ASPECTS OF STAPHYLOCOCCAL GROWTH, HAEMOLYSIS AND PHAGOCYTOSIS

by

SABAH, A.A. JASSIM, BSc, MPhil, CBiol, MIBiol

A Doctoral Thesis
Submitted in Partial Fulfilment of the
Requirements for the Award of the Degree of
Doctor of Philosophy

of

Loughborough University of Technology

May 1989

Supervisors: Dr W.G. Salt, BPharm, PhD, MPS, CBiol, MIBiol
Dr R.J. Stretton, BSc, PhD, MPS, CBiol, MIBiol

Dedication

I dedicate this work to my late father who died before seeing me through , and to my mother for her sacrifice , self-denial , unbounded love and continuous encouragement .

ACKNOWLEDGEMENTS

I would like to express my thanks to Dr W G Salt and Dr R J Stretton, my supervisors, for their constant interest and advice during the course of the research work presented here.

I am also grateful for the technical assistance rendered to me by Mr J Brennan.

For her patience in typing this thesis I would like to give my gratitude to Mrs Janet Smith.

I am greatly indebted to my dear wife Kathleen, and to my dearest brothers Saib and Dr Sahib for their constant encouragement, love, sacrifice and financial support throughout this work.

* * * * *

ABSTRACT

The effects of medium composition on the growth, biochemical and biological activities of selected coagulase positive and negative staphylococci have been investigated. The study concentrated on four major points, namely: nutrient requirements; haemolysin and enzyme production; their antigenic properties; and phagocytosis of the cells. The nutrient requirements for adequate growth of staphylococci was investigated in a chemically defined medium (CDM) for each strain to determine the supplemental, required and essential amino acids. It was found that strain/species specific differences in amino acid requirements occur amongst the six staphylococci. The growth of Staphylococcus epidermidis showed complete biotin-independence. Additionally, media derived from fish waste extract (FE) were prepared after developing a simple rapid method to digest the fish waste. FE was found a good substitute for beef extract in culture media.

The growth and alpha- and beta-haemolysin production (qualitatively or quantitatively) of staphylococci were investigated in chemically defined media varying in amino acid composition or concentration. Fish Infusion Broth (FIB), Brain Heart Infusion (BHI), yeast-extract, beef-extract, fish-extract, peptone and proteose peptone. The ability to produce a particular haemolysin was strain dependent and the quantity liberated was medium dependent. Highest yields of cells and haemolysins titres of staphylococci, were obtained in BHI and FIB. Detection of haemolysis was reliant on the species origin of the blood used. Staph. epidermidis produced haemolysis that is non-blood species specific and of a delta-haemolysin type on BHI based blood agars, but only rabbit blood was sensitive in agars based on a developed, chemically defined medium (CDM/A; thirteen amino acids). All six staphylococci grew in CDM/A. The addition of yeast extract to CDM/A suppressed beta-haemolysin formation, although beta haemolysin was detected in yeast extract/phosphate-buffered saline. The combination of proteose peptone in dilute solutions of the fish extract alone was synergistic in the production of alpha-haemolysin. A novel haemolytic profile is described for Staphylococcus aureus NCTC 8532 growing on blood agars based on CDM/A and may relate to the production of methaemoglobin during haemolysis.

Enzymes produced by staphylococci were studied. The ability to form certain enzymes was strain/species and medium dependent. The concurrent production of acetoin by staphylococci growing on lipids was investigated. Clumping occurred with Staph. aureus strain Wood 46 and NCTC 8532 when they were grown in chemically defined media.

Cells of Staph. aureus Wood 46 cultivated in the different media were immunologically (rabbit serum) similar. The phagocytosis (rabbit PMN's) of six staphylococci strains cultivated in BHI, FIB and a range of chemically defined media varying in composition or concentration of amino acids was studied in vitro. The effects of serum bactericidins and the bactericidial substances of bacteria-free and PNN's-free supernatant liquids (obtained after 120 min phagocytosis) on the survival of staphylococci (six strains) were also investigated. The intracellular-killing of Staph. aureus Wood 46 by PMN's and the effect of PMN's crude extracts (PMNCE) on the survival of Staph. aureus Wood 46 were also studied. It was found that the medium of incubation affected the susceptibility of staphylococci to phagocytosis, efficiency of intracellular killing, interaction with serum and PMNCE. Generally, the growth in the various chemically defined media, and to a lesser extent FIB, encourages intraleucocytic survival for staphylococci including Staph. epidermidis. Cells from a 5 amino acid medium (free of associated haemolysin), although still phagocytised, were more resistant to intracellular killing and to a PMNCE. Generally increasing the number of amino acids in chemically defined media from 5 to 12 or 13 increased the susceptibility of staphylococci to serum-bactericidins.

TABLE OF CONTENTS

Page No

INDEX OF TABLES

Page No

INDEX OF FIGURES

CHAPTER ONE

GENERAL INTRODUCTION

1.1 TAXONOMY

Staphylococcal taxonomy provides a good example of the problems of bacterial taxonomy in the three major aspects of classification, nomenclature and identification. The reasons for this are twofold: (i) the medical microbiologist was interested in identifying the pathogenic members of this group and their properties that enable him to trace the source of an infection; and (ii) the taxonomist was interested in how this organism fits into the general order of living things.

In classifying bacteria, taxonomists are in fact classifying their knowledge about them (Rescigno and Maccacaro, 1961). The history of staphylococcal taxonomy can be seen as differences between taxonomists and their knowledge at a particular time, as well as the application of several methods or approaches that have had a major impact on the whole practice of bacterial taxonomy (Hill, 1981).

One hundred years ago a number of workers had observed cocci in inflammations, abscesses and pus of humans. These organisms were called "micrococci" by Von Recklinghausen (1871) though Cohn (1872) has the credit for the name Micrococcus. Pasteur (1880) reached similar conclusions to those given by Ogston (1880), who showed conclusively, for the first time, that a cluster-forming coccus was the causative agent for certain pyogenic abscesses in man, and named it Staphylococcus (Ogston, 1882); deriving the name from the Greek nouns staphyle "a bunch of grapes" and coccus "a grain or berry".

Staphylococci were first classified by Rosenbach (1884) who was the first to grow isolates of Staphylococcus in pure culture, to study them in the laboratory. These organisms isolated from pus were identical to those observed and described by Ogston and therefore

Rosenbach adopted Ogston's name Staphylococcus for the genus, but he found that these were two differently coloured colonies: white ones which he called Staphylococcus pyogenes albus (Staphylococcus albus) and an orange one which he called Staphylococcus pyogenes aureus (Staphylococcus aureus). One year later, an organism producing a lemon-coloured colony was named Staphylococcus pyogenes citreus (Passet, 1885). Rosenbach caused some confusion among taxonomists by the use of both trinomials and binomials for the same organisms. Unfortunately, the trinomial system persisted for some time in the older literature. Staphylococcus aureus Rosenbach was accepted as the type species of the genus Staphylococcus by the Judicial Commission of the International Committee for Bacteriological Nomenclature (Editorial Board, 1958). The division according to the colour of the colonies is now of minor importance because it has been established that colourless variants of the so called Staph. aureus are of frequent occurrence.

Between 1884 and 1923, the year of the first edition of Bergey's Manual of Determinative Bacteriology, some 25 published classification systems did not recognise the genus Staphylococcus though 9 did (Hill, 1981). Most classifications in this period did not favour the separation of Staphylococcus from Micrococcus. Between the first edition of Bergey's Manual 1923 and 1948, when the sixth edition was published, the number of species listed under Staphylococcus varied from none to nine. The sixth edition deleted the genus Staphylococcus and relegated all Staphylococci to the genus Micrococcus. The number of species listed under Micrococcus for this same period varied from 22 to 46.

In the mid-fifties there was renewed interest in the classification of staphylococci and several publications presented views either for or against separating Staphylococcus from Micrococcus (Evans, et al, 1955; Van Eseltine, 1955; Breed, 1956; Thatcher and Simon, 1957). Evans, et al (1955) suggested that these genera could be distinguished by their ability to grow anaerobically and to produce acid from glucose. This test formed the basis for the reintroduction

of the genus Staphylococcus in the 7th edition of Bergey's Manual in 1957. In addition, two species, Staph. aureus and Staph. epidermidis, were recognised on the basis of anaerobic utilisation of mannitol and the production of coagulase by the former. Thus differentiation between micrococci and staphylococci appeared to be resolved.

However, in the proper endeavour over the years to identify pathogenic staphylococcal strains, greater attention had been paid to correlations between presumed pathogenicity and mannitol fermentation, with pigmentation, and or gelatin liquefaction, all duly reflected in Bergey's Manuals 1-6. Note was also taken of haemolysins, leucocidin and serological differences, with which Bergey's Manuals failed to keep updated until the 7th edition.

No one single, or indeed just a few tests, can be relied upon to give absolute divisions among members of a single strain. Lucas and Seeley (1955) reported a catalase-negative strain of Micrococcus pyogenes var aureus and coagulase negative variants of Staph. aureus are known. Thus different proposals, ideas, methods, fields and researches had been used to satisfy the needs for clear cut differentiation and classification. Such work includes: DNA base compositions (Rosypal, et al, 1966); numerical taxonomy technique (Hubalek, 1969); genetic transformation (Kloos and Schultes, 1969); and metabolism and cell walls (Baird-Parker, 1970).

New and good examples of methods differentiating even among strains were those based on differences in phage susceptibility antigenic structure, haemolysin pattern, different coagulase, and susceptibility to antibiotics (Meyer, 1967; Grun, 1968; Hajek and Marsalek, 1971; Oeding, Maradon, et al, 1971). Even in the 8th edition of Bergey's Manual (1974), which was reported to have abandoned most of the suprafamilial taxomomic rank's diagnostickeys of the dichotomous type were replaced in general by diagnostic-identification tables, and the enlargement of text descriptions of genera and species (Hill, 1981). This also left workers still

unsatisfied and unconvinced. Thus research continues and the application of many new tools, manuals, classification schemes including the tables of Schleifer and Kocur (1973), Schleifer and Kloos (1975), Kloos and Schleifer (1975a,b), Baird-Parker (1979), Schleifer et al, (1979), Kloos (1980), Schleifer and Stackebrandt (1983), Kloos and Jorgensen (1985), and Parisi (1985). This resulted in the description of many new species.

Now, according to the new edition of Bergey's Manual of Systematic Bacteriology (1986), the family Micrococcaceae consists of four genera: Micrococcus, Staphylococcus, Stomatococcus, and Planococcus. Their differential characteristics are listed in Table 1.1, and the genus Staphylococcus consists of 20 species. The differentiation and characteristics between them are indicated in Table 1.2.

TABLE 1.1
Differential Characteristics of the Genus Micrococcus, Stomatococcus, Planococcus, and Staphylococcus[a*]

Characteristics	Micro-coccus	Stomato-coccus	Plano-coccus	Staphy-lococcus
Irregular clusters	+	+	+	+
Tetrads	+	-	-	-
Capsule	-	+	-	-
Motility	-[b]	-	+	-
Growth on (FTO) furazolidone agar	+	-	-	-
Anaeorbic fermentation of glucose	-[c]	+	-	+
Oxidase and benzidine tests	+	-	ND	-[d]
FDP-aldolase (class)	II	ND	ND	I
Resistance to lysostaphin	R	R	R	S
Menaquinones	Hydro-genated	ND	Normal	Normal
Glycine present in peptido-glycan	-	-	-	+
Teichoic acid present in cell wall	-	-	-	+
Mol % G+C of DNA	65-75	56-60	39-52	30-39

a Symbols: +, 90% or more of strains are positive; -, 90% or more of strains are negative; ND, not determined; R, resistant; and S, susceptible.
b M. agilis is motile.
c M. kristinae produces acid from glucose anaerobically.
d Strains of S. sciuri and S. caseolyticus possess cytochrome c and are positive in the modified oxidase and benzidine tests.
* Bergey's Manual of Systematic Bacteriology (1986).

TABLE 1.2: Characteristics Differentiating the Species of the Genus Staphylococcus*

	1. Staph.aureus	2. S.epidermidis	3. S.capitis	4. S.warneri	5. S.haemolyticus	6. S.hominis	7. S.saccharolyticus	8. S.auricularis	9. S.saprophyticus	10a. S.cohnii subsp.1	10b. S.cohnii subsp.2	11. S.xylosus	12. S.simulans	13. S.carnosus	14. S.intermedius	15a. S.hyicus subsp. hyicus	15b. S.hyicus subsp.chromogenes	16. S.caseolyticus	17. S.sciuri	18. S.lentus	19. S.gallinarum	20. S.caprae
Colony diameter >5 mm	+	-	-	d	+	-	-	-	+	d	+	+	+	+	+	+	+	-	+	-	+	d
Colony pigment (carotenoid)	+w	-	-	d	d	d	-	-	d	-	d	d	-	-	-	-	+	d	d	d	d	-
Aerobic growth	+	+	+	+	+	+	-w	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anaerobic growth (thioglycolate)	+	+	(+)	+	(+)	-w	+	-w	(+)	d	(+)	d	+	+	(+)	+	+	-w	(+)	-w	+	+
Growth on NaCl agar																						
10% (w/v)	+	w	+	+	+	w	ND	+	+	+	+	+	+	+	+	+	+	ND	+	+	ND	ND
15% (w/v)	w	-	-w	w	d	-	ND	w	d	d	d	d	w	+	d	-w	-w	ND	d	-w	ND	ND
Growth at																						
15°C	+	-w	-	d	-w	-w	-w	-	+	+	+	+	+	+	+	+	+	ND	+	-w	ND	ND
45°C	+	+	+	+	+	+	+	+	d	d	d	-w	+	+	+	-w	-w	ND	-w	-	+w	+
Cytochrome c (oxidase test)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-
Lactic acid																						
L isomer	+	+	+	+	-	d	w	w	w	w	w	w	+	+	+	+	+	w	+	+	+	+
D isomer	+	-	-w	+	+	+	-	-	w	-	w	-w	d	+	-	-	-	-	-	-	-	-
Acetoin production	+	+	d	+	d	d	ND	d	+	d	d	d	-w	+	-	-	-	-	-	-	-	+
FDP-aldolase																						
Class I	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	ND	-	+	+	+	+
Class II	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	+	+	ND	+	-	-	-	-
Acid (aerobically) from																						
D-Xylose	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-w	+	-
L-Arabinose	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	ND	d	d	+	-
D-Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	+	+	+	-
D-Fucose	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-	-	-	ND	+	d	w	-
Raffinose	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-	-	-	ND	-	+	+	-
Salicin	-	-	-	-	-	-	ND	-	-	-	-	d	-	-	-	-	-	ND	+	d	+	-
Sucrose	+	+	(+)	+	+	(+)	-	d	+	-	-	+	+	-	+	+	+	d	+	+	+	-
Maltose	+	+	-	(+)	+	+	-	(+)	+	(d)	(+)	+	-w	-	(w)	-	d	+	(d)	d	+	d
D-Mannitol	+	-	+	d	d	-	-	-	d	d	d	d	+	+	(d)	-	d	-	+	+	+	d
D-Mannose	+	(+)	+	-	-	-	(+)	-	-	(d)	+	+	d	+	+	+	+	-	(d)	(+)	+	+
D-Trehalose	+	-	-	+	+	d	-	(+)	+	+	+	+	d	d	+	+	+	d	+	+	+	+
α-Lactose	+	d	-	ds	d	d	-	-	d	-	+	d	+	d	d	+	+	+	-w	d	d	+
D-Galactose	+	d	-	d	d	d	ND	-	-	-	d	d	-w	d	+	+	+	+	(+)	d	+	+
β-D-Fructose	+	+	+	+	d	+	(+)	+	+	+	+	+	+	+	+	+	+	+	+	(+)	+	-
D-Melezitose	-	(d)	-	ds	-	d	ND	-	-	-	-	-	-	-	-	-	-	ND	d	-	+	-
D-Turanose	+w	d	-	d	d	d	ND	(d)	+	-	-	d	-	-	d	-	d	-	-	-	+	-
D-Ribose	+	d	-	d	d	-	ND	-	-	-	-	d	d	ND	+	+	+	+	+	+	+	-
Xylitol	-	-	-	-	-	-	-	-	d	(d)	(d)	-w	-	-	-	-	-	-	-	-	d	-
Hyaluronidase	+	d	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	-	ND	ND	ND	-	-
Growth on $(NH_4)_2SO_4$ (nitrogen source)	-	-	-	-	-	-	ND	ND	d	d	d	+	-	ND	-	ND	ND	ND	+	+	ND	ND
Nitrate reduction	+	+w	d	-w	d	d	+	(d)	-	-	-	d	+	+	+	+	+	+	+	+	+	+
Alkaline phosphatase	+	+	-	-	-	-	ND	-	-	-	+w	d	w	+	+	+	+	ND	+w	w	+	+
Arginine dihydrolase	+w	+w	d	d	+	d	+	d	-w	-	-w	-	+	+	ds	+	+	ND	-	-	-	+
Urease	+w	+	-	+	-	+	ND	-	+	-	+	+	+	-	+	d	d	ND	-	-	+	+
Coagulase (rabbit plasma)	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	d	-	-	-	-	-	-
Clumping factor	+	-	-	-	-	-	-	-	-	-	-	-	-	-	d	-	-	-	-	-	-	-
Fibrinolysin	de	d	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	d	-	ND	ND	ND	-	-
Haemolysis	+	-w	-w	(ds)	(+)	-w	-	-	-	(d)	(d)	-w	-w	-	d	-	-	-	-	-	w	(+)
Deoxyribonuclease (DNase agar)	+	-w	w	ds	ds	-w	ND	-w	-	-w	-w	-w	w	w	+	+	w	ND	+w	+w	ND	+
Heat-stable nuclease	+	-w	-	-	-	-	ND	-	-	-	-	-	-w	d	+	+	-w	ND	ND	ND	-	-
β-glucosidase	+	(d)	-	+	d	-	ND	-	d	-	-	+	-	-	d	d	d	ND	+	+	+	-
β-glucuronidase	-	-	-	d	d	-	ND	-	-	-	+	d	d	-	-	d	-	ND	-	-	-	-
β-galactosidase	-	-	-	-	-	-	ND	(d)	d	-	+	+	+	+	ds	-	-	ND	-	-	-w	-
Novobiocin resistance (MIC ⩾ 1.6 g/ml)	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	+	+	-

Symbols: +, 90% or more strains positive; -, 90% or more strains negative; d, 11-89% strains positive; ( ) delayed reaction; w, weak reaction; -w, negative to weak reaction; +w, positive to weak reaction; ds, test differentiates subspecies (not separated out above in heating); de, test differentiates ecotypes; ND, test not determined.

* Bergey's Manual of Systematic Bacteriology (1986)

Studies on the taxonomy of staphylococci continue, and three new species were found (Staph. Kloosii, Staph. equorum and Staph. arlettae) by Schleifer (1986). Now the genus Staphylococcus comprises 23 different species, most of which are coagulase-negative, while Staph. aureus, Staph. intermedius and some strains of Staph. hyicus are coagulase-positive (see Table 1.2). As we have seen, the taxonomy of staphylococci is still difficult even with all the accumulated advances in knowledge and techniques. The need for new tools for classification and taxonomy appears to be very important.

1.2 DESCRIPTION OF MEMBERS OF GENUS STAPHYLOCOCCUS

The members of this genus are non-motile, Gram-positive, usually oxidase negative and usually catalase-positive cocci. They are 0.5-1.5 μm in diameter and are able to divide in more than one plane to form irregular clusters of cells, though some single cells and pairs are also seen as are occasional, short chains. The cells wall contains peptidoglycan and teichoic acid. The diamino acid present in the peptidoglycan is L-lysine. They are chemoorganotrophs with respiratory and fermentative metabolism. They are thus aerobic and faculatatively anaerobic organisms that require complex media for growth. On most non-selective media (Blood agar, Nutrient agar, Brain-Heart Infusion agar), abundant growth occurs in 16 to 24h at 35 to 37°C, and their doubling time (mean generation time) can be as short as 20 min. Colonies are usually 1-3 mm in diameter within 24h of incubation. They are smooth, circular, and raised to slightly convex with a "creamy" consistency. In chemically defined media, the nutritional requirements are variable (see 1.4). Most species identified require an organic source of nitrogen, i.e. certain amino acids, and B group vitamins. Some others can grow with $(NH_4)_2SO_4$ as a sole source of substrate nitrogen e.g. some strains of Staph. sciuri, some strains of Staph. saprophyticus, and some strains of Staph. cohnii. Uracil and/or a fermentable carbon source may be required by certain species for anaerobic growth (Kloos and Schleifer, 1986). They are typical mesophiles growing at

temperatures between 6.5 and 46°C with optimum 35-40°C. They can withstand temperatures of 60°C for half an hour, and can be stored at 4°C for months. They grow well at pH values between 4.5 and 9.3 (optimum 7.0-7.5).

Among nonspore-forming bacteria, staphylococci include some of the hardiest organisms. They are relatively resistant to drying and high salt concentrations (most strains grow in the presence of 10% NaCl). Sodium selenite, sodium azide, crystal violet, and gentian violet can, however, be used to inhibit the growth of staphylococci. They are susceptible to lysis by lysostaphin endopeptidase, which breaks the glycyl-glycine links in the peptide bridges of the peptidoglycan, though they are generally resistant to lysis by the muramidase lysozyme.

Some species are resistant to methicillin, novobiocin and penicillins. Natural populations are mainly associated with skin, skin glands and mucous membranes of warm-blooded animals. Some species are opportunistic pathogens of humans and/or animals.

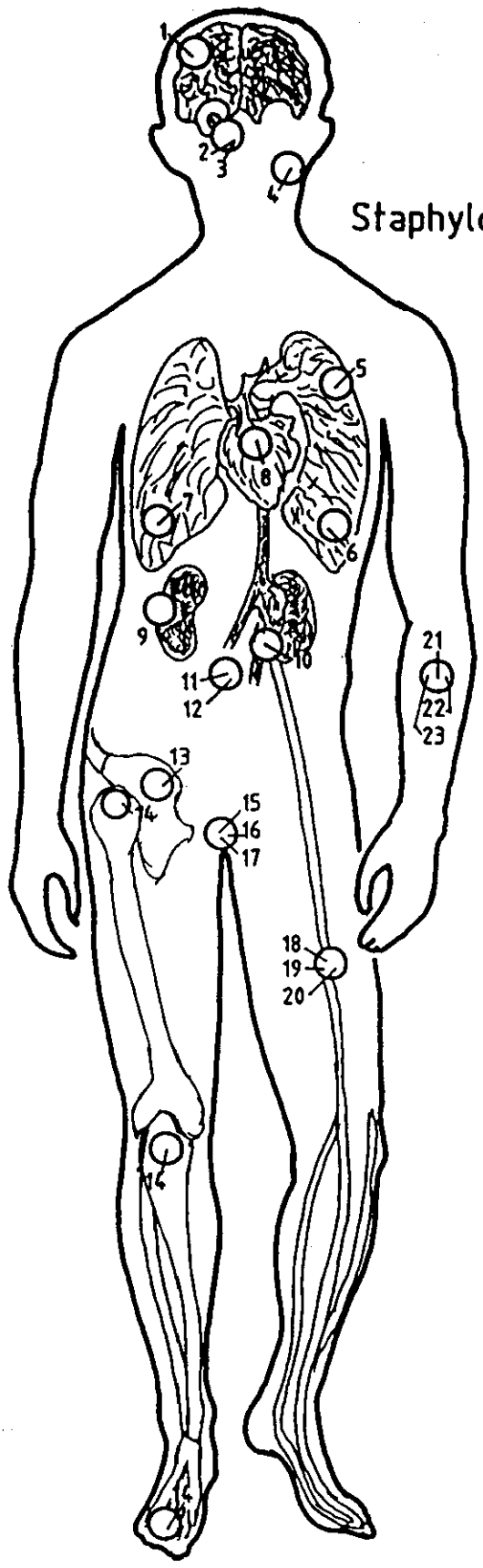
They are also more resistant than most bacteria to certain disinfectants (e.g. phenol, mercuric chloride and iodides). Carotenoid pigments may be present. Fermentation of glucose under anaerobic conditions results in the production of, mainly, lactic acid, whereas in the presence of air mainly acetic acid and small amounts of carbon dioxide are produced. Also a wide range of carbohydrates may be utilised in the presence of air, with the production of acid but no gas detectable by standard procedures. They produce acid aerobically from glycerol. Acetoin is usually formed as an end product of glucose metabolism and the terminal pH in unbuffered glucose broth is typically 4.2 to 5.4. Acid is not usually formed from arabinose, cellobiose, inositol, inulin, or raffinose. Starch and aesculin are not usually hydrolysed. The Guanine plus Cytosine (G+C) content of DNA is 30-39 moles percent.

1.3 SIGNIFICANCE OF STAPHYLOCOCCAL INFECTION

Skin infections caused by Staph. aureus are the most common human staphylococcal infections. These include (see Figure 1.1) cellulitis, pustules, boils, carbuncles, impetigo and post-operation wound infections of various sites. Also, foreign bodies enhance the likelihood that staphylococcal infection will develop by reducing the number of staphylococci needed to initiate infection. It was found that more than 10^6 virulent staphylococci were required to initiate a minor infection in healthy persons, whereas 10^2 staphylococci can infect when a foreign body was present (Elek and Conen, 1957). Bacteremia may arise from the local site and can lead to general Staph. aureus infection and abscess formation in any organ of the body (Nolan and Beaty, 1976). Sequelae include such entities as meningitis (Wellman and Senft, 1964), osteomyelitis (Waldvogel, et al, 1970), and endocarditis (Watanakunakorn and Baird 1977).

Damaged tissues, e.g. the skin in burns and the airways in influenza, are predisposed to colonization and then proliferation of Staph. aureus. A common consequence of staphylococcal infection in burn patients is septicemia (Artz and Moncrief, 1969) and in influenza patients is Staph. aureus pneumonia (Finland, et al, 1942). Other abnormalities of host defences such as neutropenia (Ladisch and Pizzo, 1978; Kilton, et al, 1979), chronic granulomatous disease (Quie, 1973), immunosuppression (Cluff, Reynolds, et al, 1968) and renal failure (Montgomerie. et al, 1968) predispose to more frequent occurrence and greater severity of Staph. aureus disease.

Staph. aureus has been isolated from infections of all organs of the body, however, the disease mechanisms are not well understood. Many factors found in Staph. aureus are considered virulence factors. These include: coagulase, thermostable nuclease (TNase), alpha-haemolysin, leucocidin, and protein A. Strains from serious infections have been shown to have more of these factors than



Staphylococcal Infection.

1 Brain abscess

2 Hypopyon

3 Endopthalmitis

4 Parotitis

5 Lung abscess

6 Pneumonia with abscess

7 Breast infections

8 Endocarditis

9 Renal carbuncle

10 Foreign bodies (grafts prostheses)

11 Peritonitis

12 Food poisoning

13 Septic arthritis

14 Osteomyelitis

15 Genitourinary tract infections

16 Diabetic gangrene

17 Toxic-shock syndrome (in females only)

18 Deep wound infection

19 Thrombophlebitis

20 Bacteremia

21 Empyema

22 Boils, Furnucles, Carbuncles

23 Skin infections: (i) Bullous impetigo

(ii) Scalded skin syndrome.

Fig. 1. 1. A diagrammatic representation showing examples of *Staph.aureus* infections in the human.

strains from carriers, but little is known about the role these factors play in initiating infections or about the interactions that may allow staphylococci to invade the host. Staph. aureus organisms isolated from fresh tissues are more virulent than desiccated organisms in air or in fomites (Maltman, et al, 1960). Some strains such as the epidemic "80/81 complex" are epidemiologically more virulent (Nahmias, Sakurai, et al, 1961; Finland, 1973). Therefore, Staph. aureus is a complex organism with a variety of bacterial products with the potential for promoting disease (Musher and McKenzie, 1977).

The extracellular products, enterotoxin (which produces food poisoning) and exfoliatin (which causes the scalded skin syndrome) clearly produce diseases directly (Morse, 1973). Toxic shock syndrome, also has been attributed to infection or colonization with Staph. aureus (Schlievent, Shands, et al, 1981). Other staphylococcal products have not been decisively related to disease (Elek, 1959; Musher and McKenzie, 1977). On the other hand, Staph. aureus caused by 1950's considerable morbidity and mortality as a pathogen of hospitalized patients and approximately 80% of hospital acquired infections were caused by pencillinase producers (Kirby, 1965). However, methicillin-resistant Staph. aureus strains (MRSA) have recently emerged as a major clinical and epidemiological problem in the USA hospitals (Boyce and Causey, 1982; Haley, Hightower, et al, 1982).

