158.

Freer, J.H. and Arbuthnott, J.P. (1982). Toxins of Staphylococcus aureus. Pharmacology and Therapeutics, 19, 55-106.

Frère, J.-M. and Joris, B. (1985). Penicillin-sensitive enzymes in peptidoglycan biosynthesis. CRC Critical Reviews in Microbiology, 11, 299-396.

Frost, A.J., Wanasinghe, D.D. and Woolcock, J.B. (1977). Some factors affecting selective adherence of micro-organisms in the bovine mammary gland. Infection and Immunity, 15, 245-253.

Fung, D.Y.C. and Petrishko, D.T. (1973). Capillary tube catalase test. Applied Microbiology, 26, 631-632.

Fuquay, J.I., Loo, D.T. and Barnes, D.W. (1986). Binding of Staphylococcus aureus by human serum spreading factor in an in vitro assay. Infection and Immunity, 52, 714-717.

Galdiero, F., Romano-Carratelli, C., Folgore, A. and Nuzzo, I. (1983). Phagocytosis in diabetic subjects: increase in hydrophobicity of granulocyte cytoplasmic membrane. Experentia, 39, 1041-1042.

Ganfield, D.J., Gray, J.H. and Esber, H.J. (1985). Stimulation of human lymphocytes with components of the Staphylococcal vaccine, Staphage lysate. Federal Proceedings, 44, Abstract No 1099, 598.

Gardner, A.D. (1940). Morphological effects of penicillin on bacteria. Nature, London, 146, 837.

Gellenbeck, S.M. (1962). Aerobic respiratory metabolism of Staphylococcus aureus from an infected animal. Journal of Bacteriology, 83, 450-455.

Gemmell, C.G. (1978). Effect of sub-growth inhibitory concentrations of antibiotics on experimental pyogenic infections in mice. In: Current Chemotherapy. Vol I. Ed: Siegenthaler, W. and Lüthy, R. Pub: American Society for Microbiology. Washington DC. 512-514.

Gemmell, C.G. (1984). Potentiation of phagocytosis of pathogenic bacteria by exposure to low concentrations of antibiotics. Journal of Antimicrobial Chemotherapy, 13, 407-415.

Gemmell, C.G. (1985). In vivo consequences of the interaction between antibiotic-damaged pathogens and host animal defences. In: The Influence of Antibiotics on the Host-Parasite Relationship. II. Eds: Adam, D., Hahn, H. and Opferkuch, W. Pub: Springer-Verlgag. Berlin. 259-266.

Gemmell, C.G. and O´Dowd, A. (1983). Regulation of Protein A biosynthesis in Staphylococcus aureus by certain antibiotics: its effect on phagocytosis by leukocytes. Journal of Antimicrobial Chemotherapy, 12, 587-597.

Gemmell, C.G., Peterson, P.K., Schmeling, D., Kim, Y., Mathews, J., Wannamaker, L. and Quie, P.G. (1981). Potentiation of opsonisation and phagocytosis of Streptococcus pyogenes following growth in the presence of clindamycin. Journal of Clinical Investigation, 67, 1249-1256.

Gemmell, C.G., Peterson, P.K., Schmeling, D. and Quie, P.G. (1982). Studies on the potentiation of phagocytosis of Streptococcus pyogenes by treatment with various antibiotics. Drugs Under Experimental and Clinical Research, 8, 235-240.

Gemmell, C.G. and Shibl, A.M. (1976). The control of toxin and enzyme biosynthesis in Staphylococci by antibiotics. In: Staphylococci and Staphylococcal Infections. Ed: Jeljaszewicz, J. Pub: Gustav Fischer. Stuttgart. 657-664.

Giesbrecht, P., Labischinski, H. and Wecke, J. (1985). A special morphogenetic wall defect and the subsequent activity of "murosomes" as the very reason for penicillin-induced bacteriolysis in Staphylococci. Archives of Microbiology, 141, 315-324.

Gillman, C.F., Bernstein, J.M. and van Oss, C.J. (1976). Decreased phagocytosis associated with increased surface hydrophobicity of neutrophils of children with chronic infections. Federal Proceedings, 35, 227.

Ginsburg, I. (1979). The role of lysosomal factors of leukocytes in the biodegradation and storage of microbial constituents in infectious granulomas. In: Lysosomes in Biology and Pathology, Vol 6. Eds. Dingle, J.T., Jacques, P.J. and Shaw, I.H. Pub. North Holland Publishing, London, 327-406.

Ginsburg, I. and Lahav, M. (1983). Cationic polyelectrolytes activate autolytic wall enzymes in Staphylococcus aureus. In: The Target of penicillin. Eds. Hakenbek, R., Holje, J.V. and Labischinski, H. Pub. Walter de Gruyter and Co. Berlin. 341-346.

Ginsburg, I., Sela, M.N., Ne´eman, N. and Lahav, M. (1983). The role of leucocyte and serum factors and of cationic polyelectrolytes in the lysis and biodegradation of Staphylococcus aureus: relation to the pathogenesis of Staphylococcal infections. In Staphylococci and Staphylococcal Infections, Vol 1. Eds. Easmon, C.S.F. and Adlam, C. Pub. Academic Press, London, 325-355.

Gladstone, G.P. and Glencross, J.G. (1960). Growth and toxin production of Staphylococci in cellophane sacs in vivo. British Journal of Experimental Pathology, 41, 313-333.

Gladstone, G.P. and Walton, E. (1970). Effect of iron on the bactericidal proteins from rabbit polymorphonuclear leucocytes. Nature, London, 227, 849-851.

Gladstone, G.P. and Walton, E. (1971). The effect of iron and haematin on the killing of Staphylococci by rabbit polymorphs. British Journal of Experimental Pathology, 52, 452-464.

Gladstone, G.P., Walton, E. and Kay, U. (1974). The effect of cultural conditions on the susceptibility of Staphylococci to killing by the cationic proteins from rabbit polymorphonuclear leucocytes. British Journal of Experimental Pathology, 55, 427-447.

Glaser, L. (1973). Bacterial cell surface polysaccharides. Annual Reviews of Biochemistry, 42, 91-112.

Glauert, A.M. and Thornley, M.J. (1969). The topography of the bacterial cell wall. Annual Reviews of Microbiology, 23, 159-198.

Godding, J.W. (1978). Use of Staphylococcal Protein A as an immunological reagent. Journal of Immunological Methods, 20, 241-253.

Gould, K., Ramirez-Ronda, C.H., Holmes, R.K. and Sandford, J.P. (1976). Adherence of bacteria to heart valves in vitro. Journal of Clinical Investigation, 56, 1364.

Grebner, J.V., Mills, E.L., Gray, B.H. and Quie, P.G. (1977). Comparison of phagocytic and chemiluminescence response of human polymorphonuclear neutrophils. Journal of Laboratory and Clinical Investigation, 89, 153-159.

Greenwood, D. (1977). In defense of turbidimetry. Journal of Antimicrobial Chemotherapy, 3, 286-287.

Greenwood, D. and O'Grady, F. (1973). Comparison of the responses of Escherichia coli and Proteus mirabilis to seven β-lactam antibiotics. Journal of Infectious Diseases, 128, 211.

Griffiths, E. (1983). Availability of iron and survival of bacteria in infection. In: Medical Microbiology, Vol 3. Eds: Easmon, C.S.F., Jeljaszewicz, J., Brown, M.R.W. and Lambert, P.A. Pub: Academic Press. London, 153-177.

Grinsted, J. and Lacey, R.W. (1973). Ecological and genetic implications of pigmentation in Staphylococcus aureus. Journal of General Microbiology, 75, 259-267.

Haas, R., Koch, H.U. and Fischer, W. (1984). Alanyl turnover from lipoteichoic acid to teichoic acid in Staphylococcus aureus. FEMS Microbiological Letters, 21, 27-31.

Hallander, H.O., Laurell, G. and Lofstrom, G. (1966). Stimulation of Staphylococcal haemolysin production by low concentrations of penicillin. Acta Pathologica Microbiologica Scandinavica, 68, 142.

Hammond, S.M., Lambert, P.A. and Rycroft, A.N. (1984). The Bacterial Cell Surface. Pub. Croom Helm Ltd., London, England.

Harboe, M. and Fölling, I. (1974). Recognition of two distinct groups of human IgM and IgA based on different binding to Staphylococci. Scandinavian Journal of Immunology, 3, 471-482.

Hartmann, B.J. and Tomasz, A. (1984). Low affinity penicillin-binding protein associated with β-lactam resistance in Staphylococcus aureus. Journal of Bacteriology, 158, 513-516.

Hauser, E.D. and Berry, L.J. (1961). The pathogenesis of Staphylococcus infections. The use of diffusion chambers in establishing the role of Staphylococcus toxins. Journal of Infectious Diseases, 109, 24-30.

Hawiger, J., Hammond, D.K., Timmon, S. and Budzynski, A.Z. (1978). Interaction of human fibrinogen with Staphylococci. Blood, 51, 799-812.

Hawiger, J., Timmons, S., Strong, D.D., Cotrell, B.A., Riley, M. and Doolittle, D.F. (1982). Identification of a region of human fibrinogen interacting with Staphylococcal clumping factor. Biochemistry, 21, 1407-1413.

Hayashi, K., Lee, D.A. and Quie, P. (1986). Chemiluminescent response of polymorphonuclear leukocytes to Streptococcus pneumoniae and Haemophilus influenzae in suspension and adhered to glass. Infection and Immunity, 52, 397-400.

Hayes, M.V., Curtis, N.A.C., Wyke, A.W. and Ward, J.B. (1981). Decreased affinity of a penicillin-binding protein for β-lactam antibiotics in a clinical isolate of Staphylococcus aureus resistant to methicillin. FEMS Microbiological Letters, 10, 119-122.

Helgeland, S., Grov, A. and Schleifer, K.H. (1973). The immunochemistry of Staphylococcus aureus mucopeptide. 1. Antigenic specificity of peptide subunits. Acta Pathologica Microbiologica Scandinavica, Section B. 81, 731-740.

Hemming, V.G., Hall, R.T., Rhodes, P.G., Shigeoka, A.O. and Hill, H.R. (1976). Assessment of Group B steptococcal opsonins in human and rabbit serum by neutrophil chemiluminescence. Journal of Clinical Investigation, 58, 1379-1387.

Heptinstall, S., Archibald, A.R., and Baddiley, J. (1970). Teichoic acids and membrane function in Bacteria. Nature, London, 225, 519-521.

Hermansson, M., Kjelleberg, A., Korhonen, T.K. and Stenstrom, T.A. (1982) Hydrophobic and electrostatic characterisation of surface structures of bacteria and its relationship to adhesion to an air-water interface. Archives of Microbiology, 131, 308-312

Heymer, B. and Reitschel, E.T.H. (1977). Biological properties of peptidoglycans. In: Microbiology. Ed. Schlessinger, D. Pub. American Society for Microbiology, Washington D.C., 344-349.

Hickling, P. and Golding, J.R. (1984). An outline of Rheumatology. Pub. Wright. Bristol.

Higgins, M.L., Coyette, J. and Shockman, G.D. (1973). Sites of cellular autolysis in Lactobacillus acidophilus. Journal of Bacteriology, 116, 1375-1385.

Hill, H.R., Quie, P.G., Pabst, H.F., Ochs, H.D., Clark, R.A., Klebanoff, S.J. and Wedgewood, R.J. (1974). Defects in neutrophil granulocyte chemotaxis in Job's syndrome of recurrent "cold" Staphylococcal abscesses. Lancet ii, 617-619.

Hill, M.J. (1968). A staphylococcal agressin. Journal of Medical Microbiology, 1, 33-43.

Hogt, A.H., Dankert, J. and Feijen, J. (1985). Adhesion of Staphylococcus epidermidis and Staphylococcus saprophyticus to a hydrophobic biomaterial. Journal of General Microbiology, 131, 2485-2491.

Höltje, J.V. and Tomasz, A. (1975). Lipoteichoic acid: a specific inhibitor of autolysin activity in pneumococcus. Proceedings of the National Academy of Sciences of USA, 72, 1690-1694.

Höltje, J.V. and Tomasz, A. (1976). Purification of the pneumococcal N-acetylmuramyl L-alanine amidase to biochemical homogeneity. Journal of Biological Chemistry, 251, 4199-4207.

Horne, D. and Tomasz, A. (1977). Tolerant response of Streptococcus sanguis to β-lactams and other cell wall inhibitors. Antimicrobial Agents and Chemotherapy, 11, 888-896.

Huff, E. and Silverman, C.S. (1968). Lysis of Staphylococcus aureus cell walls by a soluble Staphylococcal enzyme. Journal of Bacteriology, 95, 99-106.

Hughes, A.H., Hancock, I.C. and Baddiley, J. (1973). The function of teichoic acids in cation control in bacterial membranes. Biochemical Journal, 132, 83-93.

Hummell, D.S., Swift, A.J., Tomasz, A. and Wink, J.A. (198 5) Activation of the alternative complement pathway by pneumococcal lipoteichoic acid. Infection and Immunity, 47, 384-387.

Isturiz, R.E., Andriole, V.T. and Root, R.K. (1979). Effects of subinhibitory concentrations of antibiotics on killing of Staphylococcus aureus by human neutrophils. Clinical Research, 27, 347A (Abstract).

Jacob, E., Arendt, D.M., Brook, I., Durham, L.C., Falk, M.C. and Schaberg, S.J. (1985). Enzyme-linked immunosorbent assay for detection of antibodies to Staphylococcus aureus cell walls in experimental osteomyelitis. Journal of Clinical Microbiology, 22, 547-552.

James, A.M. and Brewer, J.E. (1968a). A protein component of the cell surface of Staphylococcus aureus. Biochemical Journal, 108, 257-262.

James, A.M. and Brewer, J.E. (1968b). Non-protein components of the cell surface of Staphylococcus aureus. Biochemical Journal, 107, 817-821.

Janzon, L., Löfdahl, S. and Arvidson, S. (1986). Evidence for a co-ordinate transcriptional control of alpha-toxin and Protein A synthesis in Staphylococcus aureus. FEMS Microbiological Letters, 33, 193-198.

Jarløv, J.O., Christensson, B., Espersen, F., Hertz, J.B. and Hedström, S.Ä. (1985). Antibody response against whole Staphylococcus aureus in patients with Staphylococcal septicaemia and endocarditis investigated by ELISA. Acta Pathologica, Microbiologica Immunologica Scandinavica. Section B, 93, 307-313.

Jeljaszewicz, J., Switalski, L.M. and Adlam, C. (1983). Staphyloccagulase and clumping factor. In: Staphylococci and Staphylococcal Infections, Vol 2. Eds. Easmon, C.S.F. and Adlam, C. Pub. Academic Press, London, 525-557.

Johannsen, L., Labischinski, H., Burghaus, P. and Giesbrecht, P. (1983). Acetylation in different phases of growth of Staphylococci and their relation to cell wall degradability by lysozyme. In: The Target of Penicillin. Eds. Hakenbek, R., Holje, J.V. and Labischinski, H. Pub. Walter de Gruyter and Co. Berlin. 261-266.

Johansson, S.G.O. and Inganäs, M. (1978). Interaction of polyclonal human IgE with Protein A from Staphylococcus aureus. Immunological Reviews, 41, 248-260.

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

Jones, R.J., Roe, E.A. and Gupta, J.L. (1979). Controlled trials of a polyvalent Pseudomonas vaccine in burns. Lancet, ii, 977-983.

Jonsson, P., Lindberg, M., Haraldsson, I. and Wadström, T. (1985). Virulence of Staphylococcus aureus in a mouse mastitis model. Infection and Immunity, 49, 765-769.

Jonsson, P. and Wadström, T. (1983). High surface hydrophobicity of Staphylococcus aureus as revealed by hydrophobic interaction chromatography. Current Microbiology, 8, 347-353.

Kalter, E.S., van Dijk, W.C., Timmerman, A., Verhoef, J. and Bouma, B.N. (1983). Activation of purified human plasma prekallikrein triggered by cell wall fractions of Escherichia coli and Staphylococcus aureus. Journal of Infectious Diseases, 148, 682-691.

Kanafani, H. and Martin, S.E. (1985). Catalase and superoxide dismutase activities in virulent and avirulent Staphylococcus aureus isolates. Journal of Clinical Microbiology, 21, 607-610.

Karakawa, W.W. and Vann, W.F. (1982). Capsular polysaccharides of Staphylococcus aureus. Seminars in Infectious Diseases, 4, 285.

Karawaka, W.W. and Young, D.A. (1979a). Immunological specificity of heat-stable opsonins in immune and non-immune sera and their interaction with non-encapsulated and encapsulated strains of Staphylococcus aureus. Infection and Immunity, 25, 175-186.

Karakawa, W.W. and Young, D.A. (1979b). Immunochemical study of diverse surface antigens of a Staphylococcus aureus isolate from an osteomyelitis patient and their role in in vitro phagocytosis. Journal of Clinical Microbiology, 9, 399-408.

Kefford, B., Kjelleberg, S. and Marshall, K.C. (1982). Bacterial scavenging: utilisation of fatty acids localised at a solid /liquid interface. Archives of Microbiology, 133, 257-260.

Kelly, J.A., Moews, P.C., Knox, J.R., Frère, J.-M. and Ghuysen, J.-M. (1982). Penicillin target enzyme and the antibiotic binding site. Science, 218, 479-481.

Kern, R.A., Kingkade, M.J., Kern, S.F. and Behrens, O.K. (1951). Characterisation of the action of lysozyme on Staphylococcus aureus and on Micrococcus lysodeikticus. Journal of Bacteriology, 61, 171-178.

King, B.F. and Wilkinson, B.J. (1981). Binding of human immunoglobulin G to Protein A in encapsulated Staphylococcus aureus. Infection and Immunity, 33, 666-672.

Kinsman, O.S., McKenna, R. and Noble, W.C. (1983). Association between histocompatibility antigens (HLA) and nasal carriage of Staphylococcus aureus. Journal of Medical Microbiology, 16, 215-220.

Kinsman, O.S., White, M.I. and Noble, W.C. (1981). Inflammatory reactions to Staphylococcal Protein A in mice. British Journal of Experimental Pathology, 62, 142-145.

Kjelleberg, S. and Hermansson, M. (1984). Starvation-induced effects on bacterial surface characteristics. Applied and Environmental Microbiology, 48, 497-503.

Kjelleberg, S., Humphrey, B.A. and Marshall, K.C. (1983). Initial stages of starvation and activity of bacteria at surfaces. Applied and Environmental Microbiology, 46, 978-984.

Klebanoff, S.M. (1975). Antimicrobial systems in neutrophilic PMNL's. Seminars in Haematology, 12, 117-142.

Klebanoff, S.J. (1982). Oxygen-dependent cytotoxic mechanisms of phagocytes. In: Advances in Host Defence Mechanisms. Eds. Gallin, J.I. and Fauci, A.S. Pub. Raven Press, New York, 111-162.

Knox, K.W. and Wicken, A.J. (1973). Immunological properties of ,teichoic acids. Bacteriological Reviews, 37, 215-257.

Kobayasi, A., Barnett, J.A. and Sanford, J.P. (1966). Effect of antibiotics on the in vitro production of α-haemolysin by Staphylococcus aureus. Journal of Laboratory and Clinical Medicine, 68, 890.

Koch, H.U., Döker, R. and Fischer, W. (1985). Maintenance of D-alanine ester subsitution of lipoteichoic acid by re-esterification in Staphylococcus aureus. Journal of

Bacteriology, 164, 1211-1217.

Koch, H.U., Haas, R. and Fischer, W. (1984). The role of lipoteichoic acid biosynthesis in membrane lipid metabolism of growing Staphylococcus aureus. European Journal of Biochemistry, 138, 357-363.

Koenig, M.G. (1972). The phagocytosis of Staphylococci. In: The Staphylococci. Ed. Cohen, J.O. Pub. Wiley InterScience, New York, 365-383.

Kojima, N., Araki, Y. and Ito, E. (1983). Structure of linkage region between ribitol teichoic acid and peptidoglycan in cell walls in Staphylococcus aureus H. Journal of Biological Chemistry, 258, 9043-45.

Kolawole, D.O. (1983a). Effect of mucoid growth of Staphylococcus aureus on its susceptibility to rabbit polymorph bactericidal factors. FEMS Microbiological Letters, 16, 31-33.

Kolawole, D.O. (1983b). Evaluation and use of the drug inhibition method of measuring intracellular killing in differentiating between Staphylococci grown in vivo and in vitro. Journal of Hygiene, Cambridge, 91, 211-222.

Kolawole, D.O. (1984). Mechanisms of resistance of Staphylococci grown in plasma to polymorph bactericidins. Journal of Hygiene, Cambridge, 92, 183-192.

Korpela, J. (1984). Chicken macrophages synthesise and secrete avidin in culture. European Journal of Cellular Biology, 33, 105-111.

Korpela, J., Kulomaa, M., Tuohimaa, P. and Vaheri, A. (1983). Avidin is induced in chicken-embryo fibroblasts by viral transformation and cell-damage. EMBO Journal, 10, 1715-1719.