The other coagulase-positive species Staph. intermedius and the coagulase-variable species Staph. hyicus are of particular importance to veterinary medicine. These species and Staph. aureus are serious opportunistic pathogens of animals. Staph. intermedius has been implicated in a variety of infections in dogs such as otitis externa, pyoderma, abscesses, reproductive tract infections, mastitis, and wound infections (Devriese and Hajek, 1980; Phillips and Kloos, 1981; Raus and Love, 1983). Staph. hyicus subspecies hyicus (a coagulase-variable subspecies) has been implicated in infectious exudative epidermitis and septic polyarthritis of pigs

and is occasionally isolated from the milk of cows suffering from mastitis (Devriese, 1977; Phillips, et al, 1980). Staph. hyicus subspecies chromogenes (a coagulase-negative subspecies) may be isolated from the skin of pigs and cows and it is commonly isolated from the milk of cows suffering from mastitis, although its role as an etiologic agent is not yet clear (Kloos and Schleifer, 1986).

During the past ten years, there has been increased concern for the other staphylococci that constitute a major component of our normal microflora, the coagulase-negative staphylococci. These species are endogenous to humans and certain animals and have previously been considered to be saprophytic or of low pathogenicity. Recently, more information has documented examples of infections by coagulase-negative species. These infections include bacteremia and infective endocarditis; infection or colonization of ventriculoatrial or other cerebrospinal fluid shunts, intravenous catheters, and joint prostheses; peritonitis associated with dialysis; osteomyelitis or pyoarthritis; and, prominently, genito-urinary tract infections (Holt, 1971; Wilson, Salvati, et al, 1973; Rubin, Rodgers, et al, 1980; Eng, Wang, et al, 1982; Peters, et al, 1982; Sewell, Clarridge, et al, 1982; Karchmer, et al, 1983).

However methicillin-resistant Staph. epidermidis strain (MRSE) has also recently emerged as a clinical problem, especially in patients with prosthetic heart valves (Karchmer, et al, 1983), or those who have undergone other forms of cardiac surgery (Archer and Tenenbaum, 1980).

1.4 NUTRITIONAL REQUIREMENTS FOR STAPHYLOCOCCI GROWTH AND HAEMOLYSIN PRODUCTION

Interest in the nutritional requirements of the staphylococci has greatly increased since the classic studies of Gladstone (1937). This may be attributed to the seriousness of the problem of the development of resistance to certain antibiotics and infections in both animals and humans by these organisms. The nutritive

requirements of staphylococci are complex and varied for each strain (Yamaguchi and Kurokawa, 1972). This may be due to different nutrient markers which have been realised in different staphylococci strains isolated from various sources (Tschape, 1973). However, strains of Staph. aureus should be polyauxotrophic organisms according to several authors (Edgar and Stocker, 1961; Kloos and Pattee, 1965; Smith and Pattee, 1967; Sompolinski, et al, 1969; Proctor and Kloos, 1970). They need, in their media: proteins, carbohydrates, minerals and vitamins to give good growth and to produce different types of toxins and enzymes.

There have been numerous studies of the growth of Staph. aureus to examine toxin production in chemically defined media. Dalen (1973a) showed that histidine stimulated an early appearance of alpha-lysin in a synthetic amino acid medium and that serine and glycine stimulated its production in the late growth phase. The requirements of Staph. aureus for beta-toxin production in chemically defined media was studied by Sharma and Haque (1973) who found that glutamine, proline, and cystine were required under both air and carbon dioxide incubation. Wiseman (1975) found that arginine, proline and glycine were an absolute requirement, as were cystine and methionine together, but not separately, for the production of Staph. aureus beta-toxin. He also concluded that there was no clear distinction between amino acid requirements for growth and haemolysin production. Addition of free fatty acids to the medium increased the rate of staphylococci growth, but without increasing levels of beta-toxin (Fritsche and Zitz, 1973). When growing in such media staphylococci required a number of amino acids and vitamins.

Gladstone (1938) reported that the amino acids arginine, proline, phenylalanine, glycine, and valine stimulated the production of alpha-toxin, whilst beta-toxin was only detected in small quantities. However, the amino acid requirements for alpha- and beta-toxin formation of Staph. aureus are various. Surgalla (1947) showed that strains of Staph. aureus grown in chemically defined

media required aspartic acid, glutamic acid, histidine, hydroxy-proline, leucine, lysine, and tyrosine to produce a better yield of alpha-toxin.

Fildes, Richardson, et al (1936); Gladstone (1937); Knight (1937) and Surgalla (1947) found that staphylococci grew well in media containing 14 to 16 amino acids; and for good anaerobic growth, most staphylococci strains also require the addition of uracil and/or a fermentable carbon source (e.g. pyruvate) (Richardson, 1936; Jones, et al, 1963; Evans, 1976; Kloos and Schleifer, 1986).

Staphylococci can be trained to grow (slowly) in an ammonia based, amino acid free medium (Gladstone, 1937). It is, however, usual for defined media containing as many as 18 amino acids to be used routinely in metabolic studies of the staphylococci (Pattee and Neveln, 1975; Giehl, et al, 1987).

The general vitamin requirements, for nicotinic acid, thiamine, and biotin, in the growth of staphylococci were re-established by Mah, et al, (1967), and by Cove, et al, (1980). Thiamine and nicotinic acid required for the growth of coagulase-positive staphylococci (Knight, 1937; Gretler and Evans, 1951); thiamine and nicotinamide were essential for beta-toxin production (Sharma and Haque, 1973; Wiseman, 1975); and biotin, in particular, was found essential for all coagulase-negative staphylococci strains (Gretler and Evans, 1951; Gretler, Mucciolo, et al, 1955; Cove, et al, 1980).

1.5 THE EFFECT OF MEDIA-CONSTITUENT ON THE CHEMICAL COMPOSITION OF THE CELL WALL OF STAPHYLOCOCCI

The cell wall of staphylococci is typical of the Gram-positive bacteria. Chemically, the walls of all staphylococci consist of peptidoglycan and teichoic acid (see Figure 1.2a, b, c and d). In some cases a major protein has been found in Staph. aureus which is known as staphylococcal protein A.

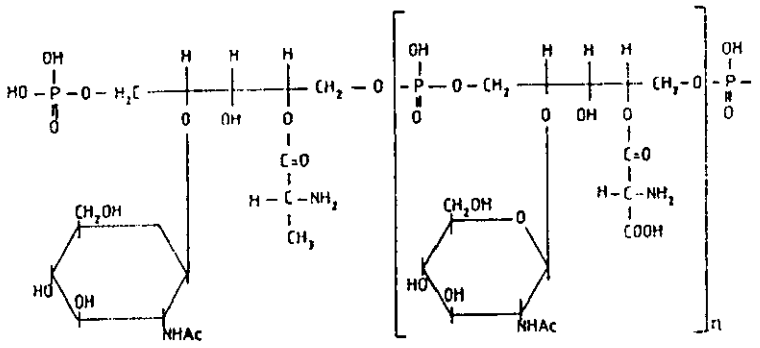


Fig. 1. 2a. Repeating unit of ribitol teichoic acid from *Staphylococcus aureus*.

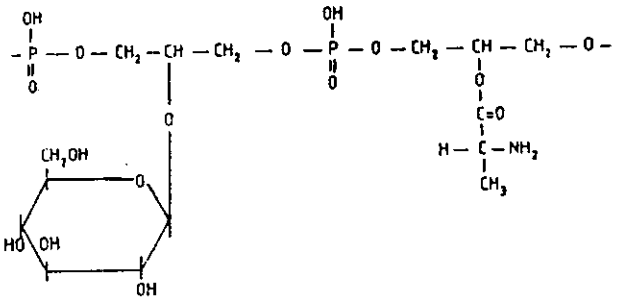


Fig. 1. 2b. Repeating unit of glucosyl substituted cell wall glycerol teichoic acid of *Staphylococcus epidermidis*.

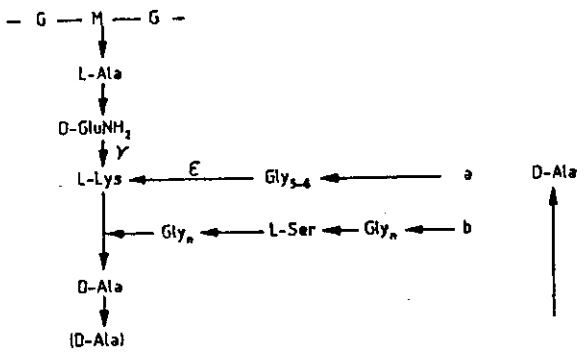


Fig. 1. 2c. Peptidoglycan types of staphylococci. (i) *Staphylococcus aureus*; (ii) *Staphylococcus epidermidis*.
Abbreviations: G, *N*-acetylglucosamine; M, *N*-acetylmuramic acid; ε, ε-amino group of lysine.
The muramyl 6-hydroxyl groups can be acetylated or to a lesser extent phosphorylated. *n*, 1-3.
L Serine can be partly replaced by glycine.

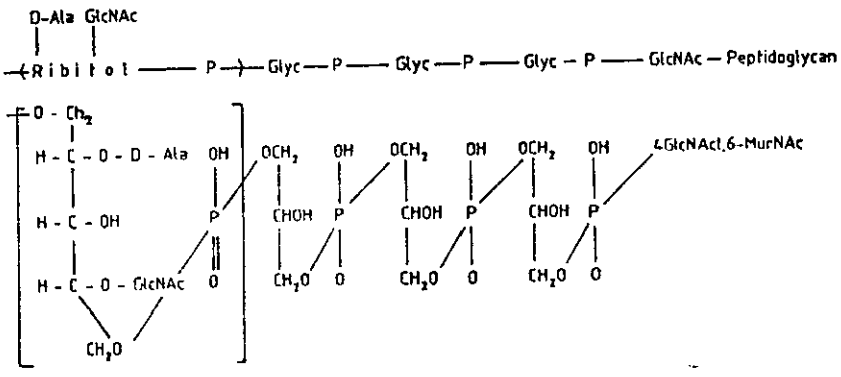


Fig. 1. 2d. Linkage unit between cell wall teichoic acid of *Staphylococcus aureus* and peptidoglycan.
Abbreviations: GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; Glyc, glycerol.

However, the chemical composition of staphylococci cell walls is subjected to distinct phenotyic variation or modifications that may markedly affect the surface properties of the bacteria (Ellwood and Tempest, 1972; Schleifer et al, 1976).

A. Teichoic Acid

The cell wall teichoic acids are the major group antigen of Staph. aureus, but they are present, as a rule, in all gram-positive bacteria either as cell wall or cell membrane components (Knox and Wicken, 1973). The wall teichoic acid of Staph. aureus is a ribitol-phosphate polymer substituted with alpha- and beta-linked N-acetyl-glucosamine (see Figure 1.2a) residues which are the main antigenic determinants (Sanderson, et al, 1962; Torii, et al, 1964). Glycerol teichoic acid with glucosyl residues have been reported in Staph. epidermidis (Oeding, and Grov, 1972) (see Figure 1.2b).

Nutrient limitations may, however, influence the content and chemical composition of teichoic acid. Walls of the Staph. aureus strain H grown in simple chemically defined medium containing four amino acids (cysteine, arginine, proline and glutamic acid) in continuous culture under potassium limitation, differ in composition from the wall of the same organism grown in rich nutrient broth in that the teichoic acid component contains a reduced proportion of N-acetylglucosaminyl substituents (Archibald and Heckels, 1975). Also, Dobson and Archibald (1978) observed that the proportion of ribitol teichoic acid present in the wall of Staph. aureus H, and the extent to which it is substituted with N-acetylglucosamine varied in Staph. aureus H grown in rich nutrient broth and in simple chemically defined media. They concluded that the degree of substitution with N-acetylglucosamine varied from about 50 to 90%, and the growth in one amino acid (cysteine) medium resulted in substantial alterations in the composition of the cell wall. Since the teichoic acid of Staph. aureus is known to be antigenic (Ellwood and Tempest, 1972), these variations in the chemical composition may also affect the biological activity of the whole organism during infections.

B. Peptidoglycan (Mucopeptide, Murein)

Peptidoglycan is the most important component structure of the bacterial cell wall, and may be the key cell wall component involved in staphylococcal opsonization (Peterson, Wilkinson, et al, 1978c).

The strength and shape of the cell wall are due primarily to peptidoglycan. These are complex macromolecules, found only in walls of microrganisms, that totally enclose the plasma membrane. Peptidoglycan is a heteropolymer consisting of glycan chains (see Figure 1.2c) which are crosslinked through short peptides (Schleifer and Kandler, 1972). The composition of staphylococci peptidoglycan has been shown to be influenced by the composition of the medium in which the bacteria are grown (Korman, 1966; Zygmunt, et al, 1967; Browder, et al, 1968; Schleifer, 1969; Schleifer and Kandler, 1972; Archibald and Heckels, 1975).

Staph. aureus strain Copenhagen and Staph. epidermidis strain 66 show substantial changes in the composition of the peptide bridge unit in response to changes in the amino-acid composition of the growth medium (Schleifer, et al, 1969). Addition of L-serine to the growth medium yielded a higher L-serine and a lower glycine content in cell walls of staphylococci (Zygmunt, et al, 1967; Browder, et al, 1968).

A chemically defined medium (Laue and MacDonald, 1968) was used to study the influence of the amino acids glycin, L-threonine and L-alanine on the composition of the peptidoglycan of Staph. aureus by Schleifer, et al, (1976). They found that the amino acid glycine and L-alanine content of the growth medium has a decisive influence on the composition of the peptidoglycan of Staph. aureus. On the other hand, the growth of Staph. aureus H under conditions of potassium, carbon or nitrogen-limitation resulted in a greatly diminished degree of crosslinking in the peptidoglycan (Dobson and Archibald, 1978). It is well known that the peptidoglycan composition of bacteria is fixed genetically. However, phenotypic changes can occur to a variable degree. For example, phenotypic modifications of

the peptidoglycan occurred also under growth-inhibiting concentrations of glycine and D-amino acids (Hammes, et al, 1973; Schleifer, et al, 1976; Trippen, Hammes, et al, 1976). Therefore, two genetically different strains of staphylococci can appear phenotypically similar if they are cultured under unusual conditions. For example, when Staph. aureus is grown in a serine-enriched medium, its peptidoglycan shows a similar chemical composition and an amino acid sequence to that of Staph. epidermidis grown in a serine-deficient medium (Schleifer, et al, 1976).

1.6 EXTRACELLULAR AND CELLULAR STAPHYLOCOCCAL PRODUCTS

Staph. aureus remains a well respected pathogen in relation to both community and hospital acquired infections in man and animals due to produce numerous extracellular and cellular substances. Staph. aureus produces up to 30 different types of extracelllular products (Wadstrom, et al, 1974; Russell, Wilkinson, et al, 1976) and these products cause many biological effects on a variety of mammalian cells and systems (Table 1.3).

The properties of staphylococcal toxins are quite complicated because none of the staphylococcal products have been shown to be directly responsible for staphyloccocal virulence.

1.7 STAPHYLOCOCCAL-HAEMOLYSINS (CYTOLYTIC TOXINS)

Staphylococci haemolysins (cytolytic toxins) cause red blood cells to lyse and they act against cell membranes with some degree of specificity that may contribute to their virulence. Haemolysins may be detected as haemolysis zones surrounding growing colonies in solid media containing suitably sensitive whole blood (Mollby, 1983) and are traditionally classified on the basis of blood species sensitivity (Bernheimer, 1965; Wadstrom, 1983). Haemolysis is also used as part of the identification protocol for staphylococci (Baird-Parker, 1979). In vitro, haemolysis is assayed, as a single event by quantifying the release of haemoglobin from lysed sensitive

TABLE 1.3: The Biological Properties of Some Staphylococcal Products

Product	Specific Biological Activities
Extracellular:	
α-haemolysin	Haemolysis, lethality, cytotoxicity.
β-haemolysin	"Sphingomyelinase C"; haemolysis, cytotoxicity.
γ-haemolysin	Haemolysis, cytotoxicity.
δ-haemolysin	Surfactant-like activity, haemolysis, cycotoxicity.
Epidermolytic toxin two types (A,B)	Epidermolysis *in vivo* (cleavage of granular layer), causative agent of Ritter-Lyell or scalded skin syndrome.
Pyrogenic toxins	Disputable as separate entities, proposed as etiologic agents of toxic shock syndrome.
Leucocidin	Leucotoxicity, lack of toxicity to other cells.
Succinic oxidase factor	Inhibition of mitochondrial respiration.
Staphylocoagulase	Prothrombin activation leading to fibrinogen polymerization and clotting without participation of other blood clotting factors.
Staphylokinase	Fibrinolysis (plasminogen activation).
Enterotoxins (A-F)	Toxicity to sympathetic nerves and smooth muscles; causative agents of staphylococcal food poisoning. Enterotoxin F proposed as causative agent of toxic shock syndrome
Bacteriocins and micrococcins	Bactericidal and bacteriostatic action *in vitro*.
Lysozyme	Hydrolysis of peptidoglycan.
Nuclease	Hydrolysis of RNA and DNA.
Mitogens	Non-specific lymphocyte activation.
Hyaluronidase	Hydrolysis of hyaluronic acid.
Lipase	May be involved in the formation of local suppurative lesions.
Cellular:	
Cell wall (peptido-glycan)	Pyrogenicity, arthrogenicity, toxicity, adjuvanticity, immuno-modulation.
Capsular substance	Antiphagocytic properties.
Clumping factor	Paracoagulation of fibrinogen.
Protein A	Reaction with Fc fragment of immunoglobulins.

Modified from Jeljaszewicz, *et al*, (1978) and Ciborowski and Jeljaszewicz (1985).

erythrocytes (Freer and Arbuthnott, 1983). Staphylococci produce at least four different haemolysins which have been recognised, chiefly, by their chemical properties, serological specificity, and by their comparative ability to haemolyse erythrocytes from different animal species (Table 1.4). There are now recognised alpha-, beta-, delta-, and gamma-haemolysins. As single strain of Staph. aureus may produce more than one (Frobisher, Hinsdill, et al, 1974). All produce beta-type haemolysis (clearing of blood agar plates), but the degree of haemolysis depends upon the organism strain and the species of red blood cells (RBC's) used, as well as the mechanism and site of action of the haemolysin at the molecular level (Lo and Fackrell, 1979).

These haemolysins are proteins produced from intact cells during the logarithmic phase of growth and are excreted into the medium. They are probably synthesized by membrane-associated, rather than cytoplasmic, ribosomes (Stanier, et al, 1977).

1.7.1 Alpha-Toxin

Since the publication of the classical studies of Burnet (1930), staphylococcal alpha-toxin is considered as one of the most important factors associated with staphylococcal infections (Ciborowski and Jeljaszewicz, 1985). It has the characteristics of an enzyme with haemolytic reaction (Cooper, et al, 1964), and possesses haemolytic, cytotoxic, dermonecrotic and lethal activities (Bernheimer, 1965; Table 1.4).

This extracellular toxin is formed during the logarithmic phase of growth (Wiseman, 1975) and excreted into the medium. Less than 1% of haemolysin is cell associated, and reaches maximal level at the early stationary phase (Duncan and Cho, 1971; McNiven and Arbuthnott, 1972). It is released by the intact cell as indicated by low levels of deoxyribonucleic acid (DNA) in the medium at a time when maximal amounts of toxin were present in the culture (Duncan and Cho, 1971). It is produced by most strains of Staph. aureus and is a toxic, immunogenic, surface-active extracellular protein with a

TABLE 1.4: Some Characteristics of Staphylococcal-Haemolysins

Serological Type	Usual Source	Properties[g]	Most Susceptible Erythrocytes	Most Susceptible Leucocytes	Animal-Toxicity lethal quantity per kg of body weight	Molecular Weight	% Occurrence among[b]	
							Coagulase-positive isolates	Coagulase-negative isolates
Alpha	Human strains	Protein binding specific receptors. Hydrophobic interaction. Inactivated at 60°C. Reactivated at 100°C.	Rabbit	Human Rabbit Mouse	Dermonecrotic for rabbit; lethal for mice 40-60 ng[e] and for rabbit 2 µg	33,000	95	2
Beta	Animal strains	Phospholipase-C specific for sphingomyelin Inactivated at 60°C	Sheep (Hot-Cold)	Human[a] Rabbit[f]	Demonecrotic 10-160 times less toxic than α-lysin. Not lethal to mice at 7 mg[e]	30,000	66	2
Delta	Human strains	Hydrophobic peptide containing 26 amino acid residues. Stable at 100°C	Cod	Human[h],[c] Rabbit, Mouse, Rat, Guinea-pig, Pigeon, and fowl	Necrosis for rabbit. Lethal for mice 110 mg and for guinea-pigs 30 mg	210,000	97	13 or 50-70[d]
Gamma	Human strains	Two protein components with unknown mode of action. Inactivated at 60°C	Rabbit	Human[h]	Partially pure is lethal for mice and rabbit in less than 1 milligram	γ_1 26,000 γ_2 29,000	-	-

Data from:

a - Gladstone and Van Heyningen (1957); b - Jeljaszewicz, (1960); c - Gladstone and Yoshida (1967); d - Gemmell, et al (1976); e - Gill (1982); f - Arbuthnott (1983); g - Mollby (1983); h - Wadstrom (1983).

molecular weight in the region of 33000 and an isoelectric point of approximately 8.5 (Freer and Arbuthnott, 1983). It contains most of the usual amino acids which vary in amount from 4 residues for histidine to 44 for aspartic acid (Bernheimer and Schwartz, 1963).

Staphylococcal alpha-toxin exists as molecules having a sedimentation coefficient of about 3S and also as aggregates having a $S_{20,w}$ of about 12S (Arbuthnott, et al, 1967).

The existence of 16S components has also been reported, Jeljaszewicz, et al, (1978). The whole spectrum of biological effects is exhibited by the 3S alpha-toxin, whereas the 12S and 16S forms are less active or inactive (Jeljaszewicz, et al, 1978). Alpha-lysin is inhibited by Ca^{+2} (Jassim, 1984; Harshman and Sugg, 1985). The haemolytic unit of purified toxin contains about 0.01 µg of toxin protein; 0.5-2.4 µg causes necrosis on intradermal injection into a rabbit, and 2 µg given intravenously will kill the rabbit (Arbuthnott, 1970). The result of injection into human skin was reported by Smith, et al, (1963) to be a biphasic reaction with an early weal and erythema and a secondary indurated area.

The toxin acts widely on animal cells; a variety of cultured cells are killed and intracellular material may be released (Artenstein, et al, 1963; Arbuthnott, 1970).

Various biological membranes are damaged (Bernheimer, 1974; Wiseman, 1975; McCartney and Arbuthnott, 1978), including those of intact human, rabbit and mouse granulocytes (Jackson and Little, 1957; Jeljaszewicz, 1972; Szmigielski, Jeljaszewicz, et al, 1976). Rabbit and human leucocytes are killed, but without gross morphological changes, and platelets are lysed (Gengou, 1935; Sigel and Cohen, 1964; Bernheimer and Schwartz, 1965) (see 1.9). Alpha-lysin may function in pathogenicity partially by its ability to lyse a variety of erythrocytes from different animal species (Arbuthnott, 1970). The susceptibility to the lytic action of alpha-haemolysin varies greatly between species, with rabbit erythrocytes being 100

times more susceptible than human erythrocytes (Bernheimer and Schwartz, 1963). However, the susceptibility of erythrocytes from different individuals of the same species may differ five fold or more (Bernheimer and Schwartz, 1963; Bernheimer, 1965).

The mechanism of action of a alpha-lysin on rabbit erythrocytes (RBC's) involves a three-step sequence: (i) binding of alpha-haemolysin to specific receptors, (ii) damage to the membranes resulting in leakage of small ions, and finally (iii) lysis of the cell (Ciborowski and Jeljaszewicz, 1985; Harshman and Sugg, 1985). Wiseman and Caird, (1970, 1972) and Wiseman, et al, (1975) suggested that the effect of alpha-lysin is to activate proteolysis of the cell membrane though Arbuthnott, et al (1973) and Freer, et al (1973) considered that the alpha-lysin becomes attached to the cell-wall lipids and directly brings about their structural disorganization with the concurrent polymerization of the toxin to an inactive form.

1.7.2 Beta-Toxin

Staphylococcal beta-haemolysin was first described as a toxin serologically distinct from the alpha-haemolysin by Glenny and Stevens (1935). This toxin is produced at a maximal rate at neutral pH during the early logarithmic phase of growth (Wiseman, 1970), and reaches maximum concentration at the end of exponential growth (Low and Freer, 1977a,b). It is produced largely by Staph. aureus strains of animal origin (Elek, 1959). Beta-toxin is an immunogenic extra-cellular protein, with a molecular weight of approximately 30,000 and an isoelectric point in the region of 9.0-9.5 (Freer, and Arbuthnott, 1983). It causes so called "hot-cold" haemolysis, a phenomenon reported in earlier studies with an undefined staphylococcal "haemolysin" by Walbum (1921) and Bigger, et al (1927).

In this phenomenon, the intensity of haemolysis is increased when incubation of erythrocytes with the toxin at 37^{o}C is followed by a transfer to a lower temperature (below 10^{o}C). It has been suggested

that this reduction in temperature causes a sudden contraction of pretreated erythrocyte membranes which leads to breaking of weak bonds and causes the structure to disintegrate (Wiseman, 1965). The mechanism of this phenomenon was also studied in more detail by Bernheimer, et al (1974) and Low, Freer, et al (1974) who reported that hydrolysis of sphingomyelin in ghost membranes is accompanied by shrinkage of the membrane and the appearance of phase-dense droplets accompanied by the formation of numerous internal membrane vesicles.

It was noticed that erythrocytes incubated at 37^{o}C with beta-haemolysin were not only sensitive to cooling but also became more sensitive to other haemolytic substances, such as the haemolysin (S) of group B streptococci (Streptococcus agalactiae) (Christie, Atkins, et al, 1944; Mollby, 1976). The latter synergy formed the basis of the so-called modified CAMP-test, which has been widely used for identification of group B streptococci (Munch-Petersen and Christie, 1947) and as the, so called, reversed CAMP-test for identification of beta-haemolysin producing isolates of Staph. aureus.

Beta-lysin is a phospholipase-C (sphingomyelinase) (see Figure 1.3) specific for sphingomyelin and lysophosphatidyl choline, requiring Mg^{2+} for activity (Doery, Magnusson, et al, 1963; Doery, Magnusson, et al, 1965) and for which Ca^{2+} acts as an inhibitor (Freer and Arbuthnott, 1983).

Beta-toxin itself plays no demonstrable role in staphylococcal lipid metabolism (Wiseman, 1975), since sphingomeylin is not found in staphylococcal cells (Fritsche, 1970). Susceptibility of erythrocytes to lysis by beta-lysin correlated with sphingomyelin content in their membranes; those of sheep and ox erythrocytes, which contained greater than 40% sphingomyelin in the membrane lipids being highly sensitive (Jeljaszewicz, 1972; Bernheimer, et al, 1974).

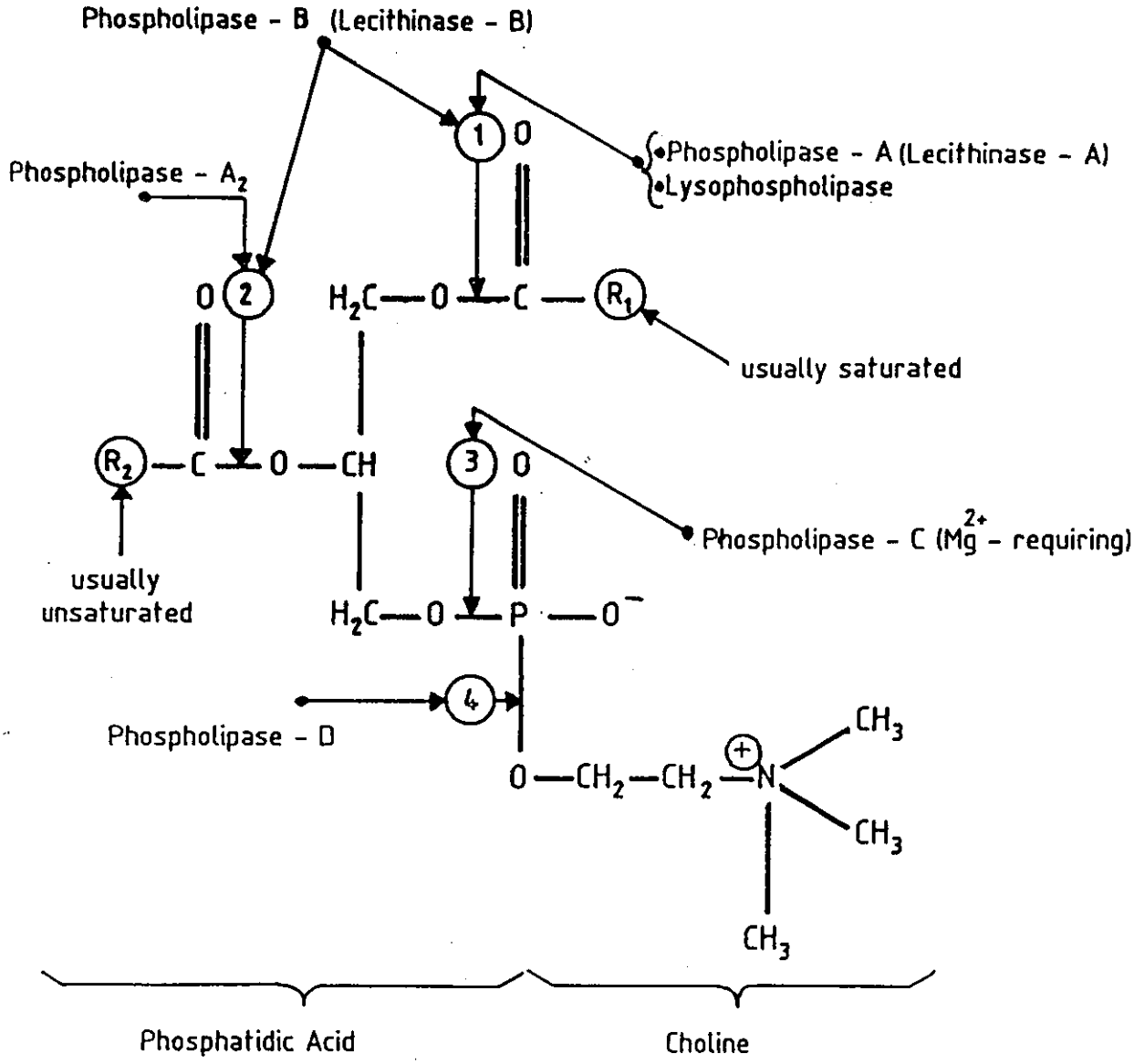


Fig. 1. 3. Showing Enzymes that degrade lecithins cleave the molecule at the numbers (1 - 4) bonds. (Modified from Bhagavan, 1974).

Moreover, it has been suggested that susceptibility of cell cultures and isolated cells to the action of beta-toxin depends on the overall lipid composition of the cellular membrane (Weinstein, Marsh, et al, 1969).

Beta-lysin also produces damage to isolated leucocytes (Gladstone and Van Heyningen, 1957; Szmigielski, Jeljaszewicz, et al, 1976), macrophages (Chesbro, Heydrick, et al, 1965), and blood platelets (Jeljaszewicz, Niewiarowski, et al, 1966; Wadstrom and Mollby, 1972). Highly purified staphylococcal beta-toxin inhibited cell locomotion of all types of human blood cells, leucocytes and monocytes being markedly affected than neutrophils (Wadstrom, 1983) (see 1.9). There are conflicting reports concerning the lethality of beta-lysin, though it would appear that it is 10 to 160 times less toxic than alpha-haemolysin (Wiseman, 1975). Beta-haemolysin is also dermonecrotic, but the mechanism of this activity is unknown (Wadstrom and Mollby, 1972).

1.7.3 Delta-Toxin

This haemolysin was observed by Williams and Harper (1947) on sheep blood agar plates. It is rather easily differentiated from other staphylococcal lysins because, generally, erythrocytes of various species are more uniformly sensitive to delta-haemolysin (Parker, 1983). Cod erythrocytes are, however, highly susceptible to delta-lysin but not to any other staphylococcal haemolysin (Chao and Birbeck, 1978).

It has been suggested that lysis of sensitive erythrocytes results from the surfactant activity of this toxin (Heatley, 1971; Rahal, 1972; Bernheimer, 1974), while alpha-, beta-, or gamma-haemolysin exhibit kinetics of cell damage resembling or indicating enzymatic reaction. Little is known about the cultural conditions controlling delta-toxin production and release (Freer and Arbuthnott, 1983). It is produced by most (97%) pathogenic strains of coagulase-positive staphylococci (Elek, 1959; Jeljaszewicz, 1960, 1972), and by a variable percentage (13-70%) of coagulase-negative isolates

(Jeljaszewicz, 1960; Gemmell, et al, 1976). Present evidence suggests that the lysins from these two groups of strains are immunologically identical, whether they are of human, sheep or rabbit origin, but that they are only partially cross-reactive with strains, (presumably of Staph. intermedius) of canine origin (Turner and Pickard, 1979).

Delta-haemolysin is an immunogenic exoprotein which has a molecular weight of approximately 210000 (Birkbeck and Whitelaw, 1980), and isoelectric points in the range of 9 to 10 (Wiseman, 1975). Delta-lysin is dermonecrotic (Marks and Vaughan, 1950), and relatively thermostable, of disputable antigenicity, high surfactant activity and hydrophobic affinity (Bernheimer, 1974). It rapidly lyses leucocytes and a variety of other tissues (Gladstone and Yoshida, 1967; Hallander and Bengtsson, 1967) and also disrupts lysosomes, and bacterial protoplasts and sphaeroplasts (Kreger, Kim, et al, 1971). It has also been shown to cause mitochondrial damage (Rahal, 1972), and may have antibacterial activity against certain microorganisms (Kreger, Kim, et al, 1971; Kantor, et al,1972).

It may also possess enterotoxin-like activity (Obrien and Kapral, 1977), due to cyclic adenosine monophosphate (cAMP) increase in intestinal cells (inhibits water absorption). Delta-toxin was reported to be lethal for laboratory animals, but only at relatively high doses (110 mg for mice, 30 mg for guinea pigs; as a single dose) (Kreger, Kim, et al, 1971). Highly purified delta-lysin is also responsible for erythema when given intradermally in rabbits, but very high doses are required to cause dermal necrosis inside the erythematous zone (Kreger, Kim, et al, 1971; Turner, 1978a). An additional property of this toxin, which differentiates it from other staphylococcal haemolysins, is its inhibition in normal serum or normal serum components, such as alpha- and beta-globulins or fibrinogen (Gladstone and Yoshida, 1967). Due to the presence of these inhibitors, it is difficult to demonstrate the appearance of specific antibodies to this toxic in serum. Removal of the inhibitors enabled determination of anti-delta toxin antibodies and

establishment of immunogenicitiy of this haemolysin (Fackrell and Wiseman, 1974). Delta-lysin was inactivated also by general serum lipid (Donahue, 1969), and by phospholipids (Kapral, 1967), including sphingomyelin, phosphatidylcholine and phosphatidylserine (Kreger, 1970). The inhibition of its activity by phospholipids and of normal serum suggests that delta-lysin is inactive *in vivo*.

1.7.4 Gamma-Toxin

The existence of gamma-haemolysin was first postulated by Smith and Price (1938) and its existence confirmed by Marks (1951). More recent studies have clearly proved that gamma-lysin is a separate entity (Guyonnet and Plommet, 1970; Taylor and Bernheimer, 1974). It is a protein with two distinct toxin components, component I (γ_1) with a molecular weight of 26,000 and an isoelectric point (pI) 9.8, whereas component II (γ_2) with a molecular weight of 29,000 and pI value of 9.9 (Taylor and Bernheimer, 1974); both components are required for toxic and haemolytic activity (Freer and Arbuthnott, 1983). Gamma-haemolysin is produced in the late stages of the logarithmic growth phase and with comparatively small amounts cell bound (Wiseman, 1975). It is active on rabbit, sheep and human erythrocytes (Wadstrom and Mollby, 1972; Fackrell and Wiseman, 1976), and horse red blood cells are most resistant (Guyonnet and Plommet, 1970). Its activity cannot be checked on standard blood agar plates, since its haemolytic action is completely inhibited by agar and heparin (Wadstrom and Mollby, 1972). It is antigenic with enzymatic activity requiring Na^+ or K^+ ions for lysis (Freer and Arbuthnott, 1983). Unlike alpha- and beta-haemolysins, gamma-toxin is inhibited by cholesterol acetate, and by a wide variety of lipids, including fatty acids (Wadstrom, 1983), and also by a variety of phospholipids, although phospholipids of the erythrocyte membranes are not attacked by gamma-haemolysin (Taylor and Bernheimer, 1974; Fackrell and Wiseman, 1976). Little is known about the biological properties of gamma-haemolysin. It does not liberate labelled nucleotides from human fibroblasts (Thelestam, *et al*, 1973), though it does cause the release of lysosomal enzymes *in vitro* and, to a lesser extent, of substances contained in specific

granules (such as alkaline, phosphatase) from isolated rabbit peritoneal granulocytes (Szmigielski, Jeljaszewicz, et al, 1976).

Fackrell and Wiseman (1976) showed that gamma-lysin injected intracardially killed guinea-pigs and produced massive haemorrhages in the kidneys and mucosal surface of the intestines. Pure gamma-toxin is also lethal for mice and rabbits at less than 1 milligram (Wadstrom and Mollby, 1972). It has been shown that gamma-toxin is produced in vivo in bone lesions caused by Staph. aureus (Freer and Arbuthnott, 1983) since elevated levels of specific anti-gamma-toxin antibodies were found in rabbits with staphylococcal osteomyelitis (Kurek, Pryjma, et al, 1977).

1.7.5 Epsilon-Toxin of Coagulase-Negative Staphylococcal

It has been suspected for a long time that coagulase-negative staphylococci produce specific haemolytic factors different from those of other staphylococci, but its separate existence has never been proved. However, Elek and Levy (1950) attributed the haemolysis surrounding colonies on rabbit blood agar plates to a separate haemolysin which they called epsilon-haemolysin, though, Marks and Vaughan (1950) reported that the haemolysin of these organisms was a delta-haemolysin. Later, Marks (1952) found that this haemolysin was formed by many strains of Staph. epidermidis (see Table 1.5).

TABLE 1.5
Production of Haemolysis by Coagulase-negative Staphylococci
(Mollby, 1983)

Species	No. strains investigated	No. strains positive (%)
Staph. epidermidis	215	104 (48)
Staph. saprophyticus	133	35 (26)
Staph. haemolyticus	67	67 (100)
Staph. hominis	46	25 (54)
Staph. cohnii	26	10 (38)
Staph. warneri	12	8 (67)
Staph. capitis	12	9 (75)
Staph. simulans	11	10 (91)
TOTAL:	522	268 (51)

Haemolysis measured on bovine blood agar plates.

On the other hand, Wadstrom, et al, (1976) found that at least three species of coagulase-negative staphylococci (Staph. haemolyticus, Staph. capitis, and Staph. simulans) produce haemolysin (S) exhibiting properties quite different from those of known haemolysins (alpha or delta) of Staph. aureus. Their haemolysin, however, consisted of two components with pIs of 5.5 and 10 on isolectric focusing and were active on a wide variety of erythrocyte species, with those from guinea-pigs and humans being the most sensitive. They were not inactivated by heat treatment at either 60°C or 100°C, nor by exposure to egg yolk lecithin. Gemmell (1983), however, separated the haemolysin produced by Staph. epidermidis as a protein with an isoelectric point of 4.25. The biological properties of this haemolysin resembled those of delta-haemolysin of Staph. aureus in that it was heat-stable and could be inhibited by egg-yolk lecithin.

If it exists as a separate entity, the haemolysin produced by coagulase-negative staphylococci (Staph. simulans, Staph. haemolyticus and Staph. epidermidis) seems to be a heat stable lysin and cytotoxin (fibroblasts; Gemmell and Schumacher-Perdreau, 1986). Most strains of Staph. epidermidis, Staph. saprophyticus, and Staph. haemolyticus which appear to produce significant amounts of this haemolysin also cause the release of (^{3}H)-uridine from human embryonic lung fibroblasts (Gemmell and Thelestam, 1981). However, beside the haemolysin produced by a number of strains of coagulase-negative staphylococci, there are other exo-enzymes which are also produced in vitro. These include DNase, proteinases, lipase, and staphylokinase (fibrinolysin), which will play a part in the pathogenicity of these bacteria (Gemmell and Schumacher-Perdreau, 1986).

However, this study has been made to discuss one of the most important staphylococcal products "haemolysins", exerting especially pronounced biological effects and possibly participating in the pathogenesis of staphylococcal infections.

1.8 PHAGOCYTOSIS OF STAPHYLOCOCCI

In 1893 Metchnikoff gave the name phagocytes to white blood cells. There are two morphologically distinct phagocytic cell populations found in animals peripheral blood: polymorphonuclear leucocytes (PMN's), composed primarily of neutrophilic granulocytes and monocytes (MN's). Both are derived from precursor cells in the bone marrow, and play an essential role in host defence against bacterial infections. PMN's are capable of more efficient bacterial phagocytosis (attachment and ingestion) than are MN's (Peterson, Verhoef, et al, 1977b).

Engulfment is not, however, the whole story in order to fulfil the defence role, phagocytes will possess a range of cytotoxic enzymes and associated pathways which are released or activated into the phagocytic vesicle formed after the engulfment of microorganisms by phagocytosis (Klebanoff and Clark, 1978; Karnovsky and Bolis, 1982).

The phagocytosis process of microorganisms comprises several separate steps (see Figure 1.4) which include: (i) alteration of the bacterial surface through opsonization; (ii) attachment and ingestion of the opsonized bacteria via membrane receptors of the leucotyes (this may be preceded by recruitment of adequate numbers of phagocytes at the focus of infection occurring by the process of chemotaxis) and, finally (iii) intracellular killing and degradation of the bacteria.

i) Opsonization and Chemotaxis

The recognition of staphylococci is mediated by opsonins that are factors from the heat labile complement system and from the heat stable antibody pool (Wright and Dougles, 1904; Cohn and Morse, 1959; Koenig, et al, 1962; Li, et al, 1963; Li and Mudd, 1965; Bryant, 1969). Cell membranes of human PMN's possess two receptors for opsonized staphylococci: a complement receptor which is utilized when bacteria are opsonized in normal serum and an Fc

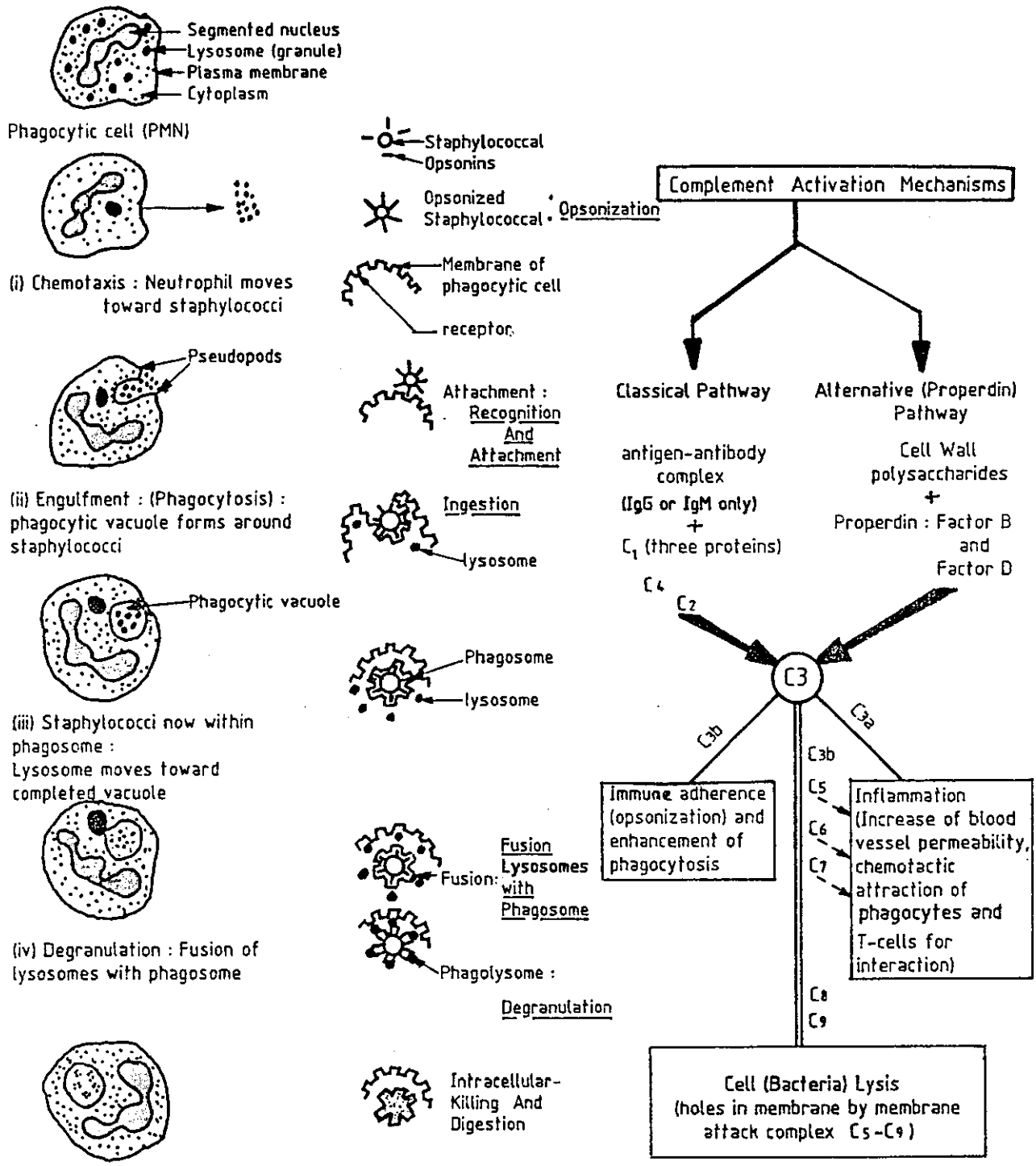


Fig. 1.4: The several separate steps of opsonization, chemotaxic, engulfment (phagocytosis), and intracellular-killing (digestion) of staphylococci.

receptor when bacteria are opsonized in heat-inactivated serum. Both receptors participate in the ingestion as well as the attachment phase of phagocytosis (Verhoef, et al, 1977b).

The heat labile serum factor (complement) supplies the majority of opsonic molecules for staphylococcal phagocytosis in healthy, non-immune individuals (Laxdal, Messner, et al, 1968; Humphreys, et al, 1974; Wheat, et al, 1974; Verhoef, et al, 1977b). The leucocytes recognise the staphylococcal cells after the opsonins (serum complement) (see Figure 1.4), are fixed to the surface of the bacterium. The complement system is the chief mediator of leucocyte chemotactic responsiveness (Russell, Wilkinson, et al, 1976).

The opsonic activity of complement residue C3b (a fragment of its third component of C3; see Figure 1.4) generated by enzymatic cleavage from native C3 (Stossel, Field, et al, 1975), and it is deposited on the bacterial cell surface (Gigli and Nelson, 1968; Stossel, Field, et al, 1975). Receptors for this component of complement (C3b) exist on the surfaces of PMN's and MN's (Stossel, 1974b), however, purified C3 alone had no opsonic activity (Koenig, 1972).

There is a direct relationship between the rate of bacterial opsonization in non-immune human serum and the rate of C3b fixation of Staph. aureus (Verbrugh, Van Dijk, et al, 1979). Also, the intracellular killing is dependent on the interaction between C3b and its receptor in the membrane (Leijh, Vanden Barselaar, et al, 1979a). However complement in normal human serum is activated by all major cell surface components of Staph. aureus (Wilkinson, Kim, et al, 1978; Verbrugh, Van Dijk, et al, 1979; Verbrugh, Van Dijk, et al, 1980) and this may contribute to staphylococcal opsonization with C3b. Some authors consider that for PMN's interacting with staphlyococci, C3b functions primarily in the adherence phase and that bound antibody serves as a trigger for ingestion (Scribner and Fahrney, 1976). Others suggest that both complement and antibody participate in both attachment and ingestion (Verhoef, et al,

1977b). On the other hand, the most important factor for the mechanisms of chemotactic activity for Staph. aureus is the bacterial cell wall (Adlam and Easmon, 1983). Staph. aureus cell wall components (peptidoglycan and teichoic acid) contributed to the generation of leucotye chemotactic C5a in normal serum (Schmeling, Peterson, et al, 1979).

In the absence of complement, staphylococci become opsonized only in immune serum containing high levels of specific IgG antibody (Laxdal, Messner, et al, 1968; Quie, et al, 1968; Wheat, et al, 1974; Verhoef, Peterson, et al, 1977a). For antibody opsonized bacteria, the probable mechanism of attachment of organisms to phagocytes is via the Fc piece of the antibody (immunoglobulins) which interacts with specific Fc receptors known to be present on both PMN's and MN's (Stossel, 1974b). The opsonization may occur by several mechanisms according to the staphylococcal strain used (Verhoef, Peterson, et al, 1977a; Verbrugh, Van Dijk, et al, 1979).

However, the immunological nature of the opsonic antibodies present in immune and non-immune serum has been investigated by many authors. They have demonstrated that the peptidoglycan moiety of the staphylococcal cell wall is capable of absorbing the heat stable opsonic activity from normal and immune human serum, and from hyperimmune serum raised in rabbits, and neither teichoic acid nor protein A is involved (Shayegani, et al, 1970; Humphreys, et al, 1974; Karakawa and Young, 1979). Moreover, the peptidoglycan-specific antibody isolated from immune rabbit serum has been shown to exert a direct opsonic effect (Karakawa and Young, 1979; Verbrugh, Van Dijk, et al, 1980). This shows that peptidoglycan of staplyococcal cell wall plays a critical role in cell opsonization by both heat stable (antibody) and heat labile (complement) serum factors (Adlam and Easmon, 1983).

In the absence of antibody, staphylococci are still opsonized but at a slower rate usually via the alternative complement pathway (Verbrugh Van Dijk, et al, 1979; Verbrugh, Van Dijk, et al, 1980;

see Figure 1.4). Therefore, the effective phagocyte/bacterium attachment may be brought about by combination of antibody and/or complement components with bacterial cell wall polymers (Adlam and Easmon, 1983).

ii) Attachment and Ingestion

After opsonization staphylococci becomes readily susceptible to phagocytosis by PMN's and MN's (Peterson, Verhoef, et al, 1977b; Verbrugh, Peters, et al, 1978; Leijh, et al, 1986). Whereas, C_3b primarily promotes attachment and IgG primarily promotes ingestion (Hed and Stenndahl, 1982). The ingestion process is also dependent on metabolic energy and requires an intact cytoplasmic microfilament system in the leucotyes (Klebanoff and Clark, 1978).

iii) Killing and Destruction

Once organisms are inside the phagocytic vacuole or phagosome (Figure 1.4) they are subjected to a powerful battery of damaging chemicals including: enzymes, oxidative mechanisms and bactericidal proteins which are released into the phagosome from lysosomal granules. The microbicidal mechanisms for intracellular killing include: low pH; myeloperoxidase; hydrogen peroxide; halide ions; cationic proteins; assorted proteases, carbohydrases and lipases; products of oxygen metabolism such as superoxide, singlet oxygen and hydroxyl radicals; lactoferrin and lysozyme (Adlam and Easmon, 1983). The bactericidal materials may differ between species, and rabbit, rat and guinea-pig PMN's have been shown to contain less myeloperoxidase than human PMN's (Paul, Strauss, et al, 1970). However, these processes involve multiple mechanisms, all of which are centred on two cellular events: (a) degranulation, i.e. fusion between the phagosome, containing the microbe, and granules present in the cytoplasm of the phagocytes (see Figure 1.4), and (b) the respiratory burst initiation, the function of which is to produce highly reactive bactericidal agents by the partial reduction of oxygen (Klebanoff and Clark, 1978).

In vitro experiments have shown that cells of Staph. aureus were apparently killed at a slower rate inside PMN's than Staph. epidermidis strains (Rogers and Tompsett, 1952). In this way, Staph. aureus may outlive the cells which have phagocytized them, emphasising that ingestion alone may be insufficient to prevent the spread of infection. On the other hand, Staph. epidermidis strain 1142 which is virulent to the mouse, showed antiphagocytic activity (Yoshida, et al, 1976). The exceptional virulence of the encapsulated staphylococci has been attributed to the (protective) antiphagocytic activity of their capsules (Morse, 1960). However, it may be concluded that pathogenic staphylococci are much more resistant than non-pathogenic ones to intracellular killing (Kapral and Shayegani, 1959; Melly, et al, 1960; Rogers and Melly, 1960; Koenig, et al, 1962).

In studies using rabbit peritoneal PMN's and extracts prepared from them, Gladstone and Walton (1970, 1971) showed that the cationic protein system was the predominant bactericidal mechanism operating. Killing of staphylococci was prevented by Fe^{2+} and haematin (but not by haemoglobin) which are known to reverse the bactericidal action of cationic proteins. They suggested that hydrogen peroxide, myeloperoxidase, and halide was not involved in rabbit PMN's under their conditions because killing was not inhibited by pretreatment with azide or catalase. Staphylococci were only susceptible to killing by the cationic proteins when growth depended on aerobic respiration (Gladstone, et al, 1974). Moreover, killing depended on an energy dependent binding of the cationic proteins to the cytoplasmic membrane of the bacteria and a functional cytochrome chain in this membrane was shown to be necessary for protein binding and killing (Walton and Gladstone, 1975, 1976).

1.9 THE INTERFERENCE OF STAPHYLOCOCCAL PRODUCTS WITH PHAGOCYTOSIS

Staphylococci produce a large number of extracellular and cellular products (see Table 1.3, Figure 1.5), many of these have shown been to have a damaging effect when injected into animals or when

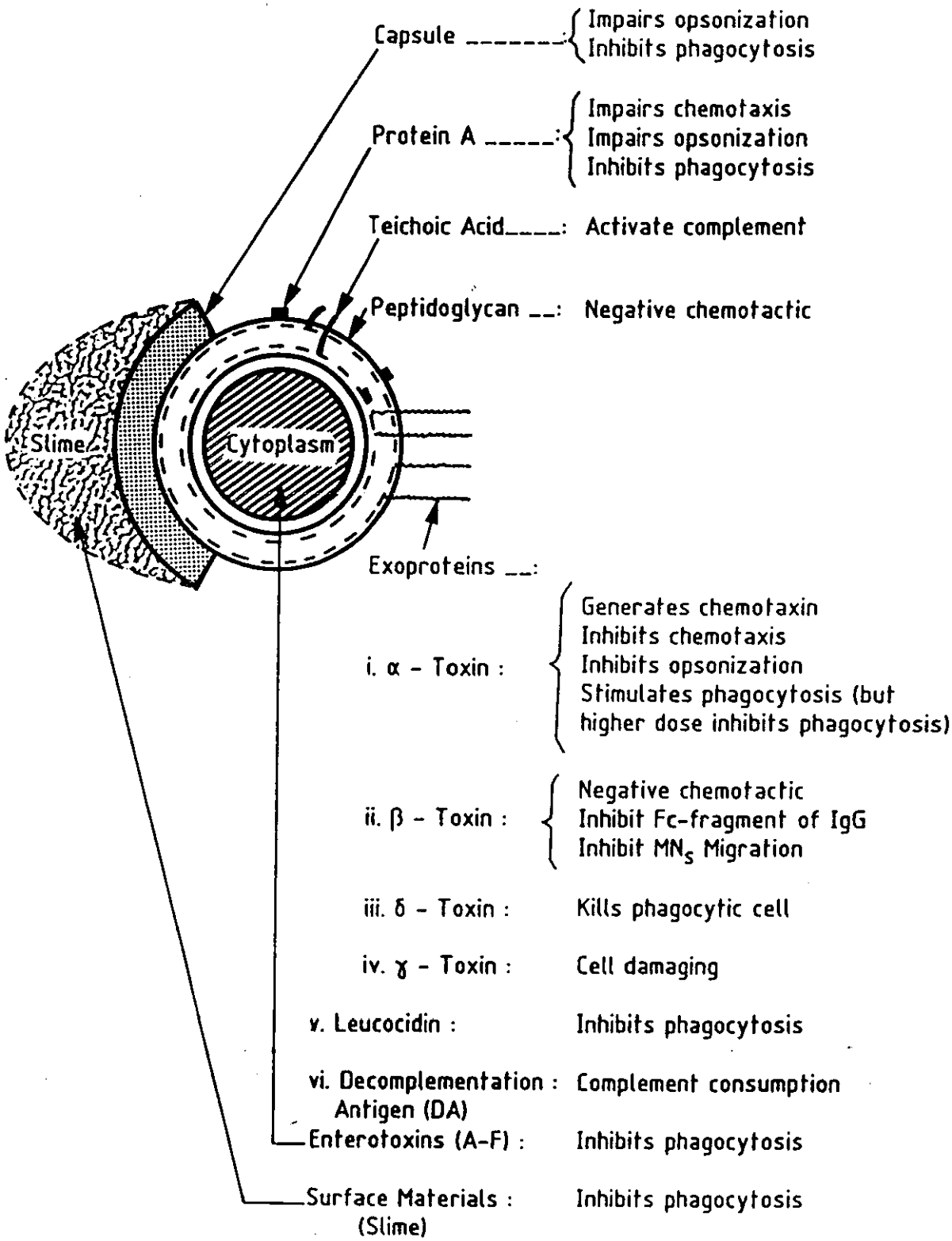


Fig. 1. 5. Staphylococcal Products and the major components of the cell wall on leucocyte function.

incubated with animal cells of various types. The earlier study on rabbit granulocytes by Maheswaran, et al, (1969) showed there was some ultrastructural effect caused by the alpha-toxin and the damage caused to the cellular lysosomes resulted in the release of various hydrolytic enzymes (Bernheimer and Schwartz, 1964).