Korpela, J., Salonen, E.-M., Kuusela, P., Sarvas, M. and Vaheri, A. (1984). Binding of avidin to bacteria and to the outer membrane porin of Escherichia coli. FEMS Microbiology Letters, 22, 3-10.

Koser, S.A. (1968). Vitamin requirements of Bacteria and Yeasts. Pub. Thomas, C.C., Illinois, USA.

Kossak, R.E., Guerrant, R.L., Densen, P., Schaedlin, J. and Mandel, G.L. (1981). Diminished neutrophil oxidative metabolism after phagocytosis of virulent Salmonella typhi. Infection and Immunity, 31, 674-678.

Kovacs, E. and Mazarean, H.H. (1967). Structuring of cell multiplication by use of inoculum with diverse catalase activity. Enzymologia, 32, 263-268.

Krikler, S.J., Pennington, T.H. and Petrie, D. (1986). Typing of strains of Staphylococcus aureus by Western Blot analysis of culture supernatants. Journal of Medical Microbiology, 21, 169-171.

Kronvall, G., Quie, P.G. and Williams, Jnr., R.C. (1970). Quantitation of Staphylococcal Protein A: determination of equilibrium constant and number of Protein A residues on bacteria. Journal of Immunology, 104, 273-278.

Kunin, C.M. (1970). A ten year study of bacteriuria in schoolgirls. Journal of Infectious Diseases, 122, 382-392.

Lacey, R.W. (1975). Antibiotic-resistance plasmids of Staphylococcus aureus and their clinical importance. Bacteriological Reviews, 39, 1-32.

Lachica, R.V.F., Genigeorgis, C.A. and Hoeprich, P.D. (1979). Occurence of Protein A in Staphylococcus aureus and closely related Staphylococcus species. Journal of Clinical Microbiology, 10, 252-253.

Lam, C., Georgopoulos, A., Laber, G. and Schutze, E. (1984). Therapeutic relevance of penicillin-induced hypersensitivity of Staphylococcus aureus to killing by polymorphonuclear leukocytes. Antimicrobial Agents and Chemotherapy, 26, 149-154.

Lam, C. and Mathison, G.E. (1982). Intraphagocytic protection of Staphylococci from extracellular penicillin. Journal of Medical Microbiology, 15, 373-385.

Lam, C., Turnowsky, F., Schwarzinger, E. and Neruda, W. (1984). Bacteria recovered without subculture from infected human urines expressed iron-regulated outer membrane proteins. FEMS Microbiological Letters, 24, 255-259.

Lambert, P.A. (1983). The bacterial surface and drug resistance. In: Medical Microbiology, Vol 3. Eds: Easmon, C.S.F., Jeljaszewicz, J., Brown, M.R.W. and Lambert, P.A. Pub: Academic Press, London, 1-19.

Lambert, P.A. and Booth, B.R. (1982). Exposure of outer membrane proteins on the surface of Pseudomonas aeruginosa PA01 revealed by labelling with ^{125}I-lactoperoxidase. FEMS Microbiology Letters, 14, 43-45.

Lambert, P.A., Hancock, I.C. and Baddiley, J. (1977). Occurence and function of membrane teichoic acids. Biochemica et Biophysica Acta, 472, 1-12.

Langone, J.J. (1982). Protein A of Staphylococcus aureus and related immunoglobulinreceptors produced by Streptococci and pneumococci. Advances in Immunology, 32, 157-252.

Langvad, E., Espersen, F. and Briand, P. (1984). Tumour growth-inhibition by Protein A and non-Protein A containing Staphylococcus aureus in a mouse mammary carcinoma model. Acta Pathologica Microbiologica Immunologica Scandinavica. Section C, 92, 255-259.

Lanser, M.E. and Saba, T.M. (1982). Opsonic fibronectin is necessary for optimal serum-mediated phagocytosis of Staphylococcus aureus

by human neutrophils. Advances in Shock Research, 8, 111-117.

Larson, H.E. and Blades, R. (1976). Impairment of human polymorphonuclear leucocyte function by influenza virus. Lancet, 1, 283.

Lee, D.A., Holdal, J.R., Clawson, C.C., Quie, P.G. and Peterson, P.K. (1983). Phagocytosis by polymorphonuclear leukocytes of Staphylococcus aureus and Pseudomonas aeruginosa adherent to plastic, agar or glass. Journal of Immunological Methods, 63, 103-114.

Leijh, P.C., van den Barselaar, M.T., Daha, M.R., and van Furth, R. (1981). Participation of immunoglobulins and complement components in the intracellular killing of Staphylococcus aureus and Escherichia coli by human granulocytes. Infection and Immunity, 33, 714-724.

Leijh, P.C., van de Barselaar, M.T., van Zwet, T.L., Dubbeldeman-Rempt, I. and van Furth, R. (1979). Kinetics of phagocytosis of Staphylococcus aureus and Escherichia coli by human granulocytes. Immunology, 37, 453-465.

Lewis, D., Reeves, D., Wiedman, B. and Zinner, S. (1985). (Eds.) Methodology and evaluation of in vitro models of antimicrobial chemotherapy. Journal of Antimicrobial Chemotherapy, 15, Suppl A.

Lichtenberg, D., Rosenberg, M., Sharfman, N. and Ofek, I. (1985). A kinetic approach to bacterial adherence to hydrocarbon. Journal of Microbiological Methods, 4, 141-146.

Lin, J.K. (1963a). Studies on Staphylococcal catalase. Report I. Effect of antibiotics on catalase biosynthesis in intact Staphylococcal cells. Journal of Formosan Medical Association, 62, 310-316.

Lin, J.K. (1963b). Studies on Staphylococcal catalase. Report II. Effect of sulphonamides and phenolic compounds on catalase biosynthesis in intact Staphylococcal cells. Ibid, 62, 368-372.

Lin, J.K. (1963c). Studies on Staphylococcal catalase. Report III. Induced formation of catalase in intact Staphylococcal cells. Ibid, 62, 440-445.

Lindahl, M., Faris, A., Wadström, T. and Hjertén, S. (1981). A new test based on "salting out" to measure relative surface hydrophobicity of bacterial cells. Biochemica et Biophysica Acta, 677, 471-476.

Lind, I., Reyn, A. and Birch-Andersen, A. (1964). Electron microscopy of specific and non-specific absorption of immunoglobulin to Staphylococcus aureus. 2nd European Regional Conference on Electron Microscopy, B535.

Lis, H., Sela, B.-A., Sachs, L. and Sharon, N. (1970). Specific inhibition by N-acetyl-D-galactosamine of the interaction between soy bean agglutinin and animal cell surfaces. Biochemica et

Biophysica Acta, 211, 582-585.

Live, I. (1972). Differentiation of Staphylococcus aureus of human and of canine origins: coagulation of human and of canine plasma, fibrinolysin activity and serological reaction. American Journal of Veterinary Research, 33, 385-391.

Ljungh, Å., Hjertén, S. and Wadström, T. (1985). High surface hydrophobicity of autoaggregating Staphylococcus aureus strains isolated from human infections studied with the salt aggregation test. Infection and Immunity, 47, 522-526.

LKB Wallace Application Note 513. The study of phagocytosis and opsonisation using luminol enhanced chemiluminescence.

Lopatin, D.E. and Kessler, R.E. (1985). Pretreatment with lipoteichoic acid sensitises target cells to antibody-dependent cellular cytotoxicity in the presence of anti-lipoteichoic acid antibodies. Infection and Immunity, 48, 638-643.

Lopes, J.D., dos Reis, M. and Brentani, R.R. (1985). Presence of laminin receptors in Staphylococcus aureus. Science, 229, 275-277.

Lorian, V. (1975). Some effects of subinhibitory concentrations of antibiotics on bacteria. Bulletin of the New York Academy of Sciences, 51, 1046-1055.

Lorian, V. (1980). Effects of subminimum inhibitory concentrations of antibiotics on bacteria. In: Antibiotics in Laboratory Medicine. Ed. Lorian, V. Pub. Williams and Wilkins, Baltimore, USA. 342-408.

Lorian, V. (1985). Phagocytosis of Staphylococci after exposure to antibiotics, mass versus colony-forming units. Journal of Antimicrobial Chemotherapy, 16, 130-132.

Lorian, V. and Atkinson, B. (1975). Abnormal forms of bacteria produced by antibiotics. American Journal of Clinical Pathology, 64, 678-688.

Lorian, V. and Atkinson, B. (1978). Effect of serum on Gram-positive cocci grown in the presence of penicillin. Journal of Infectious Diseases, 138, 865-871.

Lorian, V., Atkinson, B., Waluschka, A. and Kim, Y. (1982). Ultrastructure, in vitro and in vivo of Staphylococci exposed to antibiotics. Current Microbiology, 7, 301-304.

Lorian, V. and de Freitas, C. (1979). The minimum antibiotic concentrations (MACs) of aminoglycosides and β-lactam antibiotics from some Gram-negative bacilli and Gram-positive cocci. Journal of Infectious Diseases, 139, 599-603.

Lorian, V., Zak, O., Kunz, S. and Vaxelaire, J. (1984). Staphylococcal endocarditis in rabbits treated with a low dose of cloxacillin. Antimicrobial Agents and Chemotherapy, 25, 311-315.

Lorian, V., Zak, O., Suter, J. and Bruecher, C. (1985). Staphylococci *in vitro* and *in vivo*. Diagnostic Microbiology and Infectious Disease, 3, 433-444.

Louria, D.B. (1962). Use of quantitative analyses of bacterial population in sputum. Journal of American Medical Association, 182, 1082-1086.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, 193, 265-275.

Lugtenberg, B., Meijers, J., Peters, R., van der Hoek, P. and van Alphen, L. (1975). Electrophoretic resolution of the major outer protein of *Escherichia coli* K 12 into four bands. FEBS Letters, 58, 254-258.

Lyman, I.R., Tenery, J.G. and Basson, R.P. (1977). Correlation between decrease in bacterial load and rate of wound healing. Surgery, 130, 616-621.

MacArthur, A.E. and Archibald, A.R. (1984). Effect of culture pH on d-alanine ester content of lipoteichoic acid in *Staphylococcus aureus*. Journal of Bacteriology, 160, 792-793.

Magnusson, K.-E., Dahlgren, C., Maluszynska, G.M., Kihlström, E., Skogh, T., Stendahl, O., Söderlund, G., Öhman, L. and Walan, A. Non-specific and specific recognition mechanisms of bacterial and mammalian cell membranes. Journal of Dispersion Science and Technology, 6, 69-89.

Magnusson, K.-E., Kihlström, E., Norquist, A., Davies, J. and Normark, S. (1979). Effect of iron on surface charge and hydrophobicity of *Neisseria gonorrhoea*. Infection and Immunity, 26, 402-407.