Alpha-toxin has been reported to be leucocidic for rabbit and mouse PMN's (Table 1.4; Wright, 1936; Gladstone and Van Heyningen, 1957). However, at low concentrations, staphylococcal alpha-toxin can potentiate the phagocytic and bactericidal activity of human PMN's and it is thought that the toxin exposes more receptor sites on the PMN cell membrane for opsonized staphylococci. At higher concentrations a certain amount of cell damage was caused which resulted in a reduction of their phagocytic capacity (Gemmell, Peterson, et al, 1982a). Also, alpha-toxin found potent granulocyte aggregant and depressed chemotactic responsiveness (Schmeling, Gemmell, et al, 1981). Similarly, Russell, Wilkinson, et al, (1976) and Arbuthnott (1983), using human blood cells, shows that in vitro staphylococcal alpha-, beta-lysin, and leucocidin had significant inhibitory effects on chemotaxis of leucocytes. Thus, alpha-toxin inhibited migration of both PMN's and MN's, but beta-toxin only appeared to inhibit monocytes, while leucocidin strongly inhibited PMN migration and had a less effect on MN's. On the other hand, staphylococcal alpha-toxin has been reported to elicit complement consumption in human serum (Gemmell, Peterson, et al, 1982b); however, this was refuted by Bhakdi And Muhly (1985) who suggested that the complement consumption is due to extracellular staphylococcal decomplementation antigen (DA).

In contrast, the role of beta-toxin is in preventing opsonization as a negative chemotactic substance. In addition it has been shown to inhibit the Fc fragment of immunoglobulin from binding to monocytes, lymphocytes and polymorphonuclear leucocytes (Wilkinson, 1977). The staphylococcal enterotoxins A-F are best known as the causative agents of the foodborne disease, but evidence is now available to implicate them in preventing phagocytosis. In vitro, enterotoxins

A-F are considerably inhibited to the latex phagocytosis in human PMN granulocytes, though the inhibitory effect of the enterotoxins can be protected by a specific serum antibody (Jozefczyk and Raczka, 1985).

The formation of proteases by staphylococci whether grown in vivo or resulting from tissue damage, can attack both complement components, C3 and C5, to produce chemotactic C3a and C5a (Stossel, 1974a; see Figure 1.4). On the other hand, the coagulase enzyme of Staph. aureus has been proposed to prevent Staph. aureus phagocytosis by inducing a protective fibrin coat around the bacteria (Hale and Smith, 1945). The methodological bases of these results have, however, been criticised (Cawdery, Foster, et al, 1969).

The presence of capsules and their size were found to affect the phagocytosis of staphylococci (Morse, 1960; Yoshida and Takeuchi, 1970; Melly, Duke, et al, 1974). The capsular material interferes not only with opsonization by classical and alternative complement components, but also by the antipeptidoglycan antibodies (Peterson, Kim, et al, 1978a; Peterson, Wilkinson, et al, 1978c; Wilkinson, et al, 1979).

Protein A is a widely distributed cell wall protein among Staph. aureus. The addition of staphylococcal protein A (SPA) to fresh sera causes a marked depletion of complement (Sjoquist and Stalenheim, 1969; Kronvall and Gewurz, 1970; Stalenheim and Sjoquist, 1970; Stalenheim, 1971; Stalenheim, Gotze, et al, 1973) and inhibits the heat-labile opsonization of various bacteria by activation of the complement system (Forsgren and Quie, 1974). However, Dossett, Kronvall, et al, (1969); and Peterson, Verhoef, et al, (1977a) demonstrated that this SPA may block the opsonic effect of antibody. Their results were consistent with the binding of the Fc portion of opsonic antibody by protein A. Thus, the soluble SPA inhibits phagocytosis in vitro of Staph. aureus (Dossett, Kronvall, et al, 1969). Therefore, we can conclude that SPA after it is produced and released into the culture from the cell wall of some strains of

Staph. aureus will interfere with phagocytosis by its uniform capacity to bind immunoglobulins (IgG) and depletion of complement. The non-toxic nature of teichoic acid (Knox and Wicken, 1973), appears to play a small part in the opsonization process, and removal of teichoic acid by chemical extraction did not influence the rate of phagocytosis (Peterson, Wilkinson, et al, 1978b). Teichoic acid has, however, been shown to activate complement via the classical pathway (Verbrugh, Van Dijk, et al, 1979). In contrast many reports have shown the endotoxic potential of cell wall peptidoglycans from Gram-positive bacterial species (Heymer, 1975; Rotta, 1975). Peptidoglycan, fragment of the cell wall can also act as negative chemotactic substances (Weksler and Hill, 1969; Grov, et al, 1976); and in the presence of peptidoglycan specific antibody neutralized this effect (Grov, 1976).

So, it has been shown that staphylococcal strains have the ability to produce certain factors which act directly or indirectly on the defence system. It was suggested that the chemotaxis (complement-dependent) factor was acting at "long-range" to attract PMN's to the focus of the staphylococcal infection. The "short-range" negative-chemotaxins such as alpha-, beta-toxin and leucocidin may also be acting to protect the staphylococci (Russell, Wilkinson, et al, 1976).

1.10 THE ROLE OF SERUM-FACTORS IN THE GROWTH, PHAGOCYTOSIS AND INTRACELLULAR-KILLING OF STAPHYLOCOCCI

There are several bacteriostatic or bactericidal serum components which have been described with anti-staphylococcal activities. They comprise: alphaglobulin (Yotis and Lentino, 1973); C-reactive protein (Patterson and Higginbotham, 1965); cationic proteins released from phagocytic cells during phagocytosis (Zurier, Weissmann, et al, 1974); iron binding proteins (Schade, 1963); lysozyme (Kern, R.A; Kingkade, et al, 1951); platelet derived proteins released during clotting (Hirsch, 1960); and progesterone (Yotis and Stanke, 1966).

The bacteriostatic and bactericidal action of serum on staphylococci has been studied by Cybulska and Jeljaszewicz (1966a,b) who tested 100 strains of coagulase-positive and negative strains for their ability to grow in the serum of ten species of animals. They found that in general, saprophytic strains were more susceptible than pathogenic strains although no relationship existed between coagulase formation and the ability of the organisms to be grown in serum. It has been known for several years that in in vitro the coagulase-positive of Staph. aureus are more resistant than coagulase-negative of Staph. epidermidis strains to serum bactericidins (Tejler, 1937; Spink and Paine, 1940; Yotis and Ekstedt, 1959; Fletcher, 1962; Borowski and Tybusz, 1963; Cybulska and Jeljaszewicz, 1966a; Ehrenkranz, et al, 1971) Recently, Anderson and Williams (1985), demonstrated that the coagulase-negative encapsulated Staph. simulans strain grew significantly less well in the presence of serum or whey in gelatin-Hank's balanced salt solution (gel-HBSS) medium, than the coagulase-positive Staph. aureus strains. On the other hand, there are some exceptions such as the mouse virulent Staph. aureus (Giorgio), was found susceptible to the bactericidal action of rabbit serum at concentrations as low as 10% (Cohn and Morse, 1959). However, if beta-lysins (also called the beta bactericidal system of serum, is distinct from antibody, complement and lysozyme Muller-Eberhard, 1965) are excluded from serum, both Staph. aureus and Staph. epidermidis grow well in whole rabbit serum even from small washed inocula (Gladstone, 1973).

Gladstone (1973) showed that the addition of iron in excess of that required to saturate the transferrin also has a negligible effect and concluded that since plasma serum and blood serum would contain the same concentration of transferrin, it is unlikely that this substance is concerned in the antistaphylococcal action of serum. The protection of Staph. aureus from the killing by serum has also been suggested to be due to staphylocoagulase (Ekstedt and Nungester, 1955; Ekstedt, 1956; Yotis and Ekstedt, 1959; Ehrenkranz, et al, 1971) but this was refuted by Cybulska and Jeljaszewicz, (1966a) and Fletcher, (1962).

Bhakdi and Muhly (1985) reported that staphylococci produce an extracellular substance, designated as staphylococcal decomplementation antigen (DA). They concluded that DA production does not strictly correlate with the expression of known pathogenic factors including coagulase, clumping factor, protein A, and alpha-toxin, and it causes rapid consumption of early-reacting complement components up to and including C5 (see Figure 1.4) in human serum, the complement activation occurs as a consequence of immune complex formation between DA and specific human IgG and proceeds primarily via the classical pathway. They anticipated also that DA contributes to the pathogenicity of staphylococci through the abortive consumption of C3 in the fluid phase.

On the other hand, the role of serum factors in intracelluar-killing has been studied and the majority of these studies using human PMN's, suggested that antibody is probably not directly involved in intracellular-killing. Verhoef, Peterson, et al (1977a) showed that staphylococci opsonized with IgG deficient serum were rapidly killed. While incubation of Staph. aureus strain (502A) with either 10% fresh human serum alone or human white cells alone did not result in significant killing of staphylococci. However, when Staph. aureus was incubated with white cells in 10% unheated serum, over 80% of staphylococci were killed within one hour (Wheat, et al, 1974). A similar study by Leijh, Vanden Barselaar, et al, (1979) showed that intracellular killing by human monocytes of Staph. aureus, Staph. epidermidis and E. coli did not occur or was low in the absence of serum and when fresh serum was added maximal killing was obtained. However, Solberg, Christie, et al, (1976) reported that antibody present in normal human serum appeared to enhanced both uptake and killing of staphylococci.

It is well established that specific immune serum is able to promote phagocytosis, at a much greater rate than normal serum, by combining with specific receptor sites on the surface of the antigenic particle (Denys and Leclef, 1895; Wright and Douglas, 1904; Mudd, Lucke, et al, 1930; Mudd, et al, 1934). The study of the

phagocytosis on cold-blooded animals, such as fish, by Avtalion and Shahrabani (1975) revealed that neither the Staph. aureus growth nor the ingestion of bacteria into the cells seem to be influenced by immunization. They concluded that the phagocytic cells of immune blood are more effective in inactivating or killing the staphylococci.

In mammals, on the other hand, Shayegani, et al (1964); Shayegani and Mudd (1966), reported that leucocytes or monocytes and autologous serum from immunized rabbits are not significantly different from those of normal rabbits in their effect on non-encapsulated Staph. aureus. Moreover, Shayegani (1970), using immunized rabbit serum with non-encapsulated Staph. aureus failed to enhance phagocytosis and intracellular killing of the homologous non-encapsulated Staph. aureus by rabbit PMN's or MN's, when compared with normal rabbit serum. He concluded that acquired specific antibacterial antibody appears to play little or no role in phagocytosis of non-encapsulated Staph. aureus. However, it has been shown (Verhoef, Peterson, et al, 1977a) that the general picture of effective opsonization by antibody and complement can differ according to the strain of Staphylococcus under investigation.

In contrast to the lack of influence of antibody on intracellular killing, complement has been shown to play a role. When a comparison of phagocytosis, with normal and heated serum, was carried out, this showed that intracellular killing of staphylococci was inhibited when heated serum was used (Li, et al, 1963; Shayegani and Mudd, 1966; Wheat, et al, 1974). On the other hand, similar intra-cellular-killing has been demonstrated by others when either heated or normal serum was used with normal rabbit cells or normal human babies cells (Shayegani, et al, 1964; Adlam, et al, 1970b). However, it was concluded that phagocytosis and intracellular-killing are dissociable phenomena, in which phagocytosis required only heat stable (antibody) and was increased in the presence of heat labile (complement), while heat labile (complement) required

only for intracellular killing (Li, et al, 1963). Intracellular killing is dependent on the interaction between C3b and its receptor in the membrane (Leijh, Vanden Barselaar, et al, 1979).

In general it seems that in the presence of normal serum intracellular-killing is enhanced or is at least efficient, though, Downey and Kejima (1967) claimed that normal serum had a depressive effect on intracellular killing in a guinea-pig system. They considered that serum factors were enabling too many bacteria to be ingested and speculated that the digestive mechanisms of the cells were becoming overloaded. However, in vivo, this overloading would be controlled by continued replacement of PMN's. In contrast the encapsulated staphylococci required higher concentrations of serum and milk whey to opsonise the organisms, both serum and whey were able to support opsonization at concentrations within physiological limits (Anderson and Williams, 1985).

1.11 THE EFFECT OF CULTURAL CONDITIONS ON THE SUSCEPTIBILITY OF STAPHYLOCOCCI TO PHAGOCYTOSIS

Direct evidence suggests that staphylococcal strains grown in infected animals are different chemically and biologically from those grown in vitro (Beining and Kennedy, 1963; Ekstedt and Yoshida, 1969; Adlam, et al, 1970a,b). Therefore, when microbes are removed from infected animals and grown in vitro the change of environment may induce phenotypic different from those found in vivo; such phenotypes may lack one or more virulence determinants or even possess apparent virulence factors never found in vivo (Smith, 1964). Similarly, Staph. aureus has been shown to accumulate, under special conditions of culture, extracellular material (pseudocapsules) which could be demonstrated about the cells (Sall, et al, 1961; Sall, 1962). Also, under distinct conditions mucoid variants have been selected from many Staph. aureus strains, and possessing novel extracellular materials not detected in the absence of human gamma-globulin or immune serum (Wiley, 1961; Wiley and Wonnacott, 1962).

When staphylococcal strains were grown in media which mimic the in vivo situation or are freshly isolated from clinical materials, then they may elaborate surface materials (slime) which render them more virulent and more resistant to phagocytosis (Yoshida and Ekstedt, 1968; Brock et al, 1973; Ekstedt and Bernhard, 1973; Yoshida, Nakamura, et al, 1974; Brock and Reiter 1976). This may explain, in part, the enhanced virulence of staphylococci when grown in vivo (Adlam, et al, 1970a). Also, these surface antigens may be antigenically similar to, and in some cases identical with, true capsular antigens (Karakawa, et al, 1978).

Gladstone, et al, (1974) showed that Staph. aureus grown in a 17 amino acid chemically defined medium were resistant to the test dose of rabbit granular extract (GE), whereas those grown in a medium containing Brain Heart Infusion broth (BHI) or proteose peptone broth in the absence of a fermentable carbohydrate (glucose or lactose and to a lesser extent mannitol) are susceptible. Recently, Ciborowski, Garlinski, et al, (1988) found that the presence of 1/2 of MIC level of the antibiotics gentamycin, amikacin, mezlocillin, cefotaxime and cefamandole in the media HD + glycin and Tryptone Soya Broth (TSB) used for cultivation of staphylococci strains, can influence their sensitivity to phagocytosis. Other media such as BHI, Muller-Hinton broth and T-H broth had no influence on phagocytosis.

Staph. epidermidis grown in human peritoneal-dialysis effluent had the same opsonic requirement as cells grown in BHI (Clark and Easmon, 1986). Their data showed also that the phagocytosis rate was lower when Staph. epidermidis has grown in peritoneal-dialysis effluent than those grown in BHI. This difference in phagocytosis may be explained by the observation of Williams et al (1988) who reported that Staph. epidermidis grown in human peritoneal dialysate (HPD) shows marked differences in cell envelope properties compared with bacteria grown in nutrient broth. Smith (1977) drew attention to the importance of the cell envelope in relation to the pathogenicity of a bacterium. It seems likely that variation among

the same strains of bacteria to phagocytosis may be due to the effects of growth under various nutrient conditions, which will influence the composition and properties of the bacterial cell wall.

1.12 GROWTH AND SURVIVAL OF STAPHYLOCOCCI IN LEUCOCYTES

Most foci of infection are localised by rapid influx of PMN's and MN's, then following phagocytosis (see 1.8). Most bacteria are killed in the vacuoles of PMN or MN phagocytes in 5-10 min (Wilson, et al, 1957; Wood, 1960). In human neutrophil-associated staphylococci 88% were killed in vitro within 20 min (Verbrugh, Peters, et al, 1978). Bovine neutrophils killed about 90% of total Staph. aureus after 2h incubation (Williams, Hibbitt, et al, 1984). In general the intracellular-killing of Staph. aureus is very slow (Shayegani and Mudd, 1966). Nevertheless, it must be concluded that a high percentage of Staph. aureus are killed within phagocytes leaving only small proportions of persisting intracellular survivors. Several reports have, however, concluded that intracellular staphylococci may sometimes survive within both rabbit and human PMN's where they multiply and eventually kill the cell (Rogers and Tompsett, 1952; Tompsett, 1954; Pearce, et al, 1976). On the other hand, the virulent strains of Staph. aureus could survive, but not multiply, within leucocytes of normal rabbits and humans. Staph. epidermidis (former albus) under the same conditions was destroyed by leucocytes (Kapral and Shayegani, 1959). However, intracellular multiplication of Staph. aureus does not occur to any significant extent in normal phagocytic cells and Staph. aureus cannot, therefore, be considered to be an intracellular pathogen in the way as, for example, Mycobacterium tuberculosis or Brucella abortus (Adlam and Easmon, 1983).

1.13 THE AIM OF THIS STUDY

The aim of the present study was to investigate the growth of selected staphylococci in a range of routine and developed chemically defined media and the concurrent production of

haemolysins and other extracellular products. An integral part of the study was the development of a chemically defined medium in which all staphylococci included in the investigation would give adequate growth.

In vitro studies of the phagocytosis of staphylococci would be included to enable a clearer understanding of the importance of medium composition in phagocytosis.

In general observations would be centred on Staphylococcus aureus strain Wood 46.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 MICROORGANISMS

Six strains of staphylococci were used throughout: Staphylococcus aureus NCTC 8532; Staph. aureus NCTC 7428; Staph. aureus NCTC 5655; Staph. aureus NCIB 8625; Staph. aureus Wood 46 (kindly supplied by Dr G. Coleman, Department of Biochemistry, University of Nottingham) and Staphylococcus epidermidis NCTC 7944.

Also a selection of other organisms were used for the evaluation of the nutritional capabilities of media containing fish extract (see Appendix 1), these were: Aspergillus nidulans CMI 16643, Aspergillus niger CMI 31821, Bacillus subtilis NCIB 10073 and NCIB 3610, Byssochlamys fulva CMI 40021, Candida albicans A39, Escherichia coli NCIB 10243, Klebsiella pneumoniae NCIB 8267, Nocardia opaca NCIB 9409, Pseudomonas aeruginosa NCTC 7244, Saccharomyces cerevisiae NCYC 975 and NCYC 620, Schizophyllum commune FPRL9, Serratia marcescens NCIB 11879, and Streptomyces henetus NCIB 11003. Corynebacterium renale NCTC 7448 was used to evaluate lipase activity and acetoin production.

2.2 ROUTINE MEDIA AND SOLUTIONS

Media and solutions were prepared following the manufacturer's instructions (unless otherwise stated) and were sterilized by autoclaving at 121°C for 15 min or 115°C for 20 min or sterilized by 0.45 μm membrane filter (Whatman).

Media

Nutrient broth (NB; Oxoid CM 67).
Brain Heart Infusion broth (BHI; Oxoid CM 255).
Diagnostic Sensitivity Test Agar (DST-Agar; Oxoid CM261).
Fish Infusion broth (FIB; see Appendix 1).

Chemically defined medium with 13 amino acids (CDM/A, see Table 3.6).
Yeast extract buffered saline (YEBS; comprising 0.1% w/v yeast extract, Oxoid L21, in phosphate buffered saline, PBS).
When necessary, media were solidified by the addition of 1.5% w/v agar (Oxoid, L13).

Solutions

Ringer Solution - 1/4 strength (1/4 RS; Oxoid, BR52).
Phosphate Buffered Saline (PBS; prepared from mg/l; Na_2HPO_4, 1419.6 and NaCl, 8500; pH 7.0).

Hank's Balanced Salt Solution (HBSS; prepared according to Gibco Europe Companies and Agents, UK, 1981-1982 (does not contain calcium chloride, magnesium sulphate or phenol red), from mg/l; NaCl, 8000; KCl, 400; Na_2HPO_4, 48; $NaHCO_3$, 350; KH_2PO_4, 60; and glucose, 1000; pH 7.3-7.4.

Gelatin-Hank's Balanced Salt Solution (gel-HBSS; prepared by adding gelatin "Oxoid , L8" 0.1% w/v to HBSS; pH 7.3-7.4).

Heparin-Hank's Balanced Salt Solution (H-HBSS; heparin "Sigma", 500 I.U. into HBSS, 50 ml).

Glycogen Saline Solution (GSS: glycogen "Sigma", 0.1% w/v and NaCl, 0.9% w/v).

Electrophoresis Buffer (EB: prepared according to Leinonen, 1985 from: Na-barbitone "Fisons", 20600 mg; $NaCH_3COOH.3H_2O$, 13600 mg and 0.1 mol HCl, 280 ml made up to 2000 ml with distilled water; pH 8.2).

2.3 BLOOD PRODUCTS

Defibrinated whole blood from various animal species is available commercially: horse (Oxoid, SR50) and sheep (Oxoid, SR51). The blood not available commercially guinea pig-, rabbit-, or human-blood was collected fresh from healthy animals and humans in a sterile bottle containing glass balls, 4 mm undrilled (Fisons) with continuous gentle stirring by sterile wood stick. As soon as the bottle was half full, it was stoppered and gently turned up and down continuously for 5 min to reduce and separate the fibrin.

Defibrinated rabbit or sheep red blood cells (RBC's) were standardised as follows:

a) Isolation of RBC's

10 ml of RBC's (whole blood) were transferred under aseptic conditions to a 50 ml sterile glass tube, then 30 ml of sterile PBS was added to the RBC's. A piece of parafilm was then placed on the top of the tube. The tube was held between the palms of the hands and gently turned up and down (ten times) to reduce the risk of rupturing the erythrocytes. The tube was placed in a centrifuge and spun at 3500g for 10 min, at $4^{o}C$. The supernatant was discarded and the cells were resuspended again with 30 ml of fresh PBS and the mixing procedure repeated three times.

After the third wash a suspension of RBC's was adjusted to 2% (v/v) in PBS (see 2.3b) and stored at $4^{o}C$ for a period not exceeding three days. If a suspension with traces of lysis was observed, the suspension was discarded and a fresh one was used.

b) Calibration of RBC's suspensions

The packed volume of red blood cells was determined by centrifugation at 12000g in a micro-Haematocrit centrifuge for 5 min, so that the packed cell volume in the Benjamin Haematocrit tubes could be read directly. When necessary the red blood cells were diluted with a calculated volume of PBS to give the desired

concentration (2% v/v RBC's). The results were confirmed using a Hawksley micro-Haematocrit reader.

2.4 CULTURE MAINTENANCE

Staphylococci were maintained on Brain Heart Infusion-Agar (BHI-agar) slants, containing 7% (v/v) sterile, defibrinated sheep blood and stored at 4°C in the dark with monthly subcultures. Nutrient broth (NB) was used for all other microorganisms, for culture maintenance and inoculum development.

2.5 CHARACTERISATION OF STAPHYLOCOCCI

The identification of staphylococcal strains by the physiological, morphological and biochemical properties were based on the methods of the International Committee on Nomenclature of Bacteria (ICNB) Subcommittee on Taxonomy of Staphylococci and Micrococci (1968), unless otherwise stated. The techniques used were as described by Cowan and Steel (1974), unless otherwise stated. General investigation included:

a) **Macroscopic and microscopic examination**

These are: pigment production; Gram stain and motility.

b) **Biochemical test**

To enable comparisons of biochemical capabilities, organisms were subjected to a series of biochemical tests for the production of: coagulase; acid from carbohydrates; indole; hydrogen sulphide; oxidase; nitrite; catalase; lipase; phosphatase; deoxyribonuclease (DNase); gelatinase; caseolases; urease; lecithinase; ammonia (Harrigan and McCance, 1969).

c) Special tests

i) Lysostaphin sensitivity

The method described by Bennett (1984) was adopted.

ii) Thermonuclease (TNase) activity

Two approaches were used: the first was basically as described by Bennett (1984). The second was a modified version of the Bennett (1984) test to allow microtesting on glass slides as follows. The ability of each strain of staphylococci to produce TNase was determined from cultures incubated in BHI for 24h at 37°C before testing. Samples were heated for 15 min at 100°C in a water bath. Microscope slides were prepared by spreading 3 ml DNase-agar (Oxoid, CM321), onto the surface of each slide. When the agar had solidified, 6 mm diameter wells (3 per slide) were cut in the agar. To each of these wells 0.01 ml of heated sample was added. The slides were incubated in a moist chamber for 4h at 37°C, then each slide was covered with N-HCl. A positive result showed as a clear zone around the wells, while an opaque zone resulted with a negative result.

iii) Methyl red (MR) - Voges Proskauer (VP) test

The methods for the MRVP test described by Cowan and Steel (1974) were used. For the VP test, two extra methods were also used:

Method I: was basically as described by Devriese and Hajek (1980).
Method II: The following method was established and investigated during the study of the ability of the organisms to produce lipases and permits the concurrent testing to produce acetylmethyl-carbinol (acetoin). Plates of a standard medium containing glycerol and different lipids (Sigma) (triacetin, tributyrin, tricaproin, tricaprylin, tricaprin, trilaurin, trimyristin, tristearin, triolein, arachis oil and fish oil; see Appendix 1) were prepared separately. Basically 1 ml of lipid was added to the molten medium containing: peptone, 0.5g; yeast extract, 0.3g; agar, 1.5g; distilled water 100 ml; pH 7.5. For comparison, tributyrin was added to CDM/A-agar (see 2.2 and Table 3.6) with and without 1% mannitol. After inoculation and incubation for 48h at 37°C the plates were examined for lipase activity. These plates were then tested for acetoin production by a method based on that of Davis and Hoyling (1973).

A filter paper disc, freshly soaked in a 10% (w/v) sodium pyruvate solution, was placed on each plate. The plates were incubated again for a further 3h. Then one drop of 40% (w/v) potassium hydroxide, one drop of 1% (w/v) creatine and one drop of an alcoholic solution of 1% (w/v) alpha-naphthol are spotted successively on each disc. The development of a pink or red colour within one hour indicated acetoin production.

iv) Detection of haemolytic activity

Blood agar was prepared by adding, aseptically, 7% (v/v) sterile defibrinated guinea-pig, rabbit, sheep, horse or human blood (see 2.3) to CDM/A-agar (see 2.2 and Table 3.6) . For comparison, the same range of blood samples was added to BHI-agar. Preliminary experiments had shown that blood enriched BHI-agar encouraged both alpha- and beta-haemolysin production/detection; it was preferable to both Nutrient-agar and Diagnostic Sensitivity Test Agar (see 2.2) which had been initially considered for inclusion as a standard medium. Additionally, with chemically defined medium (CDM/A), it was found that mannitol (1% w/v) enhanced haemolysin production in comparison with glucose (1% w/v) and was added in place of the glucose. The detection of beta-haemolysin was aided by washing the sheep erythrocytes with PBS prior to adding them to the media.

In each case, inoculated plates were incubated at 37^{o}C for 24 to 48h and examined for zones of haemolysis. Plates were then incubated at 4^{o}C for a further 18 to 24h and examined for latent haemolysis (beta-haemolysin). The presence of zones of haemolysed red blood cells around the colonies was taken as indicating the production of a haemolytic toxin. The overall pattern of haemolysis (time of appearance, sharpness of zone) in conjunction with the species origin of the blood used, determined the assignment to a probable class of haemolysin (Bernheimer, 1965; Mollby, 1983).

To observe direct effects of staphylococcal growth on haemoglobin, BHI or CDM/A agar plates were enriched (7% v/v) with either lysed (freeze/thawed; -170^{o}C/37^{o}C; 3 cycles) whole, defibrinated, sheep or

rabbit blood or with the red blood cells (RBC's) free supernatant (4000g; MSE Mistral 6L, for 20 min) of similarly lysed blood. Inoculated plates were incubated and observed as above.

v) The sensitivity of Staphylococci to methicillin

The methicillin resistance of staphylococci was determined by the method of minimal inhibitory concentrations (MIC), on the plates of BHI-agar which were inoculated by spreading 0.1 ml of 10^7 cells/ml, of a standard cell suspension prepared as described below (see 2.6), over the surface of the medium. The surfaces of the plates were left to dry, until there was no visible moisture.

Place a number of single 13 mm discs, soaked in different concentrations of a sterile methicillin sodium (Beecham Pharmaceuticals BRL 1241) solutions (μg/ml): 0.4, 2, 10, 50, 100 and 200; equally spaced, onto the inoculated surface. After applying susceptibility discs, the plates were incubated for 18h at 37^{o}C, and examined for the presence or absence of the zones of inhibition around the single discs. Zones of inhibition were measured.

vi) Detection of capsule formation

The method of Smith, Parisi, et al, (1977) was carried out to detect encapsulation of six staphylococcal strains.

In this method standard cell suspensions (SCS, see 2.6) of staphylococci were diluted. Plates of BHI-agar or CDM/A-agar (see 2.2) were inoculated by spreading 0.1 ml, so as to yield about 400-500 colonies per plate. After 48h incubation at 37^{o}C a sterile molten mixture of safranine (0.02% w/v) and agar (0.8% w/v) was poured over the surfaces of the BHI- or CDM/A-agar plates and incubation was continued for another 48h at room temperature. Colonies produced by unencapsulated cells stained red, whereas colonies produced by encapsulated cells stained pink. As a staining and microscopic technique concern this method correlated with negative staining using Indian ink.