Maki, D.G. (1981). Nosocomial bacteraemia. American Journal of Medicine, 70, 719-732.

Malmqvist, T. (1983). Bacterial hydrophobicity measured as partition of palmitic acid between the two immiscible phases of cell surface and buffer. Acta Pathologica Microbiologica Immunologica Scandinavica. Section B, 91, 69-73.

Maluszynska, G.M., Stendahl, O. and Magnusson, K.-E. (1985). Interaction between human polymorphonuclear leukocytes (PMNL) and bacteria cultivated in aerobic and anaerobic conditions. Acta Pathologica Microbiologica Immunologica Scandinavica. Section B, 93, 139-143.

Mandell, G.L. (1975). Catalase, superoxide dismutase and virulence of *Staphylococcus aureus*. Journal of Clinical Investigation, 55, 561-566.

Marcelis, J.H., den Daas-Slagt, H.J. and Hoogkamp-Korstanje, J.A.A. (1978). Iron requirement and chelator production of Staphylococci, *Streptococcus faecalis* and Enterobacteriaceae.

Antonie van Leeuwenhoek, 44, 257-267.

Marrie, T.J. and Costerton, J.W. (1984). Scanning and transmission electronmicroscopy of in situ bacterial colonisation of intravenous and intra-arterial catheters. Journal of Clinical Microbiology, 19, 687-693.

Marrie, T.J. and Costerton, J.W. (1985). Mode of growth of bacterial pathogens in chronic polymicrobial human osteomyelitis. Journal of Clinical Microbiology, 22, 924-933.

Maskell, J.P. (1980). The functional interchangeability of Enterobacterial and Staphylococcal iron chelators. Antonie van Leeuwenhoek, 46, 343-351.

Masson, P., Heremans, J.F. and Dive, C. (1966). Studies of proteins of secretions from two villous tumours of rectum. Gastroenta, 105, 270-273.

McBride, B.C., Song, M., Krasse, B. and Olsson, J. (1984). Biochemical and immunological differences between hydrophobic and hydrophilic strains of Streptococcus mutans. Infection and Immunity, 44, 68-75.

McDonnell, R.W., Sweeney, H.M. and Cohen, S. (1983). Conjugational transfer to gentamicin resistance plasmids intra- and interspecifically in Staphylococcus aureus and Staphylococcus epidermidis. Antimicrobial Agents and Chemotherapy, 23, 151-160.

McDougall, J.S., Kennedy, M.S., Hubbard, M., Browning, S.W., Tsang, V.C.W., Winton, E.F. and McDuffie, F.C. (1986). Antigen detection in immune complexes by a modified Staphylococci binding assay and Western blot analysis. Clinical and Immunological Immunopathology, 38, 184-197.

Melly, M.A., Duke, L.L., Liau, D. and Hash, J.H. (1974). Biological properties of the encapsulated Staphylococcus aureus M. Infection and Immunity, 10, 389-397.

Melly, M.A., Thomison, J.B. and Rogers, D.E. (1960). Fate of Staphylococci within human leukocytes. Journal of Experimental Medicine, 112, 1121-1130.

Metchnikoff, E. (1905). Immunity in Infective Disease. Transl: Binnie, F.G. Pub: Cambridge University Press (from Russian), London.

Meynell, G.G. and Meynell, E. (1970). Theory and practice in Experimental Bacteriology, 2nd Edition. Pub. Cambridge University Press.

Milatovic, D. (1982). Effect of sub-inhibitory antibiotic concentrations on the phagocytosis of Staphylococcus aureus. European Journal of Clinical Microbiology, 1, 97-101.

Milatovic, D. (1983). Antibiotics and phagocytosis. European Journal of Clinical Microbiology, 2, 414-425.

Milatovic, D., Braveny, I. and Verhoef, J. (1983). Clindamycin enhances opsonisation of Staphylococcus aureus. Antimicrobial Agents and Chemotherapy, 24, 413-417.

Miles, A.A. and Khimji, P.L. (1975). Enterobacterial chelators of iron: their occurence, detection and relation to pathogenicity. Journal of Medical Microbiology, 8, 477-490.

Miles, A.A. and Misra, S.S. (1938). The estimation of the bactericidal power of blood. Journal of Hygiene, Cambridge, 38, 732-748.

Miller, R.D. and Fung, D.Y.C. (1973). Amino acid requirements for the production of enterotoxin B by Staphylococcus aureus S-6 in a chemically defined medium. Applied Microbiology, 25, 800-806.

Mims, C.A. (1977). The Pathogenesis of Infectious Diseases. Pub: Academic Press. London.

Miörner, H., Albertsson, P.-A. and Kronvall, G. (1982). Isoelectric points and surface hydrophobicity of Gram-positive cocci as determined by cross-partition and hydrophobic affinity partition in aqueous two phase systems. Infection and Immunity, 36, 227-234.

Miörner, H., Myhre, E., Björk, L. and Kronvall, G. (1980). Effect of specific binding of human albumen, fibrinogen and immunoglobin G on surface characteristics of bacterial strains as revealed by partition experiments in polymer phase systems. Infection and Immunity, 29, 879-885.

Mudd, E.B.H. and Mudd, S. (1933). The process of phagocytosis. The agreement between direct observation and deductions from theory. Journal of General Physiology, 16, 625-636.

Mudd, S., Taubler, J.H. and Baker, A.G. (1970). Short Communication. Delayed-Type Hypersensitivity to Staphylococcus aureus in Human Subjects. Journal of Reticuloendothelial Society, 8, 493-498.

Nagata, Y. and Burger, M.M. (1974). Wheat germ agglutinin. Journal of Biological Chemistry, 249, 3116-3122.

Nardella, F.A., Teller, D.C., Barber, C.V. and Mannik, M. (1985). IgG rheumatoid factors and Staphylococcal Protein A bind to a common molecular site on IgG. Journal of Experimental Medicine, 162, 1811-1824.

Ndulue, A.N. and Flandrois, J.P. (1983). Immunochemical studies of Staphylococcus aureus Oeding-Haukenes antigen a_5: a phosphorous-containing polysaccharide. Journal of General Microbiology, 129, 3603-3610.

Nealon, T.J. and Mattingly, S.J. (1985). Kinetic and chemical analyses of the biologic significance of lipoteichoic acids in mediating adherence of serotype III Group B Streptococci. Infection and Immunity, 50, 107-115.

Neilands, J.B. (1972). Evolution of biological iron binding centres. Structure and Bonding (Berlin) 11, 145-170.

Neilands, J.B. (1974). Iron and its role in microbial physiology. In: Microbial iron metabolism. Ed. Neilands, J.B. Academic Press, London, 4-34.

Neilands, J.B. (1981). Microbial iron compounds. Annual Reviews of Biochemistry, 50, 715-731.

Neilands, J.B. (1982). Microbial envelope proteins related to iron. Annual Reviews of Microbiology, 36, 285-309.

Nelles, M.J., Niswander, C.A., Karakawa, W.W., Vann, W.F. and Arbeit, R.D. (1985). Reactivity of type-specific monoclonal antibodies with Staphylococcus aureus clinical isolates and purified capsular polysaccharide, 49, 14-18.

Nesbitt, E.W., Doyle, R.J. and Taylor, K.G. (1982). Hydrophobic interactions and the adherence of Streptococcus sanguis to hydroxylapatite. Infection and Immunity, 38, 637-644.

Nishihara, S., Seiki, K. and Masuda, S. (1985). Resistance of a mutant with an extremely low catalase production from Staphylococcus aureus Cowan 1 strain to the bactericidal activity of human leukocytes. Microbiology and Immunology, 29, 151-155.

Nived, O., Linder, C., Odeberg, H. and Svensson, B. (1985). Reduced opsonisation of Protein A containing Staphylococcus aureus in sera with cryoglobulins from patients with active systemic lupus erythematosus. Annals of Rheumatic Diseases, 44, 252-259.

Noble, W.C. (1976). Skin as a microbial habitat. In: Selected Topics in Bacteriology. Ed. de Louvois, J. Pub. Balliere Tindal, London, 235-256.

Noble, W.C. and White, M.I. (1983). Staphylococcal skin infection in man. In: Staphylococci and Staphylococcal infections, Vol 1. Eds. Easmon, C.S.F. and Adlam, C. Pub. Academic Press, London, 166-191.

Novick, R.P. (1963). Analysis by transduction of mutations affecting penicillinase formation in Staphylococcus aureus. Journal of General Microbiology, 33, 121-136.

Nyland, H. and Aasjord, P. (1983). Humoral and cellular immune responses to Staphylococcal lipoteichoic acid in multiple sclerosis patients. Progress in Brain Research, 59, 345-354.

O'Driscoll, M., Crawford, L. and Biggar, W.D. (1985). Clinical features and abnormal neutrophil function in disseminated Staphylococcal disease. Pediatric Infectious Disease, 4, 137-141.

Oeding, P. (1983). Taxonomy and identification. In: Staphylococci and Staphylococcal Infections, Vol 1. Eds. Easmon, C.S.F. and Adlam, C. Pub. Academic Press, London, 1-32.

Oeding, P. (1984). Cellular antigens. In: Staphylococci and Staphylococcal disease in man and animals. Ed. Meyer, W. Pub. Gustav Fischer Verlag. Jena, 74-94.

Oeding, P., Wergeland, H., Endresen, C., Natas, O. and Aasjord, P. (1983). Antibodies to Staphylococcus aureus peptidoglycan and lipoteichoic acid in Staphylococcal infections. Scandinavian Journal of Infectious Diseases, Suppl. 41, 126-129.

Ofek, I. and Beachey, E.H. (1980). Bacterial adherence. Advances in Internal Medicine, 25, 503.

Ogawa, S.K., Yurberg, E.R., Hatcher, V.B., Levitt, M.A. and Lowy, F.D. (1985). Bacterial adherence to human endothelial cells in vitro. Infection and Immunity, 50, 218-224.

Ogston, A. (1882). Micrococcus poisoning. Journal of Anatomy and Physiology, 16, 526-567.

Opdebeeck, J.P. and Norcross, N.L. (1985). Antibodies in bovine serum and lacteal secretions to capsular antigens of Staphylococcus aureus. American Journal of Veterinary Research, 46, 1561-1564.

Ou, L. and Marquis, R. (1970). Electromechanical interactions in cell walls of Gram-positive cocci. Journal of Bacteriology, 101, 92-101.

Owen, P. (1981). Immunology of the bacterial membrane. In: Organisation of Procaryotic Cell Membranes. Vol I. Ed. Ghosh, B.K. Pub. CRC Press Inc. Florida, USA, 73-164.

Parker, M.T. (1983). The significance of phage typing patterns in Staphylococcus aureus. In: Staphylococci and Staphylococcal Infections, Vol 1. Eds. Easmon, C.S.F. and Adlam, C. Pub. Academic Press, London. 34-62.

Parker, M.T. (1983/1984). Staphylococcal diseases. In: Topley and Wilson's Principles of Bacteriology, Virology and Immunity. 7th Edition, Vol 3. General Eds. Wilson, G., Miles, A. and Parker, M.T. Vol 3. Ed. Smith, G.R. Pub. Edward Arnold, London, 254-278.

Pedersen, K. (1980). Electrostatic interaction chromatography, a method for assaying the relative surface charges of bacteria. FEMS Microbiology Letters, 12, 365-367.

Pereira, A.T. (1961). Antigenic loss variation in Staphylococcus aureus. Journal of Pathology and Bacteriology, 81, 151-156.

Pereira, M.E.A. and Kabat, E.A. (1974). Blood group specificity of the lectin from Lotus tetragonolobus. Annals of the New York Academy of Sciences, 234, 301-305.

Perna, P., Di Lillo, M., Andreana, A., Utili, R. and Ruggiero, R. (1984). Increased susceptibility to rat serum of Staphylococcus aureus exposed to sub-inhibitory concentrations of cefamandole and penicillin. Microbios Letters, 26, 83-87.

Peterson, P.K., Gekker, G., Shapiro, R., Freiberg, M. and Keane, W.F. (1984). Polyamino acid enhancement of bacterial phagocytosis by human polymorphonuclear leukocytes and peritoneal macrophages. Infection and Immunity, 43, 561-566.

Peterson, P.K., Kim, Y., Wilkinson, B.J., Schemling, D., Michael, A.F. and Quie, P.G. (1978). Dichotomy between opsonisation on serum complement activation by encapsulated Staphylococci. Infection and Immunity, 20, 770-775.

Peterson, P.K., Verhoef, J., Sabath, L.D. and Quie, P.G. (1977). Effect of Protein A on Staphylococcal opsonisation. Infection and Immunity, 15, 760-764.

Peterson, P.K., Wilkinson, B.J., Kim, Y., Schmeling, D., Douglas, S.D. and Quie, P.G. (1978a). The key role of peptidoglycan in the opsonisation of Staphylococcus aureus. Journal of Clinical Investigation, 61, 597-609.

Peterson, P.K., Wilkinson, B.J., Kim, Y., Schemling, D. and Quie, P.G. (1978b). Influence of encapsulation on Staphylococcal opsonisation and phagocytosis by human polymorphonuclear leukocytes. Infection and Immunity, 19, 943-949.

Pistole, T.G. (1981). Interaction of bacteria and fungi with lectins and lectin-like substances. Annual Reviews of Microbiology, 35, 85-112.

Poston, S.M. and Naidoo, J. L. (1983). Genetics and antimicrobial drug resistance in the Staphylococci. In: Staphylococci and Staphylococcal infections, Vol 1. Eds. Easmon, C.S.F. and Adlam, C. Pub. Academic Press, London, 63-119.

Powell, D.A., Duckworth, M. and Baddiley, J. (1975). A membrane-associated lipomannan in Micrococci. Biochemical Journal, 151, 387-397.

Proctor, D.E., Mosher, D.F. and Olbrantz, J. (1982). Fibronectin binding of Staphylococcus aureus. Journal of Biological Chemistry, 257, 14788-14793.

Proctor, R.A., Olbrantz, P.J. and Mosher, D.F. (1983). Subinhibitory concentrations of antibiotics alter fibronectin binding to Staphylococcus aureus. Antimicrobial Agents and Chemotherapy, 24, 823-826.

Prokesch, R.C. and Hand, W.L. (1982). Antibiotic entry into human polymorphonuclear leukocytes. Antimicrobial Agents and Chemotherapy, 21, 373-380.

Pruul, H., Wetherall, B.L. and McDonald, P.J. (1982). The effect of exposure of Staphylococcus aureus to penicillin on susceptibility to the bactericidal activity of human leukocytes. Pathology, Sidney, 14, 263-267.

Quie, P.G., Giebink, G.S. and Peterson, P.K. (1981). Bacterial mechanisms for inhibition of ingestion by phagocytic cells. In Microbial Perturbation of Host Defences. Eds. Grady, F.O. and Smith, H. Pub. Academic Press, London, 121-141.

Quie, P.G., Mills, E.L., Cates, K.L., Abramson, J.S., Regelmann, W.E. and Peterson, P.K. (1983). Functional defects of granulocytes and susceptibility to Staphylococcal disease. In: Staphylococci and Staphylococcal Infections, Vol 1. Eds. Easmon, C.S.F. and Adlam, C. Pub. Academic Press, London, 243-273.

Ramirez-Ronda, C.H. and Gutierrez, J. (1980). Decreased adherence (AD) of Streptococcus sanguis to damaged heart valves by subinhibitory concentrations of penicillin in vitro. Programme of the Abstracts of the 20th Interscience Conference. Antimicrobial Agents and Chemotherapy. American Society of Microbiology, No 641.

Räsänen, L. and Arvilommi, H. (1982). Cell walls, peptidoglycans and teichoic acids of Gram-positive bacteria as polyclonal inducers and immunomodulators of proliferative and lymphokine responses of human B and T lymphocytes. Infection and Immunity, 35, 523-527.

Reeke, G.N., Becker, J.W., Cunningham, B.A., Gunther, G.R., Wang, J.L. and Edelman, G.M. (1974). Relationships between the structure and activities of concanavalin A. Annals of the New York Academy of Sciences, 234, 369-382.

Reeves, D.S., Phillips, I., Williams, J.D. and Wise, R. (1978). Laboratory Methods in Antimicrobial Chemotherapy. Pub. Churchill Livingstone, London.

Reeves, M.W., Drummond, M.C. and Tager, M. (1981). Partial purification and characterisation of the multiple molecular forms of Staphylococcal clotting activity (coagulase). Journal of Bacteriology, 148, 861-868.

Repine, J.E., White, J.G., Clawson, C.C. and Holmes, B.M. (1974). Effects of phorbal myristate acetate on the metabolism and ultrastructure of neutrophils in chronic granulomatous disease. Journal of Clinical Investigation, 54, 83-90.

Reynolds, P.E. and Fuller, C. (1986). Methicillin resistant strains of Staphylococcus aureus: presence of identical additional penicillin-binding protein in all strains examined. FEMS Microbiological Letters, 33, 251-254.

Richmond, M.H. (1968). The plasmids of Staphylococcus aureus and their relationship to other extrachromosomal elements in bacteria. In: Advances in Microbial Physiology, Vol 2. Eds. Rose, A.H. and Wilkinson, J.F. Pub: Academic Press Inc., New York.

Robinson, P., Wakefield, D., Breit, S.N., Easter, J.F. and Penny, R. (1984). Chemiluminescent response to pathogenic organisms: normal human polymorphonuclear leukocytes. Infection and Immunity, 43, 744-752.

Rogers, D.E. and Melly, M.A. (1962). Observations on the immunology of pathogenic Staphylococci. Yale Journal of Biology and Medicine, 34, 560-581.

Rogers, D.E. and Tompsett, M.D. (1952). The survival of Staphylococci within human leukocytes. Journal of Experimental Medicine, 95, 209-235.

Rogers, H.J. (1967). Killing of Staphylococci by penicillins. Nature, London, 213, 31.

Rogers, H.J. (1979). The function of bacterial autolysins. In: Microbial Polysaccharides and Polysaccharases. Eds: Berkeley, R.C.W., Gooday, G.W. and Ellwood, D.C. Pub: Society for General Microbiology/Academic Press. London. 237-268.

Rogers, H.J., (1983). Bacterial cell structure. Aspects of Microbiology, No. 6. Pub. Van Nostrand Reinhold Co, Berkshire.

Roitt, I. (1984). Essential Immunology. Pub. Blackwell Scientific Publications, London.

Rolinson, G.N. (1977). Sub-inhibitory concentrations of antibiotics. Journal of Antimicrobial Chemotherapy, 3, 111-113.

Root, R.K., Isturitz, R., Molavi , A., Metcalf, J.A. and Malech, H.L. (1981). Interactions between antibiotics and human neutrophils in the killing of Staphylococci. Studies with normal and cytochalasin B-treated cells. Journal of Clinical Investigation, 67, 247-259.

Root, R.K. and Metcalf, J. A. (1978). Augmented killing of penicillin-treated Staphylococcus aureus by human neutrophils: effect of cytochalasin-B. 18th Interscience Conference on Antimicrobial Agents and Chemotherapy. No. 204.

Root, R.K. and Metcalf, J.A. (1979). Surface binding and enhanced killing of penicillin-treated Staphylococcus aureus 502A by human neutrophils: mechanisms of the effect. Clinical Research, 27, 480 (Abstract).

Rosdahl, V.T. (1973). Naturally-occurring constitutive B-lactamase of novel serotype in Staphylococcus aureus. Journal of General Microbiology, 77, 229-231.

Rose, H.D., Lentino, J.R., Mavrelis, P.G. and Rytel, M.W. (1982). Jaundice associated with non-hepatic Staphylococcus aureus infection: does teichoic acid have a role in pathogenesis. Digestive Diseases and Sciences, 27, 1046-50.

Rosen, H. and Klebanoff, S.J. (1976). Chemiluminescence and superoxide production by myeloperoxidase-deficient leucocytes. Journal of Clinical Investigation, 58, 50-60.

Rosen, H. and Klebanoff, S.J. (1985). Oxidation of microbial iron-sulfur centres by the myeloperoxidase-H_2O_2-halide antimicrobial

system. Infection and Immunity, 47, 613-618.

Rosenberg, M. (1984). Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. FEMS Microbiology Letters, 22, 289-295.

Rosenberg, M., Gutnik, D. and Rosenberg, E. (1980). Adherence of bacteria to hydrocarbons. FEMS Microbiology Letters, 9, 29-33.

Rosenstein, I.J., Grady, D., Hamilton-Miller, J.M.T. and Brumfitt, W. (1985). Relationship between adhesion of Escherichia coli to uro-epithelial cells and the pathogenesis of urinary infection: problems in methodology and analysis. Journal of Medical Microbiology, 20, 335-344.

Rossi, L., Tonin, E., Cheng, Y.R. and Fontana, R. (1985). Regulation of penicillin-binding protein activity: description of a methicillin-inducible penicillin-binding protein in Staphylococcus aureus. Antimicrobial Agents and Chemotherapy, 27, 828-831.

Rozgonyi, F., Szitha, K.R., Ljungh, Å., Baloda, S.B., Hjertén, S. and Wadström, T. (1985). Standardisation of salt aggregation test for reproducible determination of cell-surface hydrophobicity with special reference to Staphylococcus species. Journal of Applied Bacteriology, 59, 451-457.