2.6 INOCULUM DEVELOPMENT AND GROWTH ASSESSMENT FOR GROWTH AND HAEMOLYSIN STUDY

For the study of haemolysin production (in either solid or liquid media), Staph. aureus strains were subcultured five times on defibrinated sheep's blood enriched plates of BHI-agar (see 2.5c) before preparing the final subculture, the colonies showing a wide zone of haemolysis being chosen for the study.

Standard cell suspensions (SCS) were prepared from 18h shaken cultures of BHI as follows:

Cells from 100 ml samples were collected by centrifugation (3500g for 15 min at 25°C; MSE Mistral 6L), washed three times with and finally resuspended in 1/4 strength Ringer's solution to a final volume of 100 ml. Samples (100 ml) of the appropriate liquid medium (at pH 7.2-7.4) in 250 ml Erlenmeyer flasks fitted with cotton wool plugs, were inoculated with 1 ml of the standard cell suspensions (SCS) of the required staphylococcal strain to give start cultures of optical density (540 nm; 1 cm path) of 0.05-0.1.

All cultures were incubated at 37°C in a Gallenkamp orbital incubator at 90 rev/min. Growth (optical density; 540 nm, 1 cm path) was assessed at various times using a Pye-Unicam SP500 series 2 spectrophotometer (unless otherwise stated). When appropriate dense cultures were diluted with fresh medium immediately prior to growth assessment.

2.7 PREPARATION OF THE CHEMICALLY DEFINED MEDIA

The L-forms of the amino acids (Table 2.1) were used for the preparation of the media. Stock concentrates (1% w/v) were prepared: the amino acids cysteine or cystine and tryptophan were dissolved in 0.2 N-HCl; tyrosine, aspartic acid, and glutamic acid were dissolved in 0.2 N-NaOH; the other amino acids were dissolved in distilled water.

The inorganic salts mixture (Table 2.1), mannitol (for Staph. aureus strains) or glucose (for Staph. epidermidis NCTC 7944), and vitamins were prepared in concentrated stock solutions (10% w/v) separately. The media were prepared to the required final concentration of amino acid and basal medium Table 2.1, and autoclaved at 115°C for 20 min (mannitol or/glucose, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ were added aseptically to the media after it had been sterilized by membrane filter 0.45 μm, Whatman). The initial pH of all media was 7.2-7.4.

TABLE 2.1: Composition of the Total Medium CDM from which a Chemically Defined Medium was Derived for Each Staphylococcal Strain

Constituent	mg/100 ml
Basal Medium:	
Carbohydrate:	
Mannitol or Glucose	1000
Inorganic salts:	
NaCl	400
Na_2HPO_4	175
KH_2PO_4	100
$MgSO_4.7H_2O$	70
NH_4Cl	50
$FeSO_4.7H_2O$	1
Vitamins:	
Thiamine	0.05
Nicotinic acid	0.05
Biotin	0.0003
Amino-acids as supplement:	
alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine.	each at 3.5

2.8 DEVELOPMENT OF STRAIN SPECIFIC CHEMICALLY DEFINED MEDIA AND GROWTH STUDIES

A two step approach was adopted. The first stage was based on the single deletion method of Mah, et al, (1967). A basal medium was supplemented with 18 selected amino acids to provide a standard chemically defined medium (CDM; Table 2.1). A series of media based on CDM was prepared by deleting single amino acids in sequence so that each medium so produced contained 17 amino acids and differed only in one amino acid. Samples (100 ml) of CDM and of each defined medium under investigation were inoculated with 1 ml of the standard cell suspensions (SCS) of the required staphylococcal strain and incubated at 37°C (see 2.6). Culture optical density (OD) monitored (540 nm, 1 cm path, see 2.6), every 3h for 12h and then at 6h intervals for at least 30h. Growth curves were plotted and interpreted (Table 3.5) as follows: where the removal of an amino acid gave a prolonged lag phase (in excess of 12h) or gave markedly depressed growth (compared to the complete medium), this was taken as an indication that the amino acid was essential (E). If growth was unaffected by deletion of a particular amino acid, then that amino acid was regarded as non-essential (N). If omission of an amino acid resulted in a lag phase, longer than that of the control culture (three to six hours) or in a reduced growth rate, then this amino acid was regarded as supplemental (S). The absence of some amino acids resulted in a prolonged lag phase (nine to twelve hours) followed by growth at a reduced rate. Such amino acids were considered to be more than supplemental, but not essential, and were described as required (R).

The second stage was, using the data from Table 3.5, to group Essential amino acids for each organism and to enrich this by the addition of Required amino acids (Table 2.2). From these preliminary studies many chemically defined media containing E and R amino acids for each strain were prepared (Tables 2.2, 3.6). Media containing 10 to 13 amino acids (Table 3.6) were developed by addition of amino

acids supplemental (S) for each strain to those amino acids classified as E and R for each strain (see Table 3.5).

TABLE 2.2: Amino Acids Supplementing the Basal Medium of CDM (Table 2.1) to give Media Supporting Adequate Growth for Each of the Staphylococcal Strains

Staph. aureus					Staph. epidermidis
NCTC 8532	NCIB 8625	NCTC 5655	WOOD 46	NCTC 7428	NCTC 7944
arg (E)		arg (E)		arg (E)	arg (E)
cys (E)	cys (E)	cys (E)	cys (E)	cys (E)	cys (E)
			his (R)	his (E)	
ile (R)	ile (R)				
					glu (R)
			leu (R)	leu (R)	
					lys (R)
				meth (R)	
phe (R)				phe (R)	
	pro (E)	pro (R)	pro (E)	pro (E)	pro (R)
thr (R)	thr (R)	thr (R)			
val (E)	val (E)	val (E)	val (E)	val (E)	

E - essential; R - required

Additionally three further media were also prepared, inoculated and incubated (see 2.6), for use in studies growth and haemolysin production; Dalen's medium (Dalen, 1973a) comprising 12 amino acids (Table 3.6, used only with Staph. aureus Wood 46), BHI and YEBS (see 2.2).

Initial difficulties were encountered with Staph. aureus Wood 46 growing in chemically defined media. The organism adhered to the surface of the glass culture vessels and clumped giving rise to large "clots" of cells; a problem encountered with other organisms

(Stormonth and Coleman, 1972). It was found that this effect could be minimised by the use of plastic flasks and/or the combined use of optical density, following vigorous agitation, and pH measurement (pH-meter; PW 9409) at the same intervals as the OD. In general, pH assessment was only used to confirm that adequate growth had occurred. Growth assessment by acid production has been used by Dreizen, Mosny, et al, (1954).

2.9 MEASUREMENT OF ALPHA- AND BETA-HAEMOLYSIN PRODUCTION IN LIQUID CULTURES

The method of Jassim et al,(1988) was used as follows:

a) Preparation of Calibration Curve for Determination of Graded Degrees of Lysis

The calibration curve was prepared in the following way: 10 ml of a 2% washed rabbit or sheep defibrinated erythrocyte suspension (see 2.3) was added to a 100 ml flask with stopper containing 30 ml phosphate buffered saline (PBS). The new diluted suspension was lysed by three alternate cycles of freezing the suspension with liquid nitrogen (-170^{o}C) and thawing it rapidly in a water bath (37^{o}C) to achieve complete haemolysis. The supernatant liquid resulting from centrifugation of the haemolysed cells at 4000g for 20 min at 4^{o}C, was used as a standard fluid of 100% lysis.

A 10% dilution series was prepared using PBS diluent (100-10%) and the optical density (545 nm, 1 cm path, Pye-Unicam SP500 series 2 spectrophotometer), of each dilution assessed. A calibration curve was constructed of percentage haemolysis versus optical density (see Figure 2.1). The 50% haemolytic unit (HU50) for rabbit was 0.41 and for sheep blood was 0.48.

b) Haemolytic Unit 50 (HU50) Assay

The haemolytic titre (HU50/ml; defibrinated rabbit blood for alpha-haemolysin and sheep blood for beta-haemolysin, unless otherwise stated) of the supernatant liquid (4000g, 20 min at 25 ^{o}C) from a

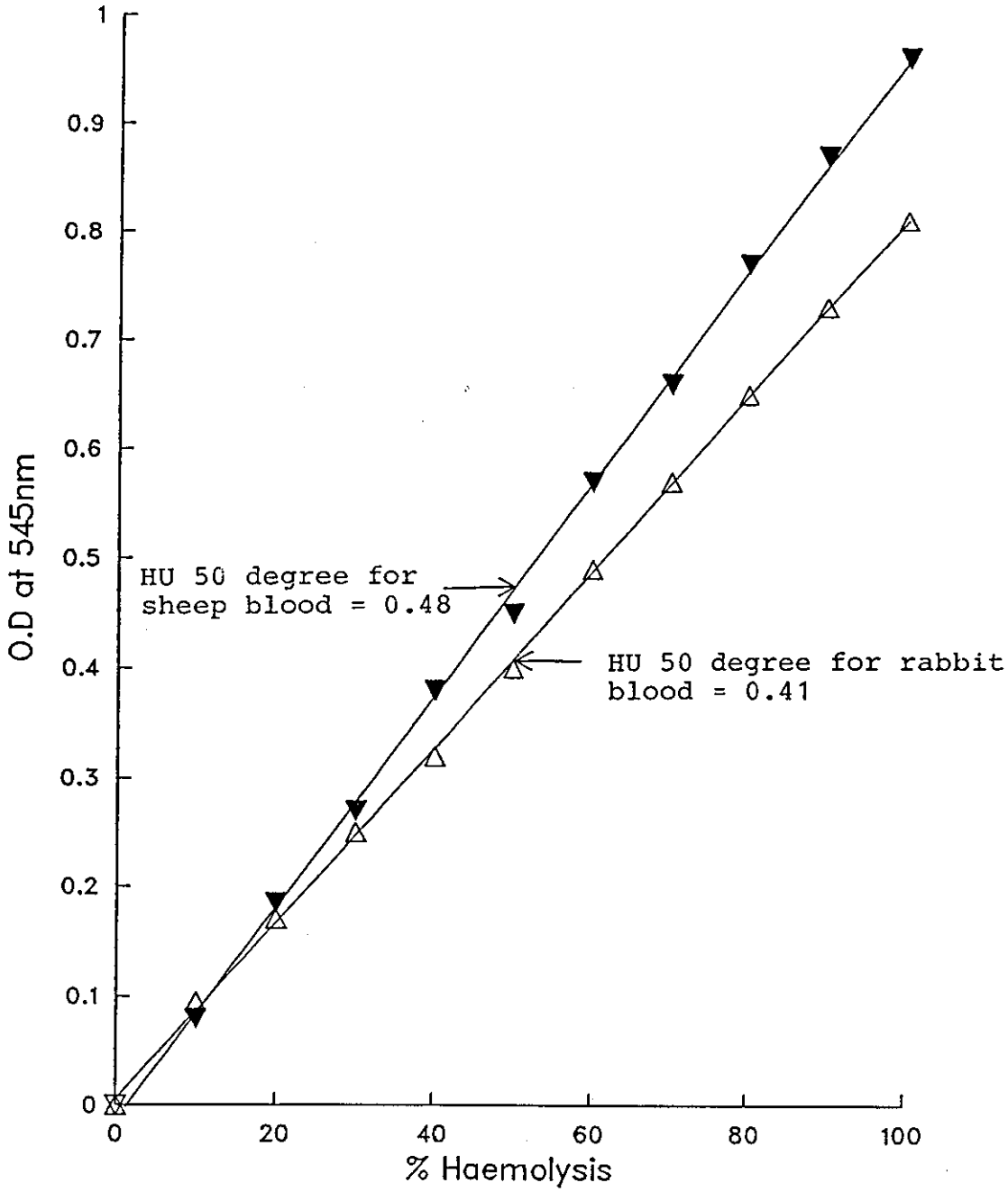


Fig. 2.1: Relation of optical density at 545 nm to % haemolysis (freeze/thaw) of a 2%: △, rabbit, and ▼, sheep RBC's suspension.

sample of the growing broth culture was assessed on the basis of 50% haemolysis of a standard erythrocyte suspension as in Table 2.3, for all organisms grown in a different range of chemically defined media (see Table 3.6), CDM/A, BHI and FIB (see 2.2) was measured at definite time intervals. Additionally, haemolytic activity was assessed for Staph. aureus NCTC 7428 and Wood 46 in medium CDM (see 2.8, Table 2.1), and a range of media containing 3.7% w/v of fish- or beef-extract and 3.7% w/v peptone or proteose peptone (see Appendix 1). In supplementary studies, haemolysin production was assessed for Staph. aureus strains NCIB 8625, NCTC 5655, NCTC 7428 and Wood 46 grown in YEBS medium (see 2.2) and in various chemically defined media to which yeast extract, 0.1% w/v had been added.

TABLE 2.3: The Haemolytic Unit 50 (HU50) Assay for Alpha- and Beta-Haemolysin

Tubes (plastic test tubes, 17 x 100 mm) Number	2 3 4 5 6 7 8 9
Tubes (plastic test tubes, 17 x 100 mm) Number	11 12 13 14 15 16 17 18 19
Phosphate buffered saline (PBS) (ml*)	1 ml added to all tubes (except 11)
Supernatant liquid (haemolysin) (ml)	1 ml serially doubling dilution of toxin in PBS (from tubes 2 to 9)
Supernatant liquid (haemolysin) (ml)	0.8 ml** serially doubling dilution of toxin in PBS (from tubes 11 to 19)
PBS (ml)	2 ml added to all tubes
2% rabbit or sheep RBC's (ml)	1 ml added to all tubes

* PBS (see 2.2) was supplemented throughout with 492.96 mg/l of $MgSO_4.7H_2O$ in case of detection of beta-haemolysin.
** 0.8 ml haemolysin, 1.2 ml PBS.

Each mixture was incubated at 37°C for 1h and then centrifuged (3500g, 10 min at 4°C) to remove intact erythrocytes. The absorbance (545 nm, 1 cm path) of the supernatant liquid was measured and the concentration giving 50% haemolysis (HU50) assessed by comparison

with that obtained by alternate rapid freezing and thawing (see 2.9a, Figure 2.1) of equivalent dilutions of the standard erythrocytes suspension. For determination of beta-haemolysin titre mixtures were held at 37°C for 1h and then 1h at 4°C prior to centrifuging off the intact erythrocytes and measuring absorbance as before.

The reciprocal of the final dilution would be within the range: 8 to 2560 for haemolysis detection and an approximate assessment of the HU50 value, further dilutions if necessary could then be performed to obtain a more accurate titre.

The 50% haemolytic unit (HU50) is defined as the reciprocal of the highest haemolysin dilution in a test series which liberates half the haemoglobin in the test erythrocyte suspension under the conditions stated.

The final dilution of haemolysin in the first tube (No 2; see Table 2.3) was considered as 1:8, the range covered was up to 1:2560. The tube No 1 considered as a 1:4 dilution (since undiluted culture supernatant was used) was also prepared. In this view of the work a visually determined observation of the degree of haemolysis (complete, almost complete, definite, doubtful or negative) was made in comparison with a negative control. However, more critical spectrophotometric readings were obtained for comparison with calibration curves.

2.10 MEDIUM AND SERUM FOR RABBIT POLYMORPHONUCLEAR LEUCOCYTES (PMNs)

The incubation-medium used in the phagocytosis studies, with rabbit granulocytes, consisted of gelatin-Hank's balanced salt solution (gel-HBSS; see 2.2) and 10% rabbit serum. The rabbit serum-rabbit normal serum (RNS) was obtained from the whole blood of a single animal in any given experiment. The animal was maintained solely for this purpose.

Blood was collected from a rabbit fasting for 18h, in a sterile glass tube from ear veins and allowed to stand at room temperature for 2h for clot formation and then placed at 4°C for 12-18h to permit clot retraction (shrinking). The tube containing the clotted blood was centrifuged at 3500g for 15 min at 4°C (MSE, Mistral 6L) and the clear serum removed by careful decanting. If red blood cells (RBCs) were still present in the serum, it was recentrifuged using the same conditions.

The serum was stored in 2 ml portions in sterile screw cap vials at -20°C for no longer than 60 days and thawed just before use. Repeated freezing and thawing can, however, be deleterious and was avoided. This serum from clotted blood contains variable amounts of bactericidal substances (Hirsch, 1960).

Rabbit heat-inactivated serum (RHS) was prepared by incubation of serum for 30 min in a water bath at 56°C.

2.11 IMMUNIZATION OF RABBITS FOR Staph. aureus WOOD 46

The chemically defined media containing 5 amino acid and 12 amino acid (CDM/7 and CDM/8 respectively; see Table 3.6) and BHI (as a control) were inoculated with Staph. aureus Wood 46 and incubated as previously described (see 2.6), for 18h. The bacterial pellets were collected by centrifugation at 3500g, for 15 min at 25°C (MSE, Mistral 6L) and diluted with 10 ml of 1/4 strength Ringers solution (see 2.2) to give approximately 10^9 bacteria/ml.

Formalised Staph. aureus Wood 46 antigen was used to immunize the rabbits with mixed-antigens and this was prepared by adding formaldehyde (to 1% v/v) to the cell suspension. The suspension was mixed well and incubated at 37°C for 3-4h. Tests for the absence of viable organisms (viable count on plates of DST-agar; see 2.2) were carried out.

The cells were harvested by centrifuging as before and washed twice with 1/4 strength Ringer's solution and resuspended to the original diluted volume in 10 ml.

A dose (0.5 ml) of the antigen of formalised Staph. aureus Wood 46, from one of each culture (different media) were injected intramuscularly and subcutaneously into each side of the rabbits (Male, Old English Rabbits weighing 3-3.5 kg) twice a week for 4 weeks. The rabbits (fasting before bleeding) were bled 7 days after the last injection, and serum was prepared as described previously (see 2.10).

2.12 SEROLOGICAL STUDY OF Staph. aureus WOOD 46 SURFACE ANTIGENS

The following methods described by Leinonen (1985) were used for the titration of rabbit anti-staphylococcal serum, in a sample of Staph. aureus Wood 46 cell suspension (not formalised) prepared as described in the first paragraph of section 2.11.

Method I: Double Immunodiffusion in Gel

Double immunodiffusion is one of the basic methods for the demonstration of the presence of a certain antigen as well as of the presence of cross-reacting antigen structures between two different preparations.

An aqueous solution containing 0.8% (w/v) purified agar (Oxoid, L28) and 3% (w/v) polyethyleneglycol (PEG, 6000) was prepared by heating for 20 min in a boiling water bath. 3 ml of the hot agar-solution was poured onto a microscope slide, cooled at room temperature and wells were punched, of size 3 mm diameter, in the arrangements of one well in the centre and others surrounding it. The distance between them was approximately 3 mm. The antigens were filled in the centre well and different antisera in the surrounding wells. They were then incubated at room temperature for 2 days in a moist chamber. The microscope slides were examined for precipitation lines: several lines between an antigen and antisera wells

indicates the presence of several separate antigen/antibody reactions; complete fusion of lines formed between antiserum and adjacent antigen wells indicates identity of antigens; partial fusion (spurring) indicates partial identity.

Method II: Antiserum Agar Method (ASA)

The ASA method (Leinonen, 1985) can be applied to the detection of bacterial colonies with certain surface antigens (capsular, slime, extracellular enzymes and toxins). The antiserum (CDM/7, CDM/8 and BHI) agar plates were prepared from 100 ml of BHI-agar autoclaved at 121°C for 15 min, cooled to 45-50°C and added the antiserum, to give the required, predetermined dilution (10% v/v). After mixing thoroughly, it was poured in a shallow layer and allowed to solidify. The plates can be stored at 4°C in tightly sealed plastic bags for up to 60 days. The bacterial suspension was diluted to produce isolated colonies, and spread onto the agar and incubated for 42h at 37°C. Then the colonies were examined for precipitation halos. The appearance of a precipitation halo around a colony demonstrated the presence of the antigen studied.

Method III: Counter Immunoelectrophoresis (CIEP)

The CIEP has been widely applied to the demonstration of acidic polysaccharide antigens. The procedure is as follows:

A solution containing 0.85% purified agar (Oxoid, L28) in the electrophoresis buffer (EB; see 2.2) diluted 1:2 with H_2O (containing 0.1% w/v sodium azide; BDH) was prepared by heating for 20 min in a boiling water bath. 25 ml of the hot agar-solution was poured onto a glass plate (7.5 x 13 cm), cooled at room temperature, and wells punched of 3 mm size diameter in two parallel rows 3 mm apart. The wells were filled with the antigen and antibody solutions so that the antigen was in the row on the cathodic side of the plate. Then the electrophoresis was run with a constant current of 40 mA across the plate for 30 min at room temperature. The plates were examined for precipitation lines immediately after the run and the next day after washing the plates overnight in PBS (see

2.2), when the washing removed non-specific precipitates. The gel was stained by 0.1% (w/v) Amido Black (Hopkin and Williams) in distilled water for 10 min and destained with 10% (v/v) acetic acid. It was then dried with a hairdryer and examined for precipitation lines.

Method IV: Slide Agglutination

Mix well one drop of bacterial suspension 10^9 bacteria/ml and one drop of antiserum diluted in 0.2% (w/v) NaCl on a glass slide. The slide was continuously tilted up and down for 2-5 min and examined against a dark background with an obliquely placed light source. Agglutination usually started in 30-60 sec, and final readings were taken after 5 min. The size of the clumps was observed (fine granular agglutination may be non-specific auto-agglutination of rough or partly rough strains). The completeness of agglutination: a milky background of non-agglutinated bacteria suggests a heterogeneous population (due to variation of the antigen). The control (4% w/v NaCl) was used instead of the antiserum to detect auto-agglutination of rough strains.

2.13 RABBIT POLYMORPHONUCLEAR LEUCOCYTES (PMN's) PREPARATION

A male Old English rabbit weighing 3-3.5 kg, used throughout the work, was lightly anaesthetised by intravenous (IV) injection of 30 $mg.kg^{-1}$ Thiopentone sodium BP (May and Baker Ltd, UK). Employing aseptic techniques, the rabbit was injected intraperitoneally (IP) with 150 ml of a sterile prewarmed freshly prepared glycogen saline solution (GSS; see 2.2), serving as a chemotactic agent to call forth circulating phagocytes. A No 19 gauge 2 inch needle (Becton, Dickinson Ltd, UK) was used.

After 12-16h rabbits (fed but not watered) were anaesthetised by intravenous (IV) injection of 80 mg kg^{-1} Pentobarbitone sodium BP (May and Baker Ltd, UK), then 50 ml of heparin-Hank's balanced salt solution (H-HBSS; see 2.2) was prewarmed to 37°C and injected (IP) through a No 16 gauge perforated plastic catheter (Becton, Dickinson

Ltd, UK). The abdomen was gently massaged and the peritoneal fluid allowed to drain by gravity from the cavity via a perforated plastic catheter into sterile 50 ml capped plastic collecting tubes. It was found possible to utilise the cells from a single rabbit in any experiment carried out. Rabbits were used with no greater frequency than every 15 days. The cells in the fluid were collected by centrifugation at 1000g for 4 min at 37°C (MSE, Mistral 6L). The cell pellet was resuspended in three volumes of 0.87% NH_4Cl in distilled water (pH 7.2) to lyse contaminating erythrocytes. Complete mixing of the cell suspension was assured by gentle inversion of the tubes every minute.

After 10 to 12 min, centrifugation was started at 800g for 10 min at 37°C. The lysate as well as the main part of the red-cell ghosts were removed by careful aspiration. The remaining NH_4Cl solution was removed by resuspending the cells in 20 ml of the washing medium (HBSS; see 2.2). After centrifugation (4 min at 800g and 37°C) the supernatant was aspirated. This was repeated three times. The washed cells were resuspended in a convenient amount of gel-HBSS (see 2.2) to a final concentration of 1-2 x 10^7 cells per millilitre. The cells were counted with a haemacytometer under a high power dry objective. Microscopic examination of the final suspension showed that granulocytes accounted for more than 95% of the total leucocytes population as assessed by Wright's (Sigma) stained smears.

2.14 GRANULOCYTE CELL COUNTING METHOD

The method of Baker and Silverton (1976) was used as follows: the cell suspension was drawn up to the 0.5 mark on the stem of a white cell pipette and Turks' solution (1% w/v gentian violet in 1% v/v glacial acetic acid) used as diluting solution drawn up to the 11 mark immediately above the bulb. The suspension was mixed by shaking the pipette for 4 min. Alternatively, a 1/20 (0.05) dilution of cell suspension was prepared and a quarter of the mixture was discarded. One to two drops were placed gently between the cover glass and

improved Neubauer counting chamber and the cells counted under a high power dry objective.

2.15 NEUTROPHIL VIABILITY

Three drops of the cell suspension were mixed with one drop of 1% w/v trypan blue (BDH) in saline. One drop of this mixture was placed on the microscope slide and covered with a cover glass. The cells were examined within 2 min. The proportion of cells that had taken up the dye was determined by a survey of at least 100 cells. Initially less than 4-5% of the granulocytes exhibited nuclear staining, and at the end of 120 min of incubation period of the phagocytosis experiment, less than 10%. Only cell suspensions containing less than 10% stained cells were considered suitably viable for experimental use.

2.16 PREPARATION OF BACTERIAL INOCULATION FOR PHAGOCYTOSIS AND INTRACELLULAR-KILLING

The staphylococcal strains were inoculated and incubated as previously described (see 2.6), for 18h in different liquid media: chemically defined media (see Table 3.6), FIB (see Appendix 1) and BHI (see 2.2). Staphylococci were harvested by centrifugation for 15 min at 3500g and 25^{o}C (MSE, Mistral 6L), washed twice with and finally resuspended in gel-HBSS (see 2.2) to a concentration of 1-2 x 10^{7} bacteria per millilitre.

2.17 VIABLE COUNTS MEASUREMENT

The viable counting method of Miles and Misra (1938) was used for bacteria.

2.18 PHAGOCYTOSIS MIXTURES AND ASSAYS

The technique employed to determine the percentage and kinetics of the phagocytic process was that of Leijh et al, (1986). An outline of the method is given in Figure 2.2.

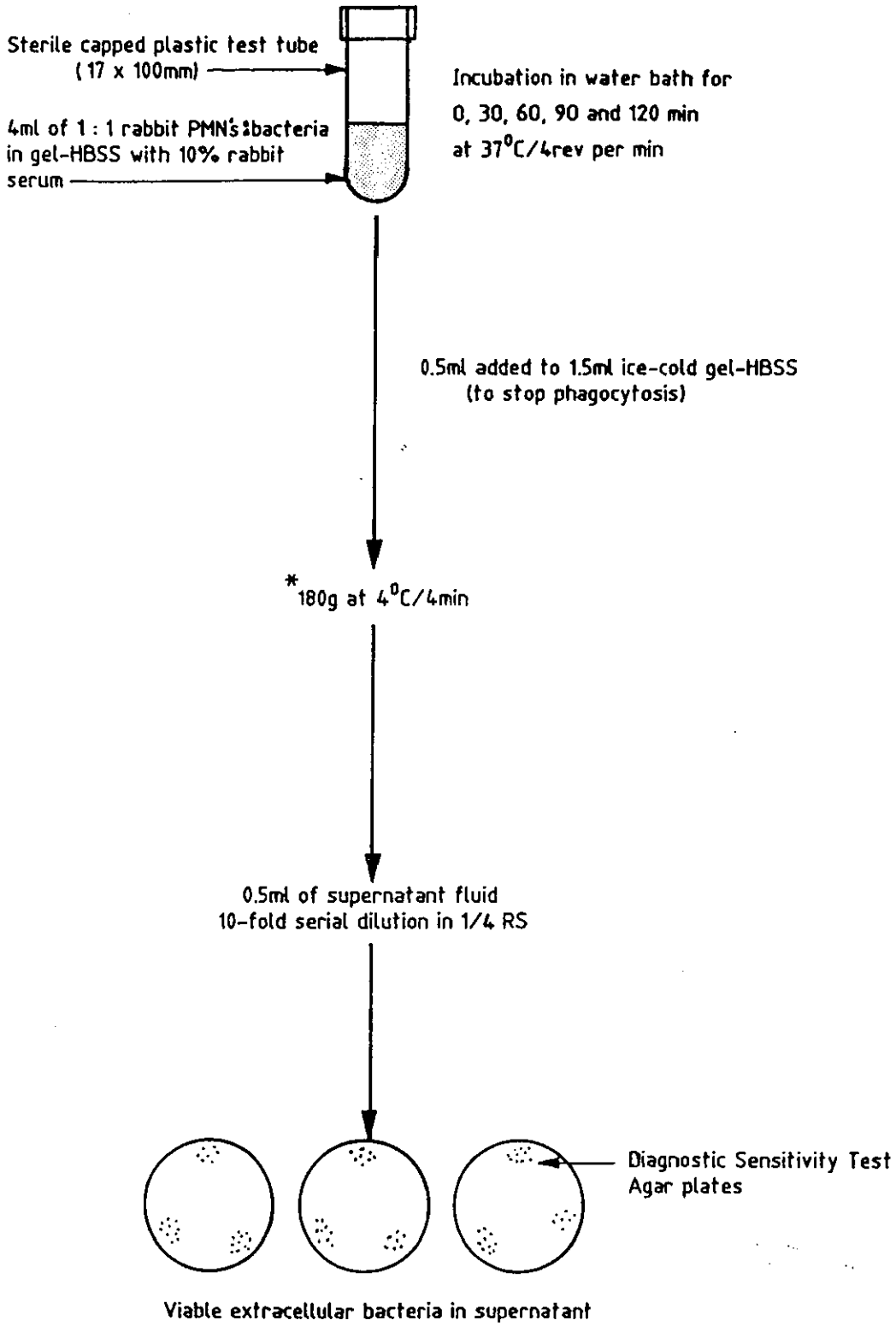


Fig. 2. 2. Outline of the technique for assessing phagocytosis *in vitro*.

*Preliminary experiments demonstrated that free bacteria were not appreciably sedimented under these conditions.

Assessment of Results

All experiments were repeated three times on different days, and the mean values of results were taken. Also each experiment had three components:

i) Incubation of PMNs, bacteria and 10% serum, in gel-HBSS for 120 min in water bath shaker (Gallenkamp, UK) at 37°C/4 reverse (rev) per minute and assayed at different time points (see Figure 2.2).

ii) Incubation of bacteria with or without 10% serum in gel-HBSS, under the same conditions as in (i). This served as a control on the initial number of bacteria, any bacterial growth during the experiment and the bactericidal effects of serum.

iii) Any bactericidal products released from PMNs were obtained as the supernatants by centrifugation 4 ml of the sample at 3500g for 15 min after 120 min of phagocytosis (as in i). Washed staphylococci (see 2.16) were incubated with the supernatant for 120 min under the same conditions as in (i). The number of viable bacteria was determined at appropriate intervals with the plating method (see 2.17; Figure 2.2).

Calculations

The phagocytic index was calculated according to the following formula:

$$P_{(t)} = (1 - \frac{N_t}{N_o}) \times 100 \qquad (1)$$

where $P_{(t)}$ is the phagocytic index at time t = t (30, 60, 90 or 120 min; see Figure 2.2). N_o and N_t are the number of viable extracellular bacteria (in the presence of PMNs and serum, see component i) initially and at time t, respectively.

The corrected phagocytic index was calculated according to the following formula:

$$P_{(t)corrected} = (1 - \frac{N_t \times B_o}{B_t \times N_o}) \times 100 \qquad (2)$$

where B_o and B_t are the number of bacteria during incubation of bacteria in serum in gel-HBSS (namely in the absence of PMNs; see component ii) initially and at time t, respectively.

2.19 MORPHOLOGICAL CHECKING OF PHAGOCYTOSIS

The ingestion of staphylococci by PMNs was also determined for each experiment. A 0.5 ml sample was taken of the staphylococci-cell suspension after 60 min incubation and then added to 1.5 ml ice-cold gel-HBSS, and washed three times with the same solution by centrifugation (4 min at 180g). Then the washed cells were resuspended in 1 ml gel-HBSS. From this, suspensions were made on microscope slides, and after staining with Wright's (Sigma) stain, the phagocytes were examined by light microscopy, and cells that had ingested bacteria were determined.

2.20 INTRACELLULAR-KILLING ASSAY

Staph. aureus Wood 46 was grown in CDM/7, CDM/8 (see Table 3.6) and BHI (see 2.16). The number of viable intracellular bacteria during incubation of phagocytic cells and bacteria was determined as described by Leijh, et al, (1986). An outline of the method is given in Figure 2.3.

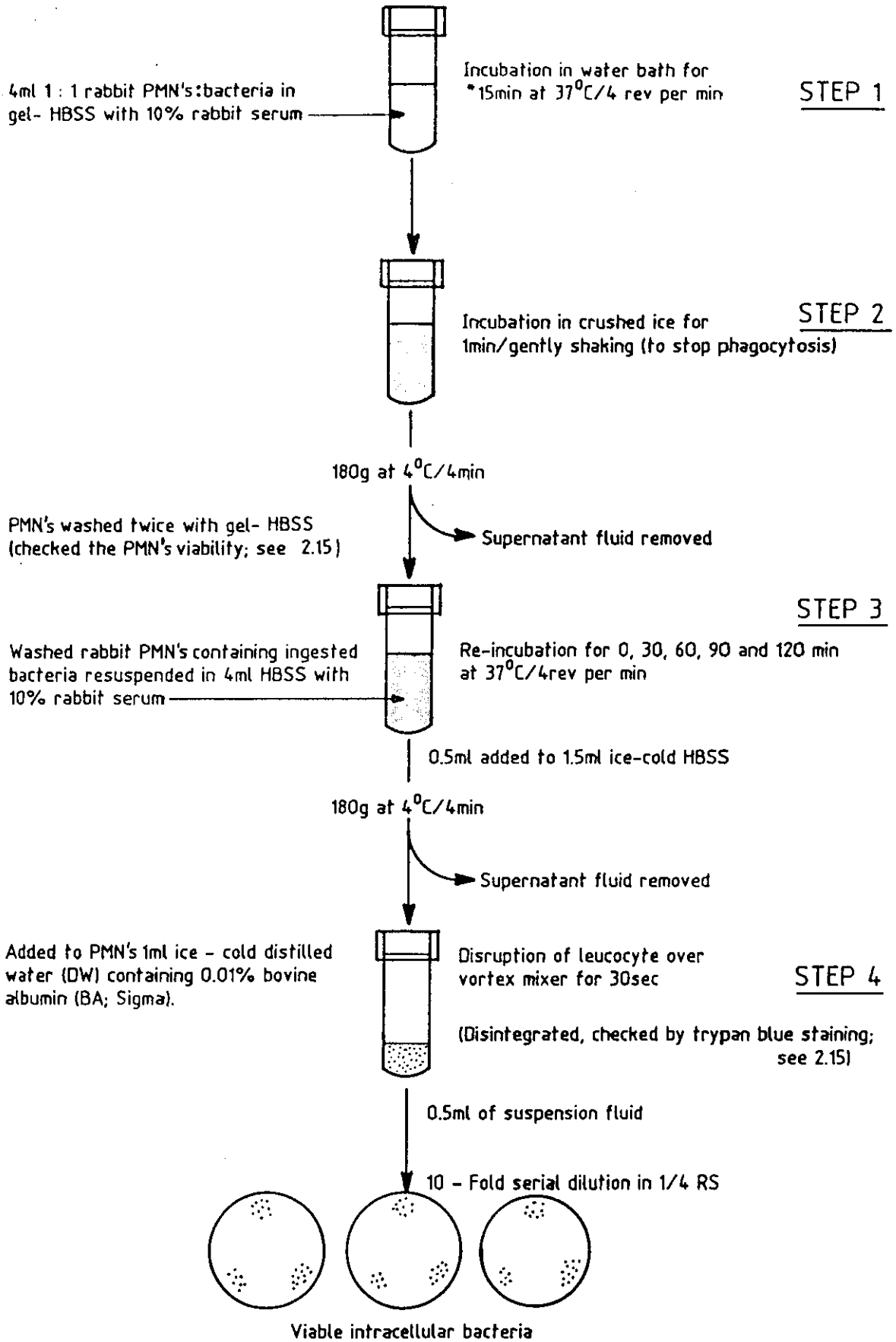


Fig. 2. 3. Outline of technique for assessing intracellular killing *in vitro*.

* Preliminary experiment gave the maximum number of viable intracellular staphylococci in the period of about 15 min.

Assessment of Results

The intracellular-killing experiments were repeated three times on different days and the mean reading of results were taken. There were two components of intracellular-killing considered in each experiment:

i) Assay at different time points of incubated cells that have ingested bacteria (see Figure 2.3, steps 3 and 4).

ii) Assay at the end of the experiment (120 min) of a similar sample (step 3; see Figure 2.3) incubated at 4°C, stationary, served as a control.

The viability of the granulocytes was checked by trypan blue staining (see 2.15), and it was found that more than 90% of the granulocytes were viable at the end of the intracellular-killing experiment.

Calculations

The intracellular-killing index was calculated according to the following formula:

$$K_{(t)} = (1 - \frac{N_t}{N_o}) \times 100$$

where $K_{(t)}$ is the killing index, N_t is the number of viable intracellular bacteria at time t = t (30, 60, 90 and 120 min; see step 3, Figure 2.3) and N_o is the number of viable intracellular bacteria at time t = 0 (obtained from step 3, see Figure 2.3).

2.21 DETERMINATION OF THE EFFECT OF BACTERICIDAL SUBSTANCES FROM RABBIT PMNs CRUDE EXTRACTS (PMNCE) ON Staph. aureus WOOD 46

Staph. aureus Wood 46 grown in CDM/7, CDM/8 and BHI was used (see 2.16). An outline of the method is given in Figure 2.4.

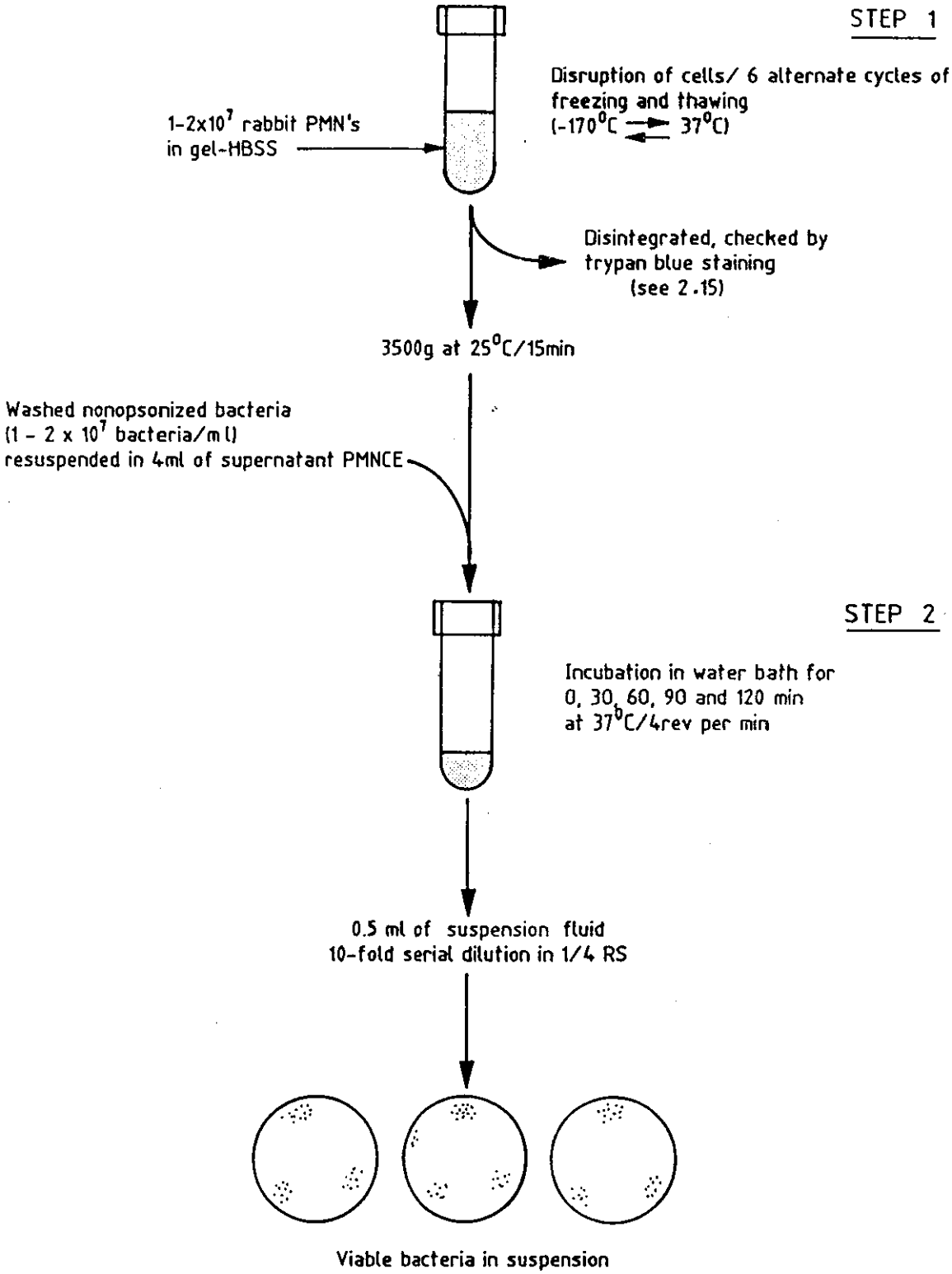


Fig. 2. 4. Outline of technique for assessing the effect of bactericidal substances from lysed PMN's(PMNCE) *in vitro*

Assessment of Results

The bactericidal effect of intracellular material from PMNs on bacteria was repeated three times on different days and the mean reading of results were taken. Each experiment had two components:

i) Assay at different time points of incubated bacteria in supernatant containing PMNs crude extracts (PMNCE) (see Figure 2.4, step 2).

ii) Assay at the end of the experiment (120 min) of bacteria (1-2 x 10^7 bacteria/ml), resuspended in 4 ml gel-HBSS (without PMNCE) incubated under the same conditions as in (i). This served as a growth control for the bacterial growth in gel-HBSS in the absence of PMNCE.

Calculations

The bactericidal index was calculated according to the following formula:

$$B_{(t)} = (1 - \frac{N_t}{N_o}) \times 100$$

where $B_{(t)}$ is the bactericidal index, N_o and N_t are the number of viable bacteria in the supernatant initially and at time t (30, 60, 90 and 120 min; see step 2, Figure 2.4), respectively.

CHAPTER THREE

RESULTS

3.1 CHARACTERIZATION OF SIX STAPHYLOCOCCI STRAINS

The morphological, biochemical and physiological characteristics that distinguish the six strains of staphylococci are given in Table 3.1.

3.1.1 General-Biochemical Tests

In general the biochemical tests showed the following: the clumping factor (bound-coagulase) results were negative for Staph. aureus NCTC 5655, Wood 46 and Staph. epidermidis NCTC 7944; weak in the case of Staph. aureus NCTC 8532. However, all five strains of Staph. aureus produced a free coagulase enzyme.

None of the staphylococci produced acid from xylose. Staph. epidermidis NCTC 7944 produced acid from mannitol (weak) both aerobically and anaerobically. All strains gave negative indole, hydrogen sulphide and oxidase production.

Ammonia production, nitrate reduction, catalase, lipase and methyl red were all positive in all six staphylococcal strains.

Only the coagulase-positive (tube test) strains of Staph. aureus produced acetoin in media containing glucose and, separately, tributyrin (see Figure 3.1), and were positive for phosphatase.

The coagulase-negative strain, Staph. epidermidis NCTC 7944, produced a weaker DNase reaction than the coagulase-positive strains. This was irrespective of the temperature used. The TNase activity was detected following modification of the method of Bennett (1984) giving a clear-zone around the wells with all strains of Staph. aureus. An opaque zone was observed with Staph. epidermidis NCTC 7944 (Figure 3.2).

TABLE 3.1: Distinguishing Characteristics of Six Staphylococci Strains

Characteristic Test	Strain of Staphylococcus					
	NCTC 8532	NCIB 8625	NCTC 7428	NCTC 5655	Wood 46	NCTC 7944
Pigmentation	W	S	P	P	N	N
Gram stain	P	P	P	P	P	P
Motility	N	N	N	N	N	N
Acid Production from Carbohydrates:						
A. Monosaccharides:						
Glucose:						
i) aerobically	P	P	P	P	P	P
ii) anaerobically	P	P	P	P	P	P
Xylose	N	N	N	N	N	N
B. Disaccharides:						
Lactose	P	P	P	P	P	P
Maltose	P	W	P	P	P	P
Sucrose	P	P	P	P	P	P
C. Sugar Alcohols:						
Mannitol:						
i) aerobically	P	P	P	P	P	W
ii) anaerobically	P	P	P	P	P	W
Indole production	N	N	N	N	N	N
Methyl red	P	P	P	P	P	P
Acetoin production:						
Method I: Voges-Proskauer (VP-test)	S	P	P	S	S	N
Method II: BHIA-Glucose	P	P	P	P	P	N
Method III: Tributyrin-agar*	P	P	P	S	P	N
Ammonia production	P	P	P	P	P	P
Nitrate reduction	P	P	P	P	P	P
Hydrogen sulphide (H_2S) production	N	N	N	N	N	N
Lysostaphin sensitivity	P	P	P	P	P	P
Enzymes production:						
A. Catalase	P	P	P	P	P	P
B. Coagulase:						
i) clumping-factor (slide-test)	W	P	S	N	N	N
ii) free-coagulase (tube-test)	P	P	S	P	S	N
C. Lipase	S	S	P	S	P	S

Table 3.1: continued

Characteristic Test	Strain of Staphylococcus					
	NCTC 8532	NCIB 8625	NCTC 7428	NCTC 5655	Wood 46	NCTC 7944
D. Lecithinase:						
i) Egg-yolk agar (EYA)	S	S	N	S	S	N
ii) EYA with 1% glucose	W	P	N	P	P	N
E. Urease	P	P	P	N	P	P
F. Phosphatase	S	P	P	S	S	N
G. Deoxyribonuclease (DNase)						
Method I - at 25°C	P	P	P	P	P	W
Method II - at 30°C	P	P	P	P	P	W
Method III - at 37°C	P	P	P	P	P	W
H. Thermonuclease production (TNase)						
Method I - Bennett (1984)	N	N	N	N	N	N
Method II - a modified method I	P	P	P	S	S	N
I. Caseolysis (Proteinases)	N	N	N	S	S	P
J. Gelatin hydrolysis						
Method I - at 22°C	S	S	P	S	S	P
Method II - at 37°C	N	P	P	S	S	P
K. Oxidase	N	N	N	N	N	N

Reactions: N - negative
W - weak
P - positive
S - strong-positive

* See also Table 3.4

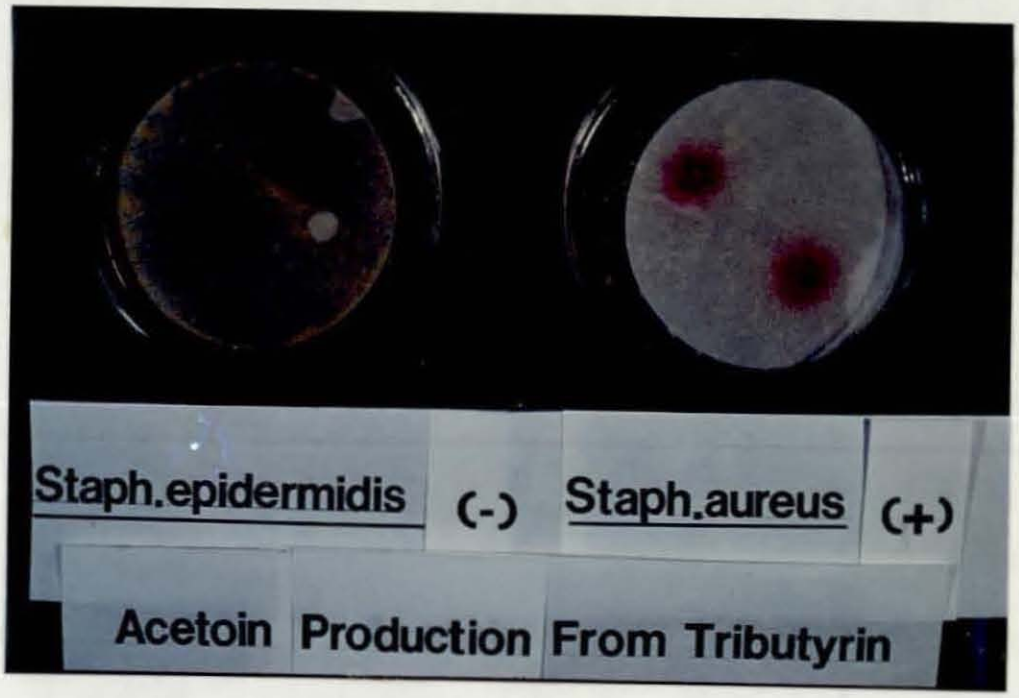


Fig. 3.1: Acetoin production of Staph. aureus Wood 46 from tributyrin, displays a pink spot, and Staph. epidermidis NCTC 7944 with negative results

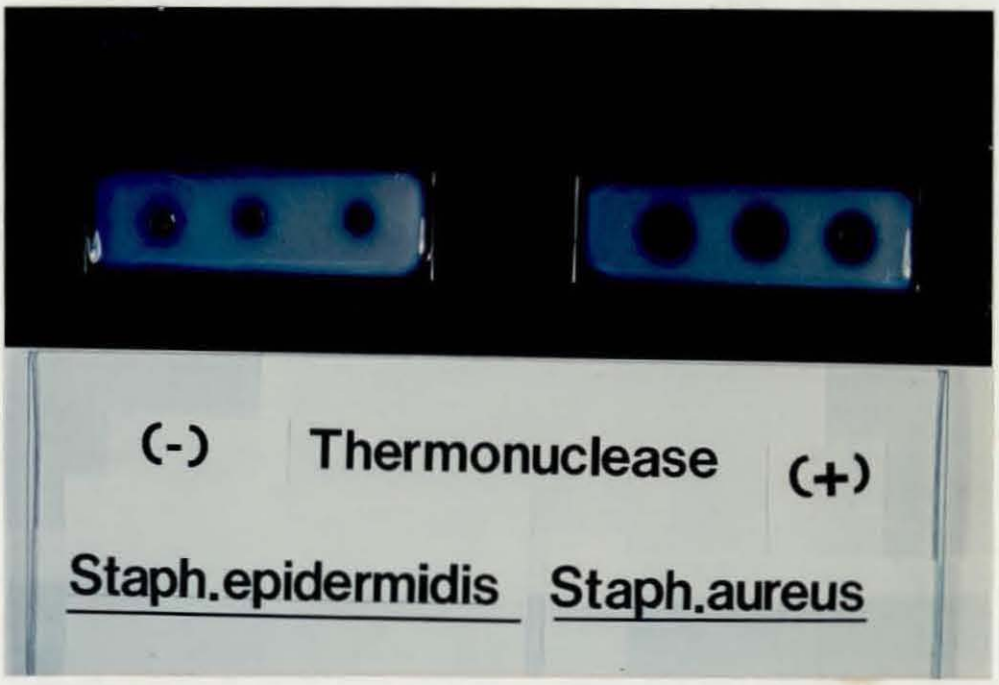


Fig. 3.2: Thermostable-nuclease (TNase) positive result of Staph. aureus Wood 46 shows a clear zone around the wells, and TNase negative of Staph. epidermidis NCTC 7944 with an opaque zone around the wells

All strains hydrolysed gelatin and it was observed that more hydrolysis occurred at 22°C than 37°C. Caseolytic activity was observed only with Staph. aureus NCTC 5655 and Wood 46 and Staph. epidermidis NCTC 7944, on both nutrient agar and BHI-agar supplemented with 10% (w/v) skimmed milk powder. The only non-urease producing strain was Staph. aureus NCTC 5655.

When the production of lecithinase enzyme was studied, it was found that the addition of 1% glucose to the egg-yolk agar (EYA) inhibited the production of lecithinase with all previously positive strains. Staph. aureus NCTC 7428 and Staph. epidermidis NCTC 7944, on EYA, with or without 1% glucose, did not produce lecithinase. Moreover, the colony morphology of egg-yolk positive strains gave a distinct star-like shape (Figure 3.3) on EYA that contained no glucose.

All six strains were sensitive to lysostaphin.

3.1.2 Studies of Some Biochemical Tests in Chemically Defined Media

Certain of the biochemical tests on the six strains of staphylococci were studied on medium CDM/A. When the results presented in Table 3.2 are compared with those in Table 3.1 it can be seen that, in general, most of the biochemical activities of the staphylococci were reduced when CDM/A was used as the basal medium. In particular, Staph. aureus NCTC 7428 appeared to be coagulase negative and its lipase activity was lost on CDM/A. Also it was not possible to demonstrate DNase activity in any of the six strains, and acetoin production from tributyrin (CDM/A containing no mannitol) by Staph. aureus NCTC 7428 and NCTC 5655 was abolished, although acetoin was produced by Staph. aureus Wood 46 and weakly produced by Staph. aureus NCTC 8532 and NCIB 8625 in 1% mannitol-deprived CDM/A. However, adding 1% mannitol to CDM/A containing 1% tributyrin induces the acetoin production of Staph. aureus strains (see Table 3.2).

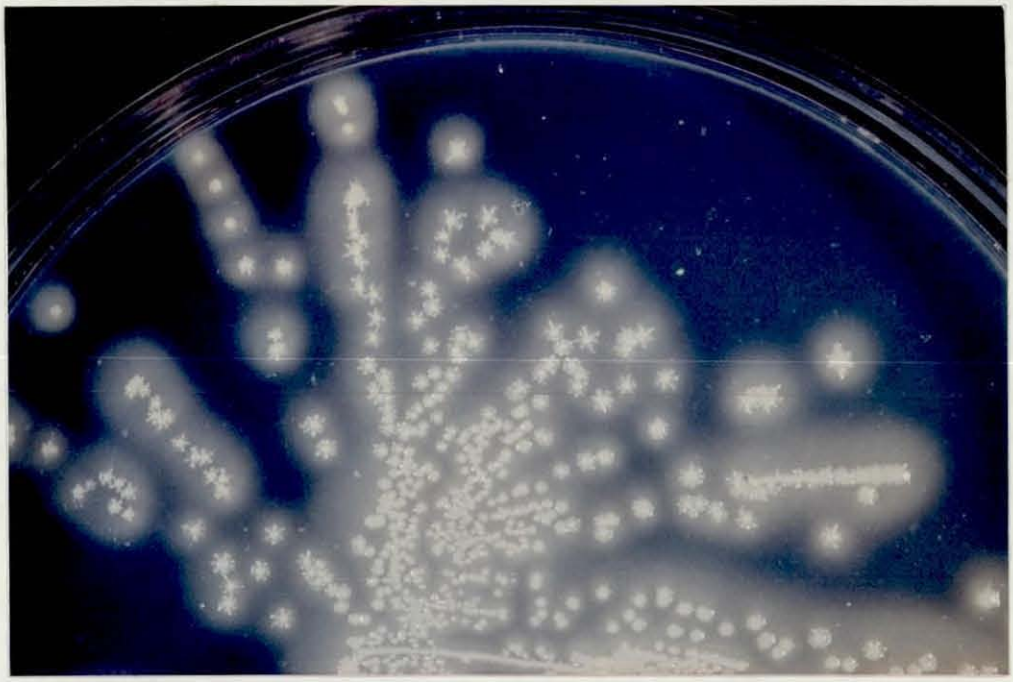


Fig. 3.3: Lecithinase production and star-like shaped colonies of Staph. aureus Wood 46 growing on egg-yolk agar (EYA) containing no glucose

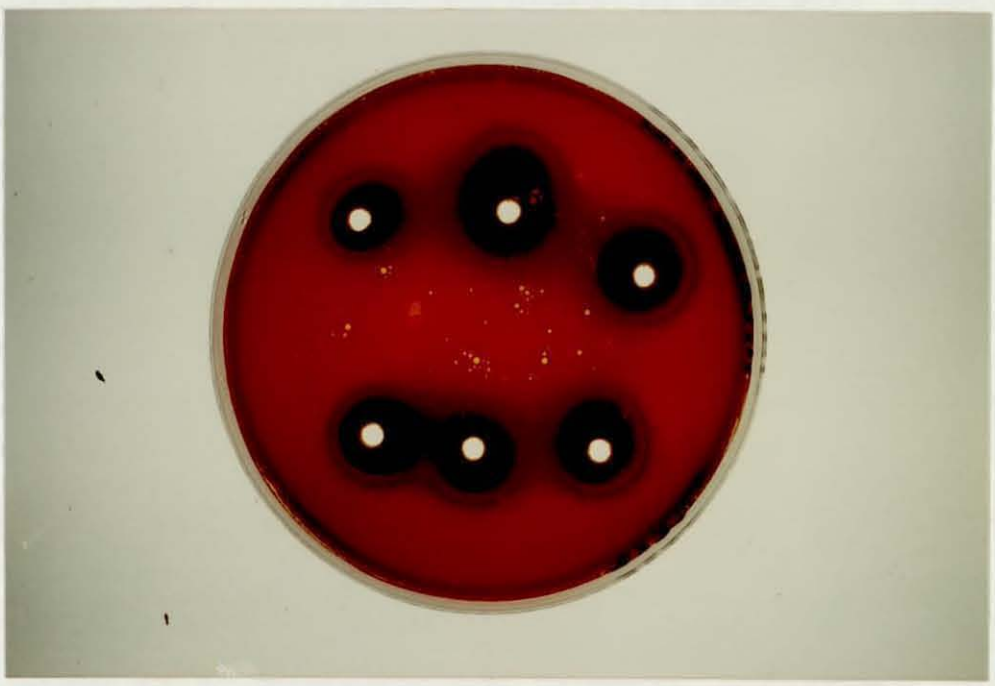


Fig. 3.4: Staph. aureus NCTC 8532 grown on CDM/A based rabbit blood agar, showing the chocolate brown colour and the halo of haemolysis accompanied by a dark, brown ring accumulated around the wells of inoculum after 36 h at 37°C

TABLE 3.2: Distinguishing some Characteristics of the Six Staphylococcal Strains in Chemically Defined Medium (CDM/A)*

Characteristic Test	*Staph. aureus* strains					*Staph. epidermidis*
	NCTC 8532	NCIB 8625	NCTC 7428	NCTC 5655	Wood 46	NCTC 7944
Catalase	P	P	P	P	P	P
Free-coagulase (tube-test)	P	P	N	P	P	N
Lipase (with tributyrin)	P	S	N	S	S	P
Phosphatase	P	P	W	S	S	N
DNase (at 37°C)	N	N	N	N	N	N
Caseolysis (Proteinases)	N	N	N	P	P	P
Acetoin production from Tributyrin:						
Method I - Absence of 1% mannitol	W	W	N	N	P	N
Method II - Presence of 1% mannitol	W	P	P	P	P	N

* For the composition of CDM/A see Table 3.6.