Russell, R.J., Wilkinson, P.J., McInroy, R.J., McKay, S., McCartney, A.C. and Arbuthnot, J.P. (1976). Effects of Staphylococcal products on locomotion and chemotaxis of human blood neutrophils and monocytes. Journal of Medical Microbiology, 9, 433-449.

Saleh, F.A.K. and Freer, J.H. (1984). Inhibition of secretion of Staphylococcal alpha-toxin by cerulenin. Journal of Medical Microbiology, 18, 205-216.

Sanders, C.C. and Sanders, W.E. (1983). Emergence of resistance during therapy with the newer beta-lactam antibiotics: role of inducible beta-lactamases and implications for the future. Review of Infectious Diseases, 5, 639-648.

Sanford, B.A., Davison, V.E. and Ramsay, M.A. (1986). Staphylococcus aureus adherence to influenza A virus-infected and control cell cultures: evidence for multiple adhesins. Proceedings of the Society for Experimental Biology and Medicine, 181, 104-111.

Schade, A.L. (1963). Significance of serum iron for the growth, biological characteristics and metabolism of Staphylococcus aureus. Biochemische Zeitschrift, 338, 140-148.

Schade, A.L. and Caroline, L. (1944). Raw hen egg white and the role of iron in growth inhibition of Shigella dysenteriae, Staphylococcus aureus, Escherichia coli and Saccaromyces cerevisiae. Science, 100, 14-15.

Schade, A.L. and Caroline, L. (1946). An iron-binding component in human blood plasma. Science, 104, 340-341.

Schade, A.L., Myers, N.H. and Reinhart, R.W. (1968). Carbohydrate metabolism and production of diffusible active substances by Staphylococcus aureus grown in serum at iron levels in excess of siderophilin iron-saturation and below. Journal of General Microbiology, 52, 253-260.

Schalén, C., Truedsson, L., Christensen, K.K. and Christensen, P. (1985). Blocking of antibody complement-dependent effector functions by Streptococcal IgG F_c-receptor and Staphylococcal Protein A. Acta Pathologica Microbiologica Immunologica Scandinavica. Section B, 93, 395-400.

Scheld, W.M., Strunk, R.W., Balian, G. and Calderone, R.A. (1985). Microbial adhesion to fibronectin in vitro correlates with production of endocarditis in rabbits. Proceedings of the Society for Experimental Biology and Medicine, 180, 474-482.

Scheld, W.M., Valone, J.A. and Sande, M.A. (1978). Bacterial adherence in the pathogenesis of endocarditis. Journal of Clinical Investigation, 61, 1394-1404.

Schindler, C.A. and Schuhardt, V.T. (1965). Purification of properties of lysostaphin — a lytic agent for Staphylococcus aureus. Biochemica et Biophysica Acta, 97, 242-250.

Schiötz, P.O., Höiby, N. and Hertz, J.B. (1979). Cross-reactions between Staphylococcus aureus and fifteen other bacterial species. Acta Pathologica Microbiologica Scandinavica. Section B, 87, 329-336.

Schleifer, K.H. (1983). The cell envelope. In: Staphylococci and Staphylococcal Infections, Vol 2. Eds. Easmon, C.S.F. and Adlam, C. Pub. Academic Press Ltd, London, 385-428.

Schmeling, D.J., Peterson, P.K., Hammerschmidt, D.E., Youngki, K., Verhoef, J., Wilkinson, B.J. and Quie, P.G. (1979). Chemotaxigenesis by cell surface components of Staphylococcus aureus. Infection and Immunity, 26, 57-63.

Seidl, P.H. and Schleifer, K.H. (1985). Secretion of fragments from bacterial cell wall peptidoglycan. In: Environmental Regulation of Microbial Metabolism. FEMS Symposium 23. Ed. Kulaev, I.S., Dawes, E.A. and Tempest, D.W. Pub. Academic Press, London, 443-450.

Seidl, P.H., Zwerenz, P. and Golecki, J.R. (1985). Streptococcus pyogenes grown under sublethal concentrations of penicillin G accumulates close to the septum, pentapeptide subunits of peptidoglycan. FEMS Microbiological Letters, 30, 325-329.

Sela, M.N., Lahav, M. and Ginsburg, I. (1977). Effect of leucocyte hydrolases on bacteria. IX. The release of lipoteichoic acid from group A Streptococci and from Streptococcus mutans by leucocyte extracts and by lysozyme: relation to tissue damage in inflammatory sites. Inflammation, 2, 151-164.

Shand, G.H., Anwar, H., Brown, M.R.W., Tranter, H., Modi, N. and Melling, J. (subm.). ELISA estimation of antibody response to human urinary tract infection due to Pseudomonas aeruginosa.

Shand, G.H., Anwar, H., Kadurugamuwa, J., Brown, M.R.W., Silverman, S.H. and Melling, J. (1985). In vivo evidence that bacteria in urinary tract infection grow under iron-restricted conditions. Infection and Immunity, 48, 35-39.

Shanson, D.C. (1982). Microbiology in Clinical Practice. Pub. Wright PSG. Bristol.

Shaw, D.J. (1969). Electrophoresis. Pub. Academic Press, London.

Shayegani, M.G. and Mudd, S. (1969). Adsorption of serum with Staphylococcal cell wall components and the effects on phagocytosis and killing. Bacteriological Proceedings, M, 104.

Sheagren, J.N. (1984). Staphylococcus aureus: the persistent pathogen. New England Journal of Medicine, 310, 1368-1373 and 1437-1442.

Sheth, N.K., Franson, T.R. and Sohnle, P.G. (1985). Influence of bacterial adherence to intravascular catheters on in vitro antibiotic susceptibility. Lancet, ii, 1266-1268.

Shibl, A.M. (1983). Effect of antibiotics on production of enzymes and toxins by micro-organisms. Review of Infectious Diseases, 5, 865-875.

Shockman, G.D. and Barrett, J.F. (1983). Structure, function and assembly of cell walls of Gram-positive bacteria. Annual Reviews of Microbiology, 37, 501-527.

Simpson, W.A. and Beachey, E.H. (1983). Adherence of group A Streptococci to fibronectin on oral epithelial cells. Infection and Immunity, 39, 275-279.

Sjöquist, J., Meloun, B. and Hjelm, H. (1972). Protein A isolated from Staphylococcus aureus after digestion with lysostaphin. European Journal of Biochemistry, 29, 572-578.

Sjöquist, J. and Stålenheim, G. (1969). Protein A from Staphylococcus aureus. Journal of Immunology, 103, 467-473.

Smith, H. (1977). Microbial surfaces in relation to pathogenicity. Bacteriological Reviews, 41, 475-500.

Smith, H. (1980). The effect of environmental conditions in vivo and in vitro on the determinants of microbial pathogenicity. In: The Molecular Basis of Microbial Pathogenicity. Eds. Smith, H., Skehel, J.J. and Turner, M.J. Pub. Verlag Chemie GmbH. Dahlem, 159-172.

Smith, H. (1984). The biochemical challenge of microbial pathogenicity. Journal of Applied Bacteriology, 47, 395-404.

Smyth, C.J., Jonsson, P., Olsson, E., Söderlind, O., Rosengren, J., Hjertén, S. and Wadström, T. (1978). Differences in hydrophobic surface characteristics of porcine enteropathogenic Escherichia coli with or without K88 Antigen as revealed by hydrophobic interaction chromatography. Infection and Immunity, 22, 462-472.

Speers, D.J. and Nade, S.M.L. (1985). Ultrastructural studies of adherence of Staphylococcus aureus in experimental acute haematogenous osteomyelitis. Infection and Immunity, 49, 443-446.

Spika, J.S., Peterson, P.K., Wilkinson, B.J., Hammerschmidt, D.E., Verbrugh, H.A., Verhoef, J. and Quie, P.G. (1982). Role of peptidoglycan from Staphylococcus aureus in leukopaenia, thrombocytopaenia, and complement activation associated with bacteraemia. Journal of Infectious Diseases, 146, 227-234.

Spink, W.W. and Vivino, J.J. (1942). The coagulase test for Staphylococci and its correlation with the resistance of the organisms to the bactericidal action of human blood. Journal of Clinical Investigation, 21, 353-356.

Spratt, B.G. (1980). Biochemical and genetical approaches to the mechanism of action of penicillin. Philosophical Transactions of the Royal Society of London, Series B, 289, 273-283.

Stendahl, O. (1983). The physiochemical basis of surface interaction between bacteria and phagocytic cells. In: Medical Microbiology, Vol. 3. Eds. Easmon, C.S.F., Jeljaszewicz, J., Brown, M.R.W. and Lambert, P.A. Pub: Academic Press, London, 137-152.

Stephen, J. and Pietrowski, R.A. (1981). Bacterial Toxins. Pub. van Nostrand Reinhold (UK) Co. Ltd., England.

Stevens, P. and Young, L.S. (1977). Quantitative granulocyte chemiluminescence in the rapid detection of impaired opsonisation of Escherichia coli. Infection and Immunity, 16, 796-804.

Stewart-Tull, D.E.S. (1980). The immunological activities of bacterial peptidoglycans. Annual Reviews of Microbiology, 34, 311-340.

Stjernholm, R.L., Allen, R.C., Steele, R.H., Waring, W.W. and Harris, J.A. (1973). Impaired chemiluminescence during phagocytosis of opsonised bacteria. Infection and Immunity, 7, 313-314.

Stossel, T.P. (1975). Phagocytosis: recognition and ingestion. Seminars in Haematology, 12, 83-116.

Strominger, J.L. and Ghuysen, J.-M. (1967). Mechanisms of enzymatic bacteriolysis. Science, 156, 213-221.

Strominger, J.L., Willoughby, E., Kamiryo, T., Blumberg, P.M. and Yocum, R.R. (1974). Penicillin-sensitive enzymes and penicillin-binding components in bacterial cells. Annals of the New York Academy of Sciences, 235, 210-224.

Styrt, B. and Klempner, M.S. (1985). Modification of interactions between neutrophils and Staphylococci by lysosomotropic weak bases. Infection and Immunity, 50, 415-419.

Sugarman, B. and Epps, L.R. (1985) Nutritional modulation of bacterial adherence. Infection, 43, (corresp).

Suginaka, H., Blumberg, P.M. and Strominger, J.L. (1972). Multiple penicillin-binding components in Bacillus subtilis, Bacillus cereus, Staphylococcus aureus and Escherichia coli. Journal of Biological Chemistry, 247, 5279-5288.

Suginaka, H., Shimatani, M., Ogawa, M. and Kotani, S. (1979). Prevention of penicillin-induced lysis of Staphylococcus aureus by cellular lipoteichoic acid. Journal of Antibiotics, Tokyo, 32, 73-77.