Reactions: N - negative
W - weak (slight positive reaction)
P - positive
S - strong-positive

3.1.3 The Haemolytic Activity of Staphylococci

The haemolytic profiles of the six staphylococci strains are given in Table 3.3. It was observed that haemolytic activity was very variable, differing from organism to organism and with the species of blood source. Activity also depended on the medium used (whether it was based on amino acid enriched mineral salts media, e.g. CDM/A or the rich, but non-defined BHI).

Preliminary experiments had shown that blood enriched BHI agar encouraged easily seen, large zones of haemolysis with both alpha- and beta-haemolysin producing strains. This medium was used in preference to diagnostic sensitivity test agar (DST-agar) which had been initially considered for inclusion as a standard medium.

Staph. aureus NCIB 8625 produced alpha-haemolysin on BHI base blood agars though only small zones of haemolysis were seen with horse blood. Typically no haemolysis was observed on agars based on CDM/A, although small zones of haemolysis were detectable with rabbit blood.

Staph. aureus NCTC 5655 alpha-haemolysin was detected in all blood (except human) agars, irrespective of basal medium; again small zones (about 1 mm from the colony) of haemolysis were observed with horse blood. Staph. aureus Wood 46 gave rise to large clear zones, typical of alpha-haemolysin with guinea-pig, rabbit and sheep blood agars (both BHI and CDM/A); with horse and human blood (BHI base) small zones of haemolysis were obtained, and no haemolysis was found on human blood CDM/A base.

Staph. aureus NCTC 7428 only with horse blood (BHI base) at 37^{o}C exhibited zones of haemolysis after 24h incubation. Small zones were, however, observed following subsequent storage at 4^{o}C with rabbit and human blood (BHI base). Similarly stored agars containing sheep blood (BHI base) showed large clear zones. However, no haemolysis was observed with this strain growing in the presence of all blood types in CDM/A based agars for 24h at 37^{o}C, although small

zones of haemolysis were found with sheep blood agar after subsequent storage at 4°C.

TABLE 3.3: Haemolytic activity of strains of Staph. aureus on solid media, chemically defined (CDM/A)** and Brain Heart Infusion (BHI) containing blood from different species. Staph. epidermidis NCTC 7944 is included.

Animal species	Medium	Strain of Staphylococcus					
		NCTC 8532	NCIB 8625	NCTC 7428	NCTC 5655	Wood 46	NCTC 7944
Guinea pig	CDM/A	N[b]	N	N	S	S	N
	BHI	N	S	N	S	S	V
Rabbit	CDM/A	W[b]	W	N*	S	S	W
	BHI	W	S	W*	S	S	V
Sheep	CDM/A	V[b]	N	W*	V	S	N
	BHI	S	S	S*	S	S	W
Horse	CDM/A	V[b]	N	N	W	S	N
	BHI	W	W	V	W	W	V
Human	CDM/A	N	N	N*	N	N	N
	BHI	N	S	W*	N	W	N

N - no detectable haemolytic zones
W - zones ca 1 mm maximum, from edge of colony
S - zones >3 mm, from edge of colony
V - positive haemolysis, zone of variable size
* - beta-haemolysin observed after incubation at 37°C for 24h, followed by 18 to 24h at 4°C.
b - chocolate brown colouration (2-3 cm) around inoculum
** - for the composition of CDM/A see Table 3.6.

No haemolysis was detected with Staph. aureus NCTC 8532 in (BHI or CDM/A) agars containing guinea pig or human bloods and only small zones with rabbit blood. Distinct, clear zones were observed around colonies growing on sheep and horse blood agars (BHI and CDM/A) after 24h incubation at 37°C with sheep blood being the most sensitive (Table 3.3).

In addition, with strain NCTC 8532 growing on blood agars based on CDM/A (with the exception of those containing human blood) extensive, diffuse, chocolate-brown areas, extending 2 to 3 cm from the colony, were observed. Except for guinea pig blood this colouration was seen in combination with haemolysis. In such cases, prolonged incubation resulted in a clear halo of haemolysis ca 1 cm from the colony, often accompanied by a dark, brown ring (Figure 3.4). The brown colour, produced after 24h incubation, could be changed back to pink by washing the agar surface with 1.0M sodium dithionite solution. The brown coloured material, when washed from the agar with PBS and examined spectrophotometrically (UV-160, Shimadzu), gave rise to a spectrum similar to that of a standard solution of methaemoglobin prepared by treating haemoglobin (freeze/thaw-lysed rabbit erythrocytes) with potassium ferricyanide, 0.0152 mol (Figure 3.5).

The brown colouration was also detected on haemoglobin and RBC's-free haemoglobin plates (CDM/A) inoculated with strain NCTC 8532. Other strains including Wood 46 did not induce any visually apparent changes in haemoglobin on either type of haemoglobin enriched plates.

A similar colour shift could be induced by incubating uninoculated blood agar plates on which a drop of either acidic (pH < 4) or alkaline (pH > 9) buffer had been placed. In such cases the brown colour was restricted to 2 ∿ 3 mm from the drop.

Staph. epidermidis gave clear, sharp zones of haemolysis (24h) on BHI based blood agars, except when using human blood. Zone size was, however, variable. Guinea pig, rabbit and horse bloods showed similar sensitivities and sheep blood was generally less sensitive. In the presence of 1% mannitol in CDM/A based blood agars, small zones of haemolysis were only observed when using rabbit blood; no haemolysis was detected with blood of other species in this medium.

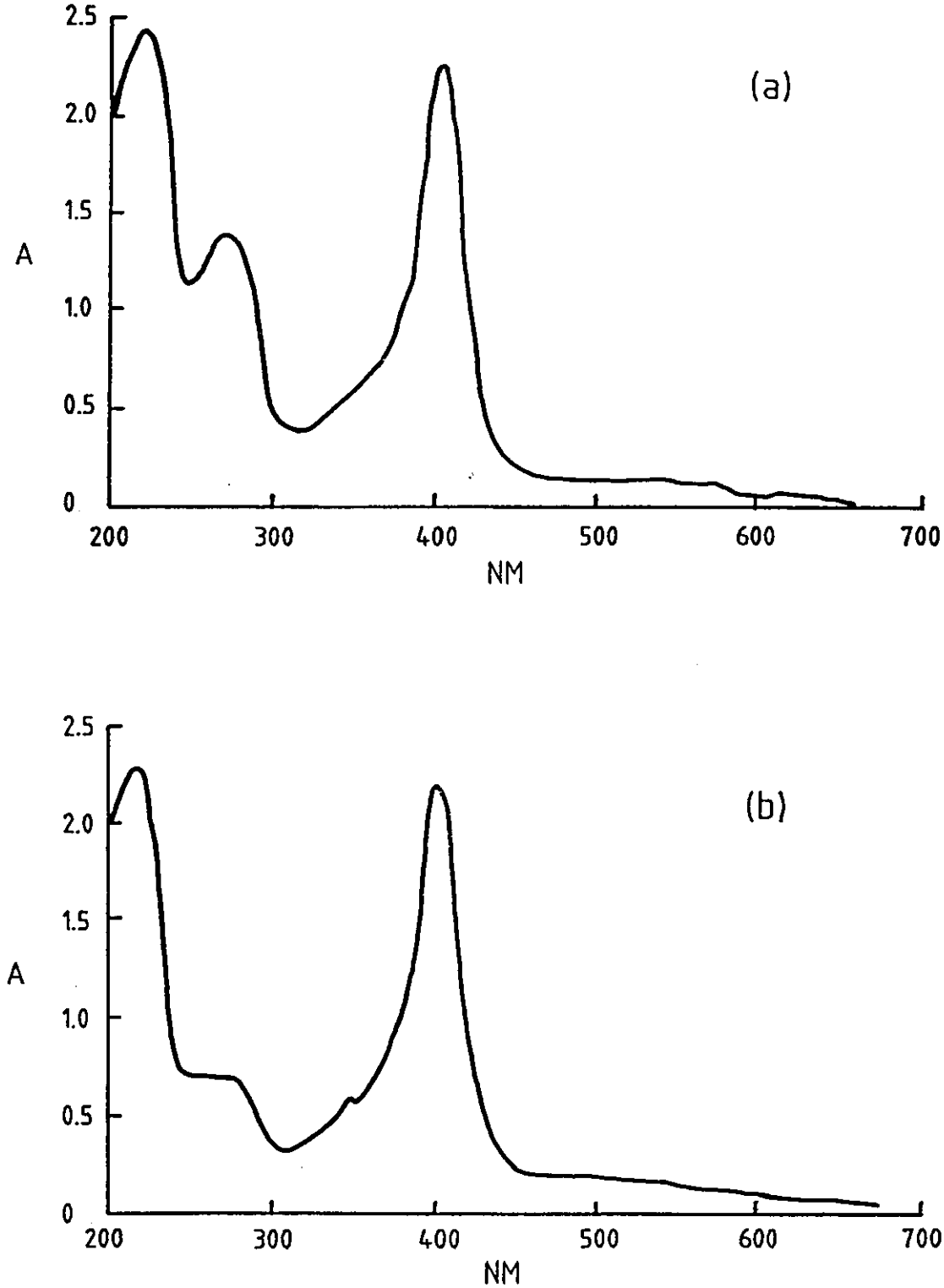


Fig. 3. 5. ..... UV / visible spectra of :
(a) the chocolate brown colour produced by *Staph-aureus* NCTC8532 on CDM / A based agar with rabbit blood
(b) a standard solution of methaemoglobin prepared by treating rabbit - haemoglobin with potassium ferricyanide (0.0152mol).

Preliminary experiments had, however, shown that the presence of 1% mannitol in CDM/A enhanced the production/detection of the haemolysins in comparison with 1% glucose with all staphylococci, including Staph. epidermidis, on blood agars.

3.1.4 The Susceptibility of Staphylococci to Antibiotic Methicillin

The resistance of the staphylococcal strains to different concentrations of methicillin was studied in BHI-agar by the agar diffusion method. The results are presented in Figure 3.6 and show that, in terms of zones of growth inhibition Staph. epidermidis NCTC 7944 and Staph. aureus NCIB 8625 were highly affected by all levels of methicillin solutions. Staph. aureus strains NCTC 5655, NCTC 8532 and NCTC 7428 showed similar susceptibility to 100 µg/ml methicillin solution. Staph. aureus Wood 46 had the highest resistance to the antibiotic methicillin.

Calculated MIC values varied from 7.62 µg/ml for Staph. epidermidis NCTC 7944 (most sensitive strain) to 38.30 µg/ml for Staph. aureus Wood 46 (most resistant strain) and others between these values.

Furthermore, preliminary studies were carried out to investigate the influence of CDM/A on the susceptibility of staphylococcal strains to different concentrations of methicillin, however, it was observed that all strains grown in CDM/A agar were more susceptible to killing by methicillin than that grown in BHI agar.

3.1.5 The Influence of Lipids on the Lipase Activity and Acetoin Production of Different Microorganisms

Different kinds of lipids (see Table 3.4) were used to study the lipase activity and additionally the concurrent production of acetoin of Staph. aureus strains NCIB 8625 and NCTC 7428, C. renale NCTC 7448 and Ps. aeruginosa NCTC 7244 were assessed.

Lipase activity

The lipase activity of Staph. aureus strains NCIB 8625 and NCTC 7428 were clearly demonstrated on the plates containing tributyrin and

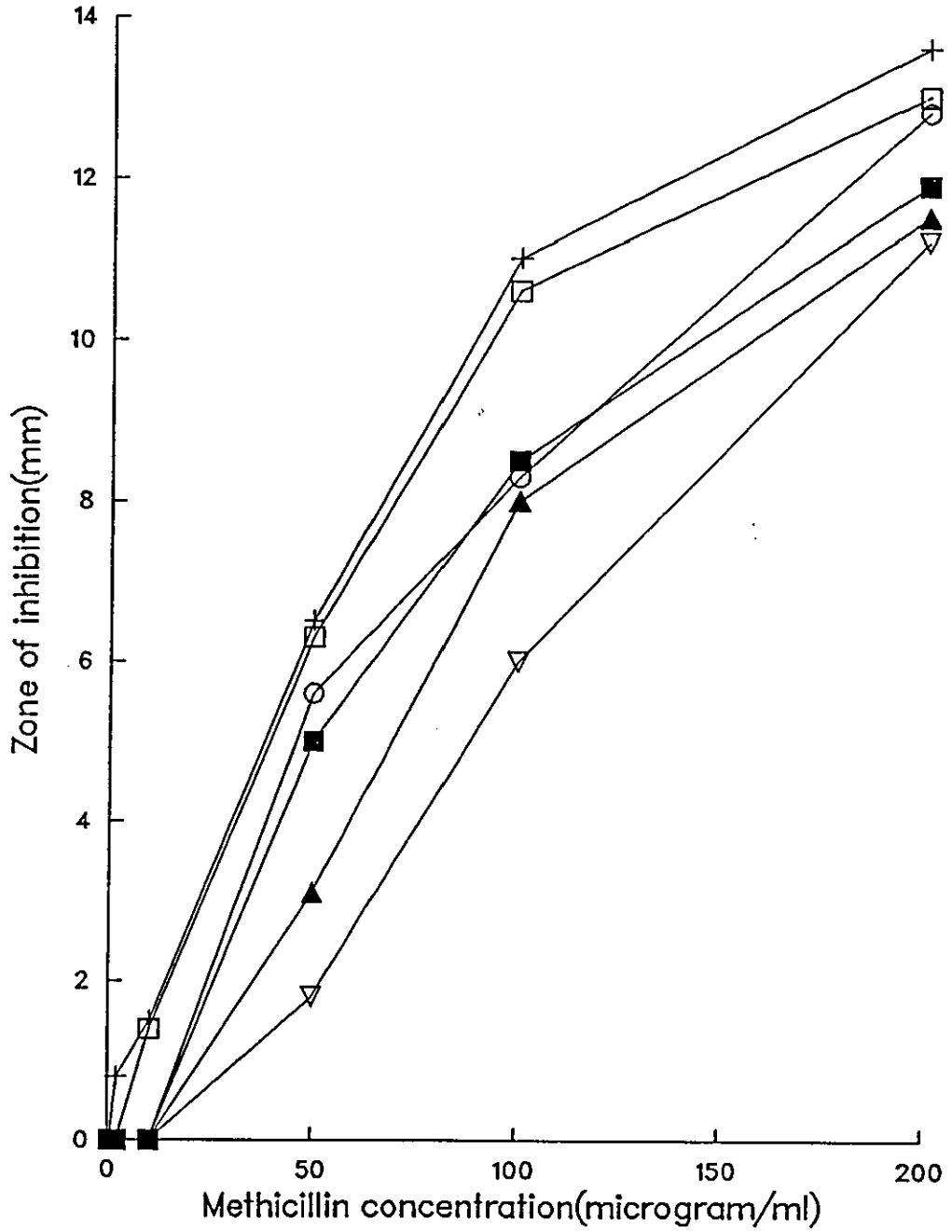


Fig. 3.6: Zones of inhibition (mm) interpreting the susceptibility of Staph. aureus strains:
▽ , Wood 46; ▲ , NCTC 7428; ■ , NCTC 8532; ○ , NCTC 5655; □ , NCIB 8625 and +, Staph. epidermidis NCTC 7944; to methicillin sodium at different concentrations (2, 10, 50, 100 and 200 µg/ml) in BHI agar

fish oil (see also Appendix 1), though weak lipase activities occurred with C. renale NCTC 7448 and Ps. aeruginosa NCTC 7244 (Table 3.4). Tricaproin and tricaprylin gives weak lipase activity with Staph. aureus NCIB 8625, whilst with others negative results were obtained.

The presence of triacetin in the media abolished the growth and lipase activity of all bacteria, except for Staph. aureus NCIB 8625 with which only weak growth was observed. Similarly, the lipid-triolein abolished the growth of Staph. aureus NCTC 7428, though it reduced the growth of Staph. aureus NCIB 8625 and no lipase was obtained.

In general, the lipase activity of different bacteria was not demonstrated with the other lipids (Table 3.4).

The effect of glucose on the lipase activity was studied in preliminary experiments and it was found that the addition of 1% glucose to tributyrin agar abolished the lipase activity with C. renale NCTC 7448 and it reduced it in the case of Staph. aureus strains and Ps. aeruginosa NCTC 7244.

Acetoin production

All Staph. aureus strains were able to produce acetoin in the presence of the lipid tributyrin (Tables 3.1, 3.4), though weak acetoin was produced by Staph. aureus NCIB 8625 in the presence of tricaproin and tricaprylin (Table 3.4).

In general, acetoin was not formed from any lipids including tributyrin with C. renale NCTC 7448 or Ps. aeruginosa NCTC 7244 (Table 3.4). However, acetoin was produced in the presence of glycerol with Staph. aureus strains NCIB 8625 and NCTC 7428, and C. renale NCTC 7448 (Table 3.4). A preliminary experiment had also shown that C. renale NCTC 7448 produced acetoin in a medium containing glucose (VP-test) whilst Ps. aeruginosa NCTC 7244 did not.

TABLE 3.4: Detection of lipase activity and acetoin production on a solid media containing different kinds of lipids Triglyceride

Micro-organisms	Kind of Tests	Kind of Lipids Triglyceride									Natural Lipids		Carbon source
		Triacetin C2:0	Tributyrin C4:0	Tricaproin C6:0	Tricaprylin C8:0	Tricaprin C10:0	Trilaurin C12:0	Trimyristin C14:0	Tristearin C18:0	Triolein C18:1	Arachis Oil	Fish Oil	Glycerol
Staph. aureus	Lipase	N†	P	W	W	N	N	N	N	N†	N	P	N
NCIB 8625	Acetoin	N	P	W	W	N	N	N	N	N	N	N	P
Staph. aureus	Lipase	‡	P	N	N	N	N	N	N	‡	N	P	N
NCTC 7428	Acetoin	N	P	N	N	N	N	N	N	N	N	N	P
C. renale	Lipase	‡	W	N	N	N	N	N	N	N	N	W	N
NCTC 7448	Acetoin	N	N	N	N	N	N	N	N	N	N	N	P
Ps. aeruginosa	Lipase	‡	W	N	N	N	N	N	N	N	N	W	N
NCTC 7244	Acetoin	N	N	N	N	N	N	N	N	N	N	N	N

Reactions: N - negative
W - weak
P - positive

† Gives a weak growth

‡ No growth occurred

3.2 GROWTH AND HAEMOLYSIN PRODUCTION OF THE STAPHYLOCOCCI IN CHEMICALLY DEFINED MEDIA

3.2.1 Determination of the Required Amino Acids for the Growth of the Six Staphylococci Strains

All the staphylococci studied grew well in BHI (Figure 3.7), and adequately in the basal medium supplemented with 18 amino acids (CDM, Figure 3.8). Using the single deletion procedure, to obtain adequate growth in a minimum number of amino acids, cysteine was found to be essential for all six staphylococcal strains and with all Staph. aureus strains valine was also essential (Table 3.5). Arginine was essential only for the three strains of Staph. aureus (i.e. NCTC 8532, NCTC 7428, NCTC 5655) and also for Staph. epidermidis NCTC 7944. All the strains studied had proline as either essential or required, except for Staph. aureus NCTC 8532 for which it was supplemental (Table 3.5). Only one strain of Staph. aureus, NCTC 7428, had histidine as an essential amino acid. Only Staph. epidermidis NCTC 7944 had a requirement for lysine. On the other hand, the amino acids, leucine, methionine, phenylalanine and threonine were regarded as required or supplemental for the growth of all six staphylococci strains except Staph. aureus NCTC 8532 for which the amino acid leucine was not required.

There was no absolute requirement for the amino acid alanine, or for aspartic acid with all the six strains, though aspartic acid was found to be supplementary for the adequate growth of Staph. aureus NCTC 8532. Other general requirements etc for the various amino acids are as shown in Table 3.5.

3.2.2 Media Development for Growth and Haemolysin Production of Staphylococci

Using the results from Table 3.5, the second stage of adding amino acids (essential + required + supplemental) was carried out and media were developed to give adequate growth for each strain of staphylococci (Table 3.6).

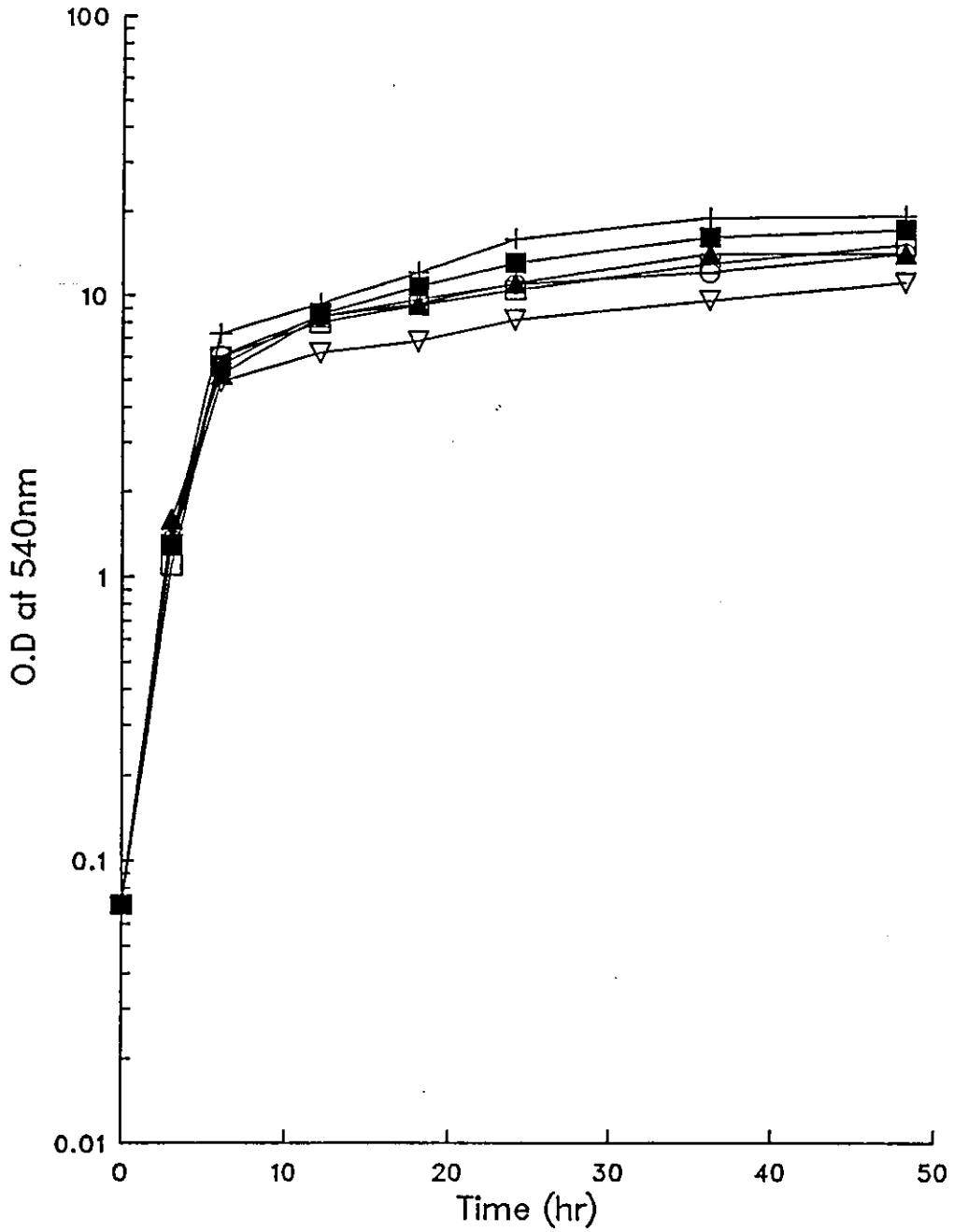


Fig. 3.7: Growth curves (O.D. at 540 nm), in shaken culture (90 rev/min) at 37°C for staphylococci (six strains) in BHI. Staph. aureus strains: ▽ , Wood 46; ○ , NCTC 5655; □ , NCIB 8625; ▲ , NCTC 7428; ■ , NCTC 8532; and Staph. epidermidis NCTC 7944, +.

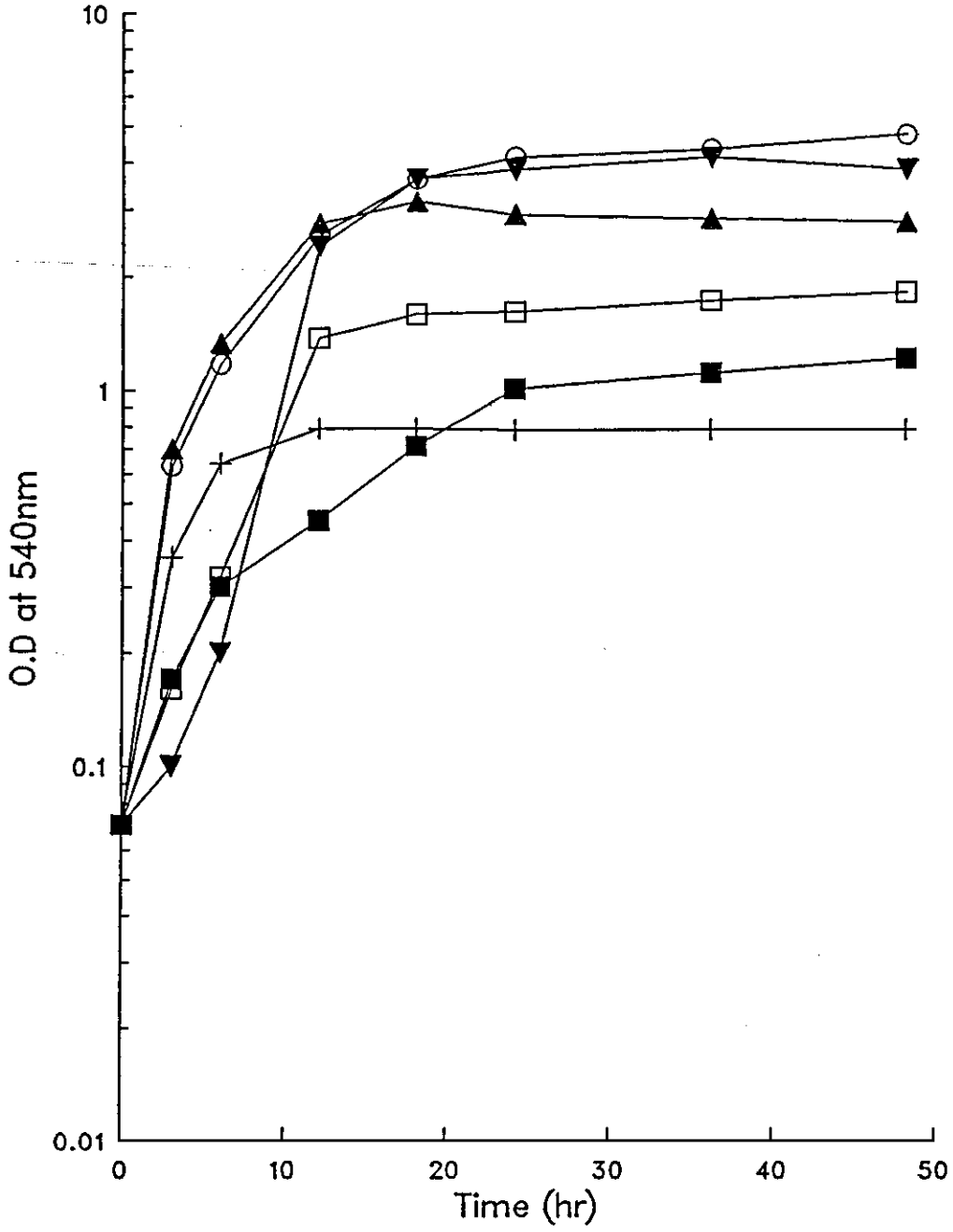


Fig. 3.8: Growth curves (O.D.at 540 nm), in shaken culture (90 rev/min) at 37°C for staphylococci (six strains) in medium CDM. Staph. aureus strains, ▼, Wood 46; ○, NCTC 5655; □, NCIB 8625; ▲, NCTC 7428; ■, NCTC 8532; and Staph. epidermidis NCTC 7944, +.