Sutherland, I.W. (1977). Surface carbohydrates of the procaryotic cell. Pub. Academic Press, London.

Switalski, L. (1976). Isolation and purification of Staphylococcal clumping factor. In: Staphylococci and Staphylococal Diseases. Proceedings of the 3rd International Symposium on Staphylococci and Staphylococcal Infections. Warsaw. Pub. Gustav-Fischer Verlag, Stuttgart.

Switalski, L.M., Rydén, C., Rubin, K., Ljungh, Å., Höök, M. and Wadström, T. (1983). Binding of fibronectin to Staphylococcus strains. Infection and Immunity, 42, 628-633.

Symington, F.W., Klebanoff, S.J., Waltersdorph, A. and Hakomori, S. (1984). Bacterial binding to neutrophil cell surface glycoconjugates triggers phagocytosis. Federal Proceedings, 43, 1097 (Abstracts).

Szewczyk, E. and Mikucki, J. (1983). Protein A as a substrate of endogenous metabolism in Staphylococci. FEMS Microbiological Letters, 19, 55-58.

Tewari, R., Tripathi, K.K., Nangia, A. and Gupta, K.G. (1984). Comparison of the bio-physical and biological properties of Staphylococcal Protein A extracted by salt-washing and conventional procedure. Zentrallblatt fur Bakteriologie, Mikrobiologie Hygiene. Series A, 258, 213-222.

Theodore, T.S. and Schade, A.L. (1965a). Growth of Staphylococcus aureus in media of restricted and unrestricted inorganic iron availability. Journal of General Microbiology, 39, 75-83.

Theodore, T.S. and Schade, A.L. (1965b). Carbohydrate metabolism of iron-rich and iron-poor Staphylococcus aureus. Journal of General Microbiology, 40, 385-395.

Thomas, V.L., Sanford, B.A., Jones, T.O., Triplett, R.G. and Keogh, B.S. (1986). Humoral immune response to Staphylococcus aureus in experimental osteomyelitis using immunoblot and gel diffusion. Abstracts of the Annual Meeting of the American Society of

Microbiology. 91. D-154.

Tipper, D.J. (1969). Mechanisms of autolysis of isolated cell walls of Staphylococcus aureus. Journal of Bacteriology, 97, 837-847.

Tipper, D.J. and Berman, M.F. (1969). Structures of the cell wall peptidoglycans of Staphylococcus epidermidis Texas 26 and Staphylococcus aureus Copenhagen. Biochemistry, 8, 2183-2192.

Tipper, D.J. and Strominger, J.L. (1965). Mechanisms of action of penicillins: A proposal based on their structural similarity to acyl-D-alany D-alanine. Proceedings of the National Academy of Sciences, USA, 54, 1133-1141.

Tomasz, A. (1979a). The mechanisms of the irreversible antimicrobial effects of penicillins: how the β-lactam antibiotics kill and lyse bacteria. Annual Reviews in Microbiology, 33, 113-137.

Tomasz, A. (1979b). From penicillin-binding proteins to the lysis and death of bacteria: a 1979 view. Reviews of Infectious Disease, 1, 434-467.

Tomasz, A. and Westphal, M. (1971). Abnormal autolytic activity in a pneumococcus with altered teichoic acid composition. Proceedings of the National Academy of Sciences, USA, 68, 2627-2630.

Tono-Oka, T., Ueno, N., Matsumoto, T., Ohkawa, M. and Matsumoto, S. (1983). Chemiluminescence of whole blood. Clinical Immunology and Immunopathology, 26, 66-75.

Towbin, H. and Gordon, J. (1974). Immunoblotting and dot immunoblotting. Current status and outlook. Journal of Immunological Methods, 72, 313-340.

Towbin, H., Stachelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proceedings of the National Academy of Sciences, USA, 76, 4350-4354.

Toy, P.T.C.Y., Lai, L.-W., Drake, T.A. and Sande, M.A. (1985). Effect of fibronectin on adherence of Staphylococcus aureus to fibrin thrombi in vitro. Infection and Immunity, 48, 83-86.

Tuazon, C.U., Sheagren, J.N. and Quie, P.G. (1981). Viability in the degree of opsonisation and phagocytosis of strains of Staphylococcus aureus isolated from patients with disseminated intravascular coagulation. Journal of Laboratory and Clinical Medicine, 98, 949-955.

Tufano, M.A., Romano-Carratelli, C., Sommese, L., Bentivoglio, C. and Galdiero, F. (1985). Modifications of surface properties in some enteropathogenic serogroups of Escherichia coli. Microbiologica, 8, 181-190.

Turnowsky, F., Brown, M.R.W., Anwar, H. and Lambert, P.A. (1983). Effect of iron limitation of growth rate on the binding of penicillin G to the penicillin binding proteins of mucoid and

non-mucoid strains of Pseudomonas aeruginosa. FEMS Microbiology Letters, 17, 243-245.

Ubukata, K., Yamashita, N. and Konno, M. (1985). Occurrence of a beta-lactam-inducible penicillin-binding protein in methicillin resistant Staphylococci. Antimicrobial Agents and Chemotherapy, 27, 851-857.

Uhlén, M., Guss, B., Nilsson, B., Gatenbeck, S., Philipson, L. and Lindberg, M. (1984). Complete sequence of the Staphylococcal gene encoding protein A. A gene evolved through multiple duplications. Journal of Biological Chemistry, 25a, 1695-1702.

Utsui, Y., Ohya, S., Takenouchi, Y., Tajima, M., Sugawara, S., Deguchi, K.and Suginaka, H. (1983). Release of lipoteichoic acid from Staphylococcus aureus by treatment with cefmetazole and other β-lactam antibiotics. Journal of Antibiotics, Tokyo, 36, 1380-1386.

Utsui, Y. and Yokota, T. (1985). Role of an altered penicillin-binding protein in methicillin and cephem-resistant Staphylococcus aureus. Antimicrobial Agents and Chemotherapy, 28, 397-403.

Valenti, P., Antonini, G., Rossifanelli, M.R., Orsi, N. and Antonini, E. (1982). Antibacterial activity of matrix-bound ovotransferrin. Antimicrobial Agents and Chemotherapy, 21, 840-841.

Valenti, P., De Stasio, A., Mastromerino, P., Seganti, L., Sinibaldi, L. and Orsi, N. (1981). Influence of bicarbonate and citrate on the bacteriostatic action of ovotransferrin towards Staphylococci. FEMS Microbiological Letters, 10, 77-79.

van Asbeck, B.S., Marx, J.J.M., Struyvenberg, A., van Kats, J.H. and Verhoef, J. (1984). Effect of iron (III) in the presence of various ligands on the phagocytic and metabolic activity of human polymorphonuclear leukocytes. Journal of Immunology, 132, 851-856.

van Oss, C.J. (1978). Phagocytosis as a surface phenomenon. Annual Reviews of Microbiology, 32, 19-39.

van Oss, C.J., Gillman, C.F. and Neuman, A.W. (1975). Phagocytic engulfment and cell adhesiveness. Pub. Marcel Dekker, New York.

van Snick, J.L., Masson, P.L. and Heremans, J.F. (1974). The involvement of lactoferrin in the hyposideremia of acute inflammation. Journal of Experimental Medicine, 140, 1068-1084.

van de Rijn, I., George, M., Bouvet, A. and Roberts, R.B. (1986). Enzyme-linked immunosorbent assay for the detection of antibodies to nutrionally variant Streptococci in patients with endocarditis. Journal of Infectious Diseases, 153, 116-121.

Vandenbroucke-Grauls, C.M.J.E., Thijssen, H.M.W.M. and Verhoef, J. (1985). Phagocytosis of Staphylococci by human polymorphonuclear leukocytes is enhanced in the presence of endothelial cells. Infection and Immunity, 50, 250-254.

Vaudaux, P.E., Zulian, G., Huggler, E. and Waldvogel, F.A. (1985). Attachment of Staphylococcus aureus to polymethyl methacrylate increases its resistance to phagocytosis in foreign body infection. Infection and Immunity, 50, 472-477.

Verbrugh, H.A., van Dijk, W.C., Peters, R., van der Tol, M.E. and Verhoef, J. (1979). The role of Staphylococcus aureus cell-wall peptidoglycan, teichoic acid and Protein A in the processes of complement activation and opsonisation. Immunology, 37, 615-621.

Verbrugh, H.A., Peters, R., Goessens, W.H.F. and Michel, M.F. (1986). Distinguishing complicated from uncomplicated bacteraemia caused by Staphylococcus aureus: the value of "new" and "old" serological tests. Journal of Infectious Diseases, 153, 109-115.

Verbrugh, H.A., Peters, R., Peterson, P.K. and Verhoef, J. (1978). Phagocytosis and killing of Staphylococci by human polymorphonuclear and mononuclear leucocytes. Journal of Clinical Pathology, 31, 539-545.

Verbrugh, H.A., Peters, R., Rozenberg-Arska, M., Peterson, P.K. and Verhoef, J. (1981). Antibodies to cell wall peptidoglycan of Staphylococcus aureus in patients with serious Staphylococcal infections. Journal of Infectious Diseases, 144, 1-9.

Verbrugh, H.A., Peterson, P.K., Nguyen, B.-Y.T., Sisson, S.P. and Kim, Y. (1982). Opsonisation of encapsulated Staphylococcus aureus: the role of specific antibody and complement. Journal of Immunology, 129, 1681-1687.

Vercellotti, G.M., Lussenhop, D., Peterson, P.K., Furcht, L.T., McArthy, J.B., Jacob, H.S. and Moldow, C.F. (1984). Bacterial adherence to fibronectin and endothelial cells: a possible mechanism for bacterial tissue tropism. Journal of Laboratory and Clinical Medicine, 103, 34-43.

Vercellotti, G.M., McCarthy, J.B., Lindholm, P., Peterson, P.K., Jacob, H.S. and Furcht, L.T. (1985). Extracellular matrix proteins (fibronectin, laminin and type IV collagen) bind and aggregate bacteria. American Journal of Pathology, 120, 13-21.

Verhoef, J. (1982). The role of the phagocyte in resistance to infection. Antonie van Leeuwenhoek, 48, 189-191.

Verhoef, J., Peterson, P.K., Kim, Y., Sabath, L.D. and Quie, P.G. (1977a). Opsonic requirements for Staphylococcal phagocytosis: Heterogeneity among strains. Immunology, 33, 191-197.

Verhoef, J., Peterson, P.K., and Quie, P.G. (1977b). Human polymorphonuclear leucocyte receptors for Staphylococcal opsonins. Immunology, 33, 231-239.

Verhoef, J. and Verbrugh, H.A. (1981). Host determinants in Staphylococcal disease. Annual Reviews of Microbiology, 32, 107-122.

Vosbeck, K. and Mett, H. (1983). Bacterial adhesion: influence of drugs. In: Medical Microbiology, Vol 3. Eds. Easmon, C.S.F., Jeljaszewicz, J., Brown, M.R.W. and Lambert, P.A. Pub. Academic Press, London, 21-62.

Wadström, T., Björnberg, S. and Hjertén, S. (1985a). Hydrophobised wound dressings in the treatment of experimental Staphylococcus aureus infections in the young pig. Acta Pathologica Microbiologica Immunologica Scandinavica. Section B, 93, 359-363.

Wadström, T., Brown, A., Ljungh, Å., Rozgonyi, F. and Hjertén, S. (1985b). Comparative hydrophobicity studies of Staphylococcus aureus and coagulase-negative Staphylococci in the salt aggregation test (SAT) and binding to various hydrophobic biomaterials. Abstracts of the Annual Meeting of the American Society of Microbiology, 26. B48.

Wadström, T., Hjertén, S., Jonnson, P. and Tylewska, S. (1981). Hydrophobic surface properties of Staphylococcus aureus, Staphylococcus saprophyticus and Streptococcus pyogenes. In: Staphylococci and Staphylococcal Infections. Ed. Jeljaszewicz, J. Pub. Gustav-Fischer Verlag. Stuttgart, 441-447.

Wadström, T., Thelestam, M. and Möllby, R. (1974). Biological properties of extracellular proteins from Staphylococcus. Annals of the New York Academy of Sciences, 236, 343-361.

Walton, E. and Gladstone, G.P. (1976). Factors affecting the susceptibility of Staphylococci to killing by the cationic proteins from rabbit polymorphonuclear leucocytes: the effects of alteration of cellular energetics and of various iron compounds. British Journal of Experimental Pathology, 57, 560-570.

Warren, G.H. and Gray, J. (1967). The influence of nafcillin on enzymic lysis of Staphylococcus aureus. Canadian Journal of Microbiology, 13, 321-328.

Warren, G.H. and Stasny, J.T. (1968). Electron microscopy of the action of nafcillin on Staphylococcus aureus. Chemotherapy, 13, 356-361.

Waterlot, Y., Cantinieaux, B., Hariga-Muller, C., De Maertelaere-Laurent, E., Vanherweghem, J.L. and Fondu, P. (1985) Impaired phagocytic activity of neutrophils in patients receiving haemodialysis: the critical role of iron overload. British Medical Journal, 291, 501-504.

Watson, D.L. (1975). Enhancement of in vitro phagocytosis of Staphylococcus aureus by polymorphonuclear leucocytes. Research in Veterinary Sciences, 19, 288-292.

Watson, D.L. (1976). The effect of cytophilic IgG2 on phagocytosis by ovine polymorphonuclear leucocytes. Immunology, 31, 159-165.

Watson, D.L. (1982). Virulence of Staphylococcus aureus grown in vitro or in vivo. Research in Veterinary Science, 32, 311-315.

Watson, D.L. and Lee, C.G. (1978). Immunity to experimental staphylococcal mastitis - comparison of live and killed vaccines. Australian Veterinary Journal, 54, 374-378.

Watson, D.L. and Prideaux, J.A. (1979). Comparisons of Staphylococcus aureus in vitro or in vivo. Microbiology and Immunology, 23, 543-547.

Waxman, D.J. and Strominger, J.L. (1983). Penicillin-binding proteins and the mechanism of action of β-lactam antibiotics. Annual Reviews of Biochemistry, 52, 825-69.

Weerkamp, A.H. and Handley, P.S. (1986). The growth rate regulates the composition and density of the fibrillar coat on the surface of Streptococcus salivarius K^+ cells. FEMS Microbiological Letters, 33, 179-183.

Weidel, W. and Pelzer, H. (1964). Bag shaped macromolecules - a new outlook on bacterial cell walls. Advances in Enzymology, 26, 193-232.

Weinberg, E.D. (1974). Iron and susceptibility to infectious diseases. Science, 184, 952-956.

Weinberg, E.D. (1978). Iron and infection. Microbiological Reviews, 42, 45-66.

Weinberg, E.D. (1984). Iron withholding: a defense against infection and neoplasia. Physiological Reviews, 64, 65-102.

Weir, D.M. (1980). Surface carbohydrates and lectins in cellular recognition. Immunology Today, August, 45-51.

Welch, W.D. (1980). Correlation between measurements of the luminol-dependent chemiluminescence response and bacterial susceptibility to phagocytosis. Infection and Immunity, 30, 370-374.

Wheat, L.J., Humphreys, D.W. and White, A. (1974). Opsonisation of Staphylococci by normal human sera: the role of antibody and heat-labile factors. Journal of Laboratory and Clinical Medicine, 83, 73-78.

Wheat, L.J., Kohler, R.B. and White, A. (1983). Diagnosis and management of deep-seated infection. In: Staphylococci and Staphyloccocal Infections, Vol 1. Eds. Easmon, C.S.F. and Adlam, C. Pub. Academic Press, London, 121-148.

White, A., Wheat, L.J. and Kohler, R.B. (1983). Diagnostic and therapeutic significance of Staphylococcal teichoic acid antibodies. Scandinavian Journal of Infectious Diseases,

Suppl 41, 105-116.

Wicken, A.J. and Knox, K.W. (1975). Lipoteichoic acids: a new class of bacterial antigen. Science, 187, 1161-1167.

Wicken, A.J. and Knox, K.W. (1977). Biological properties of lipoteichoic acids. In: Microbiology 1977. Ed. Schlessinger, D. Pub. American Society for Microbiology, Washington, 360-365.

Wicken, A.J. and Knox, K.W. (1980). Bacterial cell surface amphiphiles. Biochemica et Biophysica Acta, 604, 1-26.

Wilkinson, B.J. (1983). Staphylococcal capsules and slime. In: Staphylococci and Staphylococcal Infections, Vol 2. Eds. Easmon, C.S.F. and Adlam, C. Pub. Academic Press, London, 482-523.

Wilkinson, B.J., Peterson, P.K. and Quie, P.G. (1979). Cryptic peptidoglycan and the antiphagocytic effect of the Staphylococcus aureus capsule. Infection and Immunity, 23, 502-508.

Wilkinson, J.F. (1958). The extracellular polysaccharides of bacteria. Bacteriological Reviews, 22, 46-73.

Williams, A.J., Hastings, M.J.G., Easmon, C.S.F. and Cole, P.J. (1980). Factors affecting the in vitro assessment of opsonisation: a study of the kinetics of opsonisation using the technique of phagocytic chemiluminescence. Immunology, 41, 903-911.

Williams, M.R., Craven, N., Field, T.R. and Bunch, K.J. (1985). The relationship between phagocytosis and intracellular killing of Staphylococcus aureus by bovine neutrophils. British Veterinary Journal, 141, 362-371.

Williams, P., Lambert, P.A., Haigh, C.G. and Brown, M.R.W. (1986). The influence of the O and K antigens of Klebsiella aerogenes on surface hydrophobicity and susceptibility to phagocytosis and antimicrobial agents. Journal of Medical Microbiology, 21, 125-132.

Wilson, C.B. and Weaver, W.M. (1985). Comparative susceptibility of group B Streptococci and Staphylococcus aureus to killing by oxygen metabolites. Journal of Infectious Diseases, 152, 323-329.

Wiseman, GM. (1975). The haemolysins of Staphylococcus aureus. Bacteriological Reviews, 39, 317-356.

Wood, J.M. (1980). The interaction of micro-organisms with iron exchange resins. In: Microbial Adhesion to Surfaces. Ed. Berkley, R.C.W., Lynch, J.M., Melling, J., Rutter, P.R. and Vincent, B. Pub. Ellis Horwood, Chichester, UK, 163-185.

Wood, Jnr., W.B., Smith, M.R. and Watson, B. (1946). Studies on the mechanism of recovery in pneumococcal pneumonia. IV. The mechanism of phagocytosis in the absence of antibody. Journal of

Experimental Medicine, 84, 387-401.

Wright, T.A.E. and Douglas, S.R. (1904). An experimental investigation of the role of the blood fluids in connection with phagocytosis. Proceedings of the Royal Society (London), 72, 357-370.

Wyke, A.W. (1984). Isolation of the five penicillin-binding proteins from Staphylococcus aureus. FEMS Microbiological Letters, 22, 133-138.

Wyke, A.W., Ward, J.B. and Hayes, M.V. (1982). Synthesis of peptidoglycan in vivo in methicillin-resistant Staphylococcus aureus. European Journal of Biochemistry, 127, 553-558.

Xiu, J.H., Magnusson, K.-E., Stendahl, O. and Edebo, L. (1983). Physiochemical surface properties and phagocytosis by polymorphonuclear leucocytes of different serogroups of Salmonella. Journal of General Microbiology, 129, 3075-3084.

Yanai, M. and Quie, P.G. (1981). Chemiluminescence by polymorphonuclear leukocytes adhering to surfaces. Infection and Immunity, 32, 1181-1186.

Yoshida, K., Takahashi, M., Ohtomo, T., Minegishi, Y., Ichiman, Y., Kono, E., Haga, K. and San Clemente, C.L. (1979). Application of fluorescent antibody for detecting capsular substances in Staphylococcus aureus. Journal of Applied Bacteriology, 46, 147-152.

Young, S.E.J. (1982). Bacteraemia 1975-1980: a survey of cases reported to the PHLS Communicable Disease Surveilance Centre. Journal of Infection, 5, 19-26.

Yourtree, E.L., Metcalf, J.A., and Root, R.K. (1980). Augmented killing of penicillin-treated Staphylococcus aureus by human neutrophils: role of complement. Clinical Research, 28, 383A.

Yourtree, E.L. and Root, R.K. (1982). Antibiotic-neutrophil interactions in microbial killing. In: Advances in Host Defense Mechanisms. Eds. Gallin, J.I. and Fanci, A.S. Pub. Raven Press, New York, 187-209.

Zak, O. and Kradolfer, F. (1979). Effects of subminimal inhibitory concentrations of antibiotics in experimental infections. Review of Infectious Diseases, 1, 862-879.

Zeiger, A.R., Tuazon, C.U. and Sheagren, J.N. (1981). Antibody levels to bacterial peptidoglycan in human sera during the time course of endocarditis and bacteraemic infections caused by Staphylococcus aureus. Infection and Immunity, 33, 795-800.

Zierdt, C.H., MacLowry, J.D. and Robertson, E.A. (1982). Quantitation of pathogenic potential of Staphylococcus aureus. Journal of Clinical Microbiology, 16, 700-703.