TABLE 3.5: Amino acid requirements of the six staphylococcal strains grown in chemically defined media, as determined by the single deletion method

Amino Acid	Strain of Staphylococcus:					
	NCTC 8532	NCIB 8625	NCTC 7428	NCTC 5655	Wood 46	NCTC 7944
Ala	N	N	N	N	N	N
Arg	E	S	E	E	S	E
Asp	S	N	N	N	N	N
Cys	E	E	E	E	E	E
Glu	S	N	N	N	S	R
Gly	S	N	N	N	S	S
His	N	S	E	S	R	S
Ile	R	R	N	N	N	N
Leu	N	S	R	S	R	S
Lys	S	N	S	N	N	R
Met	S	S	R	S	S	S
Phe	R	S	R	S	S	S
Pro	S	E	E	R	E	R
Ser	N	S	S	N	N	N
Thr	R	R	S	R	S	S
Trp	N	N	S	S	S	N
Tyr	N	S	S	N	N	N
Val	E	E	E	E	E	S

E - the amino acid is essential for the organism

R - the organism has a requirement for the particular amino acid

S - the amino acid is supplemental

N - there is no absolute requirement for the amino acid.

TABLE 3.6: Amino acid composition of various chemically defined media used to cultivate staphylococci

Staph. aureus NCTC 7428		Staph. aureus NCTC 8532	Staph. aureus NCIB 8625		Staph. aureus NCTC 5655	Staph. aureus Wood 46						Staph. epidermidis NCTC 7944	
CDM/A	CDM/B	CDM/1	CDM/2	CDM/3	CDM/4	CDM/5	CDM/6	CDM/7	CDM/8	CDM/9 (mg/100ml)	Dalen*-CDM (mg/100ml)	CDM/10	CDM/11
Cys	Cys	Cys	Cys	Cys	Cys	Cys	Cys	Cys	Cys	Cys(2)	Cys(1.2)	Cys	Cys
Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro	Pro(3)	Pro(3.4)	Pro	Pro
Val	Val	Val	Val	Val	Val	Val	Val	Val	Val	Val(6)	Val(5.8)		Val
Arg	Arg	Arg		Arg	Arg	Arg			Arg	Arg(2)	Arg(1.7)	Arg	Arg
Thr		Thr	Thr	Thr	Thr	Thr			Thr	Thr(3.5)			Thr
Phe		Phe		Phe		Phe			Phe	Phe(5)	Phe(4.9)		Phe
Met		Met		Met		Met			Met	Met(3.5)			Met
Leu				Leu		Leu	Leu	Leu	Leu	Leu(7)	Leu(6.5)		Leu
His	His			His		His		His	His	His(16)	His(15.5)		His
Trp						Trp			Trp	Trp(3.5)			
Lys		Lys									Lys(9.1)	Lys	Lys
Ser				Ser									
Tyr				Tyr									
		Gly							Gly	Gly(4)	Gly(3.7)		Gly
		Glu							Glu	Glu(7)	Glu(7.3)	Glu	Glu
		Ile	Ile	Ile									
		Asp									Asp(13.3)		
											Ala(4.4)		

* Dalen (1973a)

Two strains of Staph. aureus (NCTC 7428 and NCTC 8532) were found to be particularly fastidious. A medium (CDM/A) was developed by the addition of amino acids supplemental for strain NCTC 7428. All six strains grew in medium CDM/A and it was adopted as a common defined medium for all the staphyococci. This medium (CDM/A) was used to make comparisons of growth, haemolysin production and some biochemical tests (see Table 3.2) for all organisms. It contained the basal medium (Table 2.1) with thirteen amino acids (Table 3.6). An additional range of amino acid supplemented media (Table 3.6) giving adequate growth of the other staphylococcal strains, namely Staph. aureus strains NCIB 8625, NCTC 8532, NCTC 5655, Wood 46 and Staph. epidermidis NCTC 7944 were also prepared. These were used in studies of staphylococcal growth, or haemolysin production in the case of coagulase positive Staph. aureus strains. These media comprised: a four amino acid medium CDM/6 for Staph. aureus Wood 46; five amino acid media CDM/2 for Staph. aureus NCIB 8625, CDM/4 for Staph. aureus NCTC 5655; CDM/7 for Staph. aureus Wood 46, and CDM/10 for Staph. epidermidis NCTC 7944; ten amino acid medium CDM/5 for Staph. aureus NCTC 5655; and twelve amino acid media CDM/1 for Staph. aureus NCTC 8532, CDM/3 for Staph. aureus NCIB 8625, CDM/8 for Staph. aureus Wood 46, and CDM/11 for Staph. epidermidis NCTC 7944.

Two further media were also used for Staph. aureus Wood 46, namely that of Dalen (1973a) comprising of twelve amino acids (Dalen-CDM; see Table 3.6), and a variation on CDM/8 with altered amino acid levels CDM/9. Growth assessment with Staph. aureus Wood 46 was particularly difficult in such a minimal medium as cell clumping and adhesion to glassware was considerable. Indications were, however, that although unevenly distributed, adequate cellular growth was taking place. There was no single, low (five or six) amino acid supplemented medium capable of supporting good growth of all the staphylococci investigated.

Study of the growth of Staph. aureus NCIB 8625 in CDM/3 (see Table 3.6) indicate that there was no significant difference in growth if cystine was replaced with cysteine (Figure 3.9a).

The effect of different carbohydrates such as glucose and mannitol on the growth of both coagulase positive and negative staphylococci were also studied. Experiments carried out with Staph. aureus NCIB 8625 and Staph. epidermidis NCTC 7944 grown in CDM/3 and CDM/11 (see Table 3.6) respectively, supplemented with 1% of glucose or with mannitol show that there was no significant difference in the growth of Staph. aureus NCIB 8625 if glucose was replaced with mannitol as the principal source of carbon (Figure 3.9a). With Staph. epidermidis NCTC 7944, however, a slightly better growth was obtained in the CDM/11 supplemented with glucose (Figure 3.9b).

On the other hand, the influence of glucose or mannitol on alpha-lysin production of Staph. aureus Wood 46 in liquid cultures of CDM/7 (containing five amino acids) and CDM/8 (containing twelve amino acids) (see Table 3.6) were also studied in preliminary experiments. It was found that the presence of 1% mannitol in the basal CDM/7 and CDM/8 enhance the appearance of a good yield of alpha-haemolysin formation than 1% glucose. Similar results also found that 1% mannitol enhanced the production/detection of beta-lysin of Staph. aureus NCTC 7428 and the delta-type haemolysin of Staph. epidermidis NCTC 7944 than 1% glucose in CDM/A-agar (see 3.1.3). Therefore, the works in chemically defined media were carried out by using cysteine instead of cystine and mannitol instead of glucose with all six strains of staphylococci, except for Staph. epidermidis NCTC 7944 for which glucose was used in its basal media, except that using mannitol only in the case of detection for its haemolytic activity on blood agar, and unless otherwise stated.

3.2.3 Growth and Haemolysin Production of Staphylococci

The type of blood used to estimate haemolysin activity gave measurably different sensitivities. For example, there was a 32-fold difference in the sensitivity between sheep and rabbit blood to

Fig. 3.9(a): Growth curves (O.D. at 540 nm) in shaken cultures (90 rev/min) at 37°C, for Staph. aureus NCIB 8625 grown in CDM/3; O , supplemented with 3.5 mg/100 ml cysteine and 1% mannitol; x, supplemented with 3.5 mg/100 ml cystine and 1% mannitol; and ∇ , supplemented with 3.5 mg//100 ml cysteine and 1% glucose.

Fig. 3.9(b): Growth curves (O.D. at 540 nm), in shaken cultures (90 rev/min) at 37°C for Staph. epidermidis NCTC 7944 grown in CDM/11: O , supplemented with 1% mannitol; and ●, supplemented with 1% glucose.

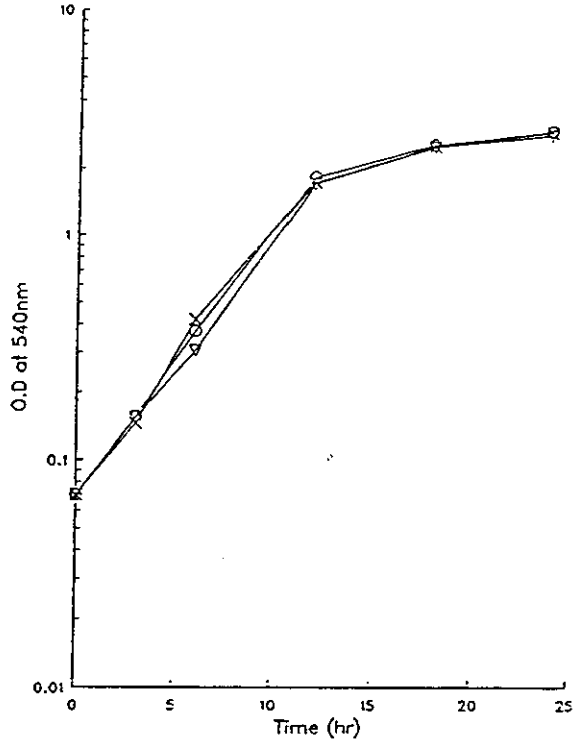


FIGURE 3.9(a)

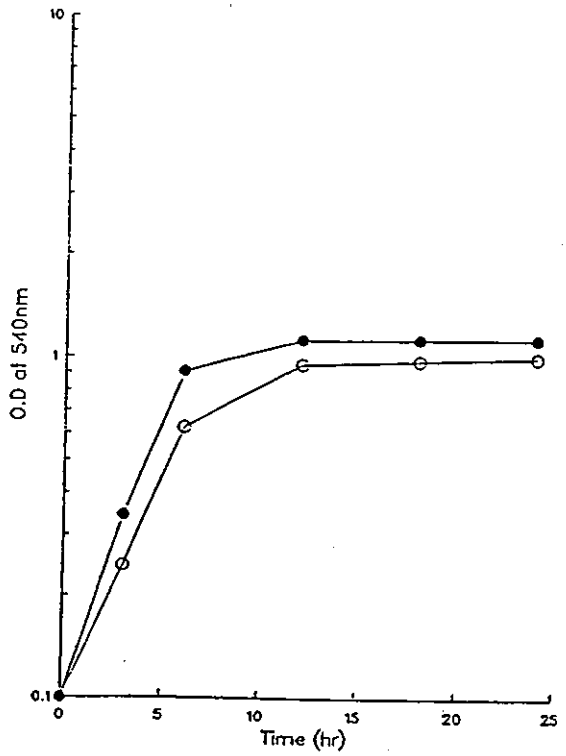


FIGURE 3.9(b)

alpha-haemolysin produced by Staph. aureus Wood 46 (grown in BHI) with rabbit blood being the most sensitive (Figure 3.10). Alpha-haemolysin was assumed on this species sensitivity and on the appearance of the haemolysis zone (clear with slight haze distant from colony). Similarly, beta-haemolysin was assumed with hot-cold haemolysis with sheep blood being the most sensitive. Delta-haemolysin was detected via sharp zones with little or no species variation in sensitivity (see Table 3.3).

Each strain of staphylococci was studied for growth and haemolysin production in chemically defined media and BHI as follows:

i) Staph. aureus Wood 46

The growth of Staph. aureus Wood 46 in BHI (Figure 3.7) was rapid and alpha-lysin was formed in BHI early, being detectable after 6h, with the highest titre, 2560 HU50/ml after 24h incubation (Figure 3.10).

The study of Wood 46 in chemically defined media, showed that 4 amino acid medium CDM/6 (containing three essential and one required amino acid; see Tables 3.5, 3.6) gives small but detectable growth (as represented by a small decline in pH values, Figure 3.11). No alpha-haemolysin was produced during the 72h of incubation in this medium. However, adding the second required amino acid (histidine) at 3.5 mg/100 ml to CDM/6 medium, as represented as CDM/7 (see Table 3.6), increased the cellular growth rate Figure 3.11 (as was indicated by the large fall in the pH values) and Figure 3.12. Again no detectable levels of alpha-haemolysin were found in CDM/7 after 24h incubation, but with the increasing growth rate, low levels, 10 HU50/ml, were seen after 36h (Figure 3.13). A four fold increase in the level of histidine (14 mg/100 ml) in CDM/7 did not affect the growth rate or the level of alpha-toxin produced (Figures 3.11, 3.12 and 3.13). Increasing the number of amino acids to twelve (CDM/8) resulted in a good yield of cells (Figures 3.11 and 3.12), and an increased titre (64 HU50/ml after 24h incubation) of alpha-haemolysin (Figure 3.13). If the levels of alpha-toxin measured at 18h are considered, then the highest titre of 128 HU50/ml was

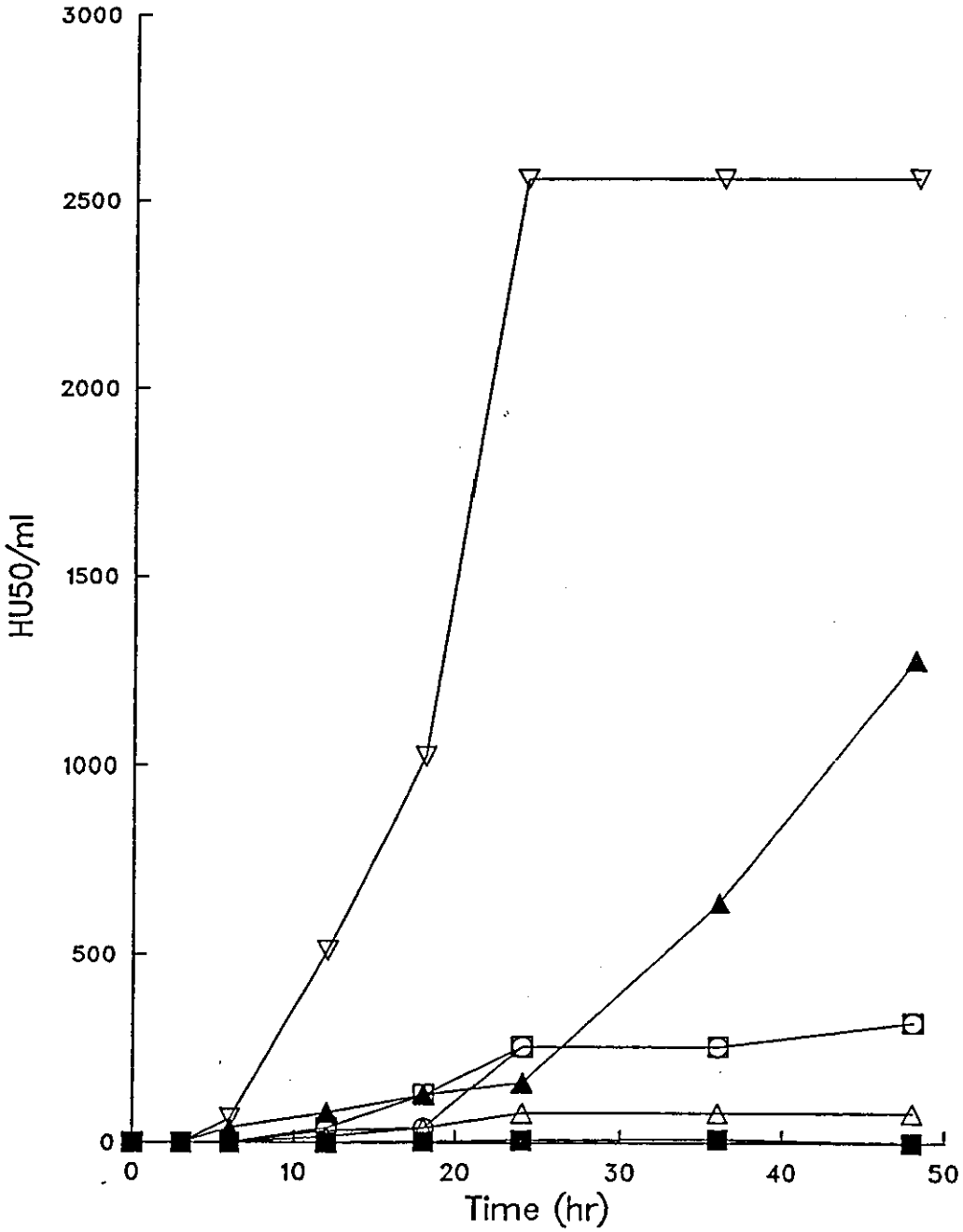


Fig. 3.10: Haemolytic titre (HU50/ml) of the supernatant liquid from cultures of staphylococci grown in shaken culture (90 rev/min) at 37°C in BHI. Titre estimated after 1h contact in PBS at 37°C using rabbit erythrocytes; Staph. aureus strains; ○ , NCTC 5655; □, NCIB 8625; ▽, Wood 46 and for ■ , NCTC 8532 and △ , Wood 46 using sheep erythrocytes. Beta-haemolysin titre estimated using sheep erythrocytes suspended in PBS, supplemented with 0.002 mol/L magnesium sulphate, after 1h at 37°C and then 1h at 4°C; ▲ , NCTC 7428 .

Fig. 3.11: The pH-values curves (as indicators of growth), in shaken cultures (90 rev/min) at 37 °C, for Staph. aureus Wood 46 grown in various amino acid containing chemically defined media and with media supplemented with 0.1% (w/v) YE: x, CDM/6; △ , CDM/7; ▼ , CDM/7 supplemented with 14 mg/100 ml histidine; ■, CDM/8; □ , CDM/8 plus YE; ● , CDM/9; ○ , CDM/9 plus YE; ▽ , CDM/A; and ▲ , Dalen-CDM .

Fig. 3.12: Growth curves (O.D. at 540 nm), in shaken culture (90 rev/min) at 37°C, for Staph. aureus Wood 46 grown in various amino acid containing chemically defined media and with media supplemented with 0.1% (w/v) YE: △ , CDM/7; ▼, CDM/7 supplemented with 14 mg/100 ml histidine; ■ , CDM/8; □, CDM/8 plus YE; ● , CDM/9; ○ , CDM/9 plus YE; ▽ , CDM/A; ▲ , Dalen-CDM. YEBS, + .

Fig. 3.13: Alpha-haemolysin titre (HU50/ml) of supernatant liquids from cultures of Staph. aureus Wood 46 grown at 37 °C in shaken culture (90 rev/min) in various amino acid containing chemically defined media and with media supplemented with 0.1% (w/v) YE. Titre estimated after 1h contact in PBS at 37 °C using rabbit erythrocytes: △, CDM/7; x, CDM/7 supplemented with 14 mg/100 ml histidine; ■, CDM/8; □, CDM/8 plus YE; ● , CDM/9; ○ , CDM/9 plus YE; ▽ , CDM/A; ▲ , Dalen-CDM; ▼ , CDM. YEBS, +.

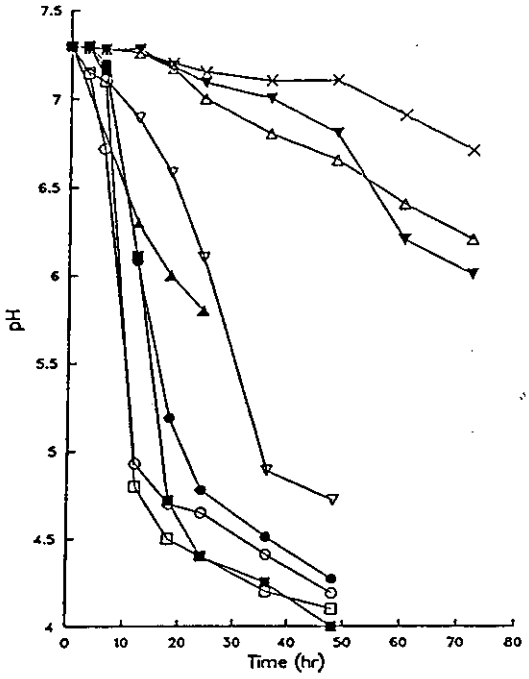


FIGURE 3.11

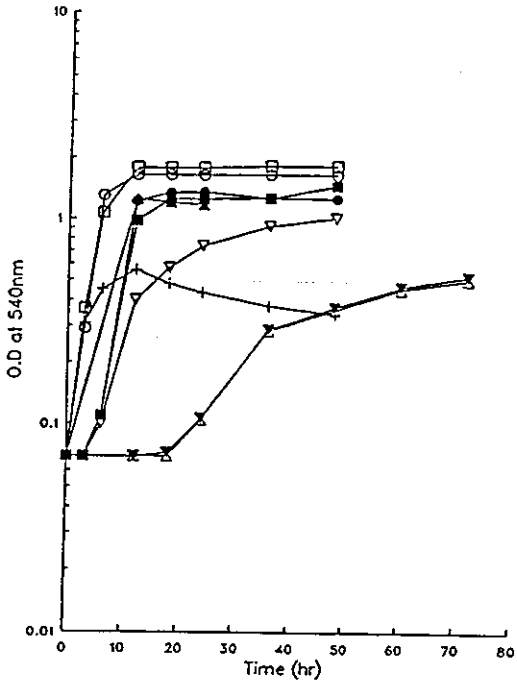


FIGURE 3.12

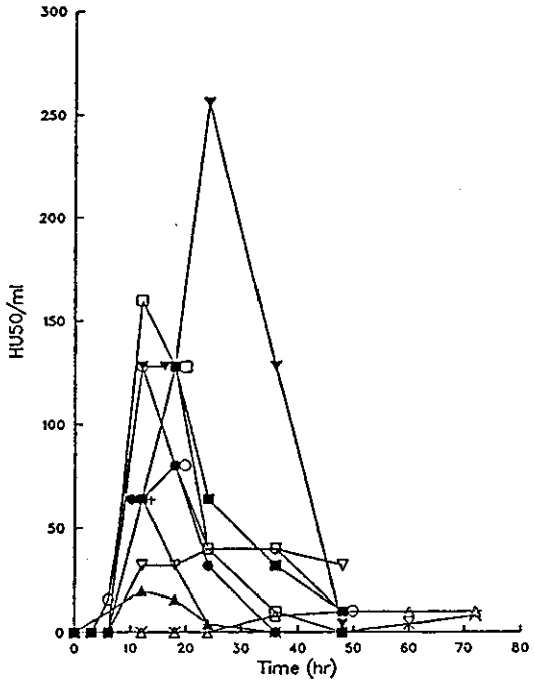


FIGURE 3.13

obtained in twelve amino acid medium, CDM/8 (see Table 3.6), where all the amino acids were presented at 3.5 mg/100 ml. When the levels of the twelve amino acids present in CDM/8 was altered (CDM/9; Table 3.6) the growth rate was similar to CDM/8 (Figures 3.11 and 3.12), but the alpha-lysin titre was reduced to 80 HU50/ml (Figure 3.13). In the case of the chemically defined medium of Dalen (1973a), Dalen-CDM (see Table 3.6), the growth rate of Staph. aureus Wood 46 during the 24h incubation was similar to CDM/8 and CDM/9 (Figures 3.11 and 3.12). The alpha-lysin titre was, however, markedly reduced, only 16 HU50/ml at 18h of incubation (Figure 3.13). In the thirteen amino acid medium CDM/A, both the growth rate and alpha-lysin titre of Staph. aureus Wood 46 were reduced (Figures 3.11, 3.12 and 3.13). The alpha-lysin titre was 32 HU50/ml at 18h. Medium CDM (containing all amino acid; see Table 2.1) gave good rpaid growth of Staph. aureus Wood 46 (Figure 3.8) with less adhesion on the surface of the glass culture vessels, or clumping of cells. The production of alpha-lysin was increased and gave the highest titre of 256 HU50/ml at 24h (Figure 3.13).

In the medium containing 0.1% yeast extract in PBS (YEBS), the growth of Staph. aureus Wood 46 was initially rapid with no lag phase of growth, but in general it obtained low overall growth rate (Figure 3.12). After 12h incubation a low titre of 64 HU50/ml of alpha-lysin (Figure 3.13) was observed.

The influence of yeast extract (YE) on the growth and alpha-haemolysin production in chemically defined media was studied for Staph. aureus Wood 46, and found that the addition of 0.1% YE to the media CDM/8 and CDM/9 gave similar good growth patterns of Staph. aureus Wood 46 (Figures 3.11 and 3.12). It abolished the lag-phase of the growth, and it diminished both the "clotes" of cells and the adhesion of bacteria to the surface of the glass culture vessels. On the other hand, the production of alpha-lysin was influenced after 12h incubation giving elevated titres of 160 and 128 HU50/ml respectively. Thereafter, the level fell to almost the same order as the unsupplemented media (Figure 3.13).

ii) Staph. aureus NCTC 5655

There was good rapid growth observed of Staph. aureus NCTC 5655 in BHI (Figure 3.7). Alpha-haemolysin was produced and gave titres of 256 HU50/ml after 24h incubation (Figure 3.10).

The growth and alpha-lysin production of strain NCTC 5655 in chemically defined media showed that the five amino acid medium, CDM/4 (containing three essential and two required amino acids; see Tables 3.5 and 3.6) gave an adequate growth profile, at least a 7-fold overall increase in culture optical density within 12h incubation, and by 36h, growth was approximately equivalent to that of the CDM/5 and CDM/A (Figure 3.14). Alpha-lysin was not produced in CDM/4 during the 48h incubation. However, adding the supplementary amino acids phenylalanine, methionine, histidine, leucine, and tryptophan at 3.5 mg/100 ml (see Tables 3.5 and 3.6) to CDM/4, as represented as CDM/5 (ten amino acid medium) increased the growth rate markedly during the first 18h (Figure 3.14). Overall, alpha-haemolysin was produced with a maximum titre of 10 HU50/ml after 18h (Figure 3.15).

The addition of three non-supplemental amino acids for this strain (lysine, serine, and tyrosin at 3.5 mg/100 ml) (see Table 3.5) to CDM/5, as presented in the thirteen amino acid medium, CDM/A (see Table 3.6), did not affect the overall growth rate (Figure 3.14), but the level of alpha-toxin was reduced to a titre of 4 HU50/ml detected at 18h (Figure 3.15).

In the case of 0.1% YE in PBS (YEBS) the growth of Staph. aureus NCTC 5655 was similar to CDM/4 during the first 6h of incubation, after which a poor growth rate was obtained (Figure 3.14). After 12h incubation, alpha-lysin was produced at a low titre of 4 HU50/ml (Figure 3.15).

Fig. 3.14: Growth curves (O.D. at 540 nm), in shaken cultures (90 rev/min) at 37°C, for Staph. aureus NCTC 5655 grown in various amino acid containing chemically defined media and with media supplemented with 0.1% (w/v) YE: Δ , CDM/4; O, CDM/5; ●, CDM/5 plus YE; +, CDM/A. YEBS, □.

Fig. 3.15: Alpha-haemolysin titre (HU50/ml) of supernatant liquids from cultures of Staph. aureus NCTC 5655 grown at 37°C in shaken culture (90 rev/min) in various amino acid containing chemically defined media and with media supplemented with 0.1% (w/v) YE. Titre estimated after 1h contact in PBS at 37°C using rabbit erythrocytes: O, CDM/5; ●, CDM/5 plus YE; +, CDM/A. YEBS, □.

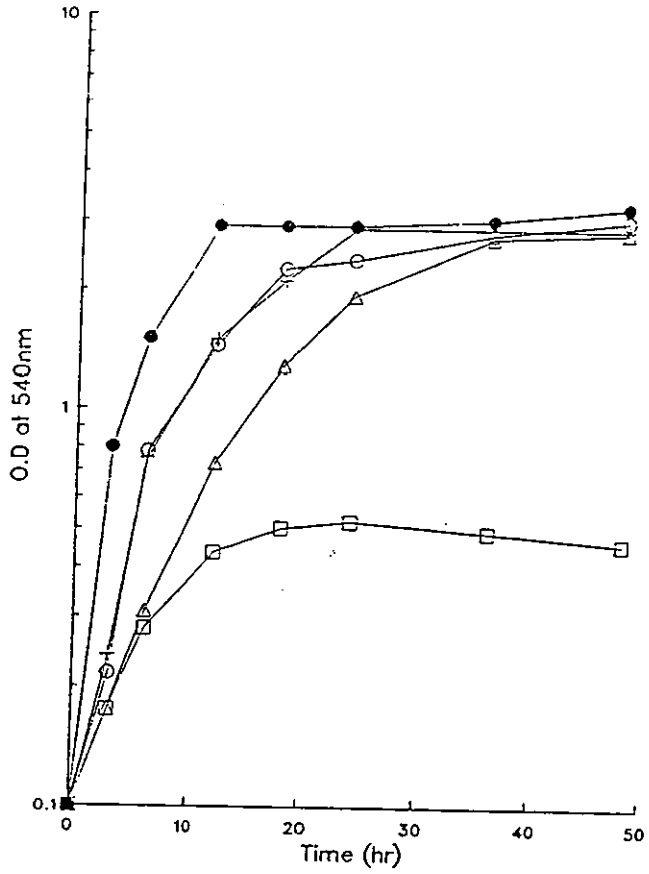


FIGURE 3.14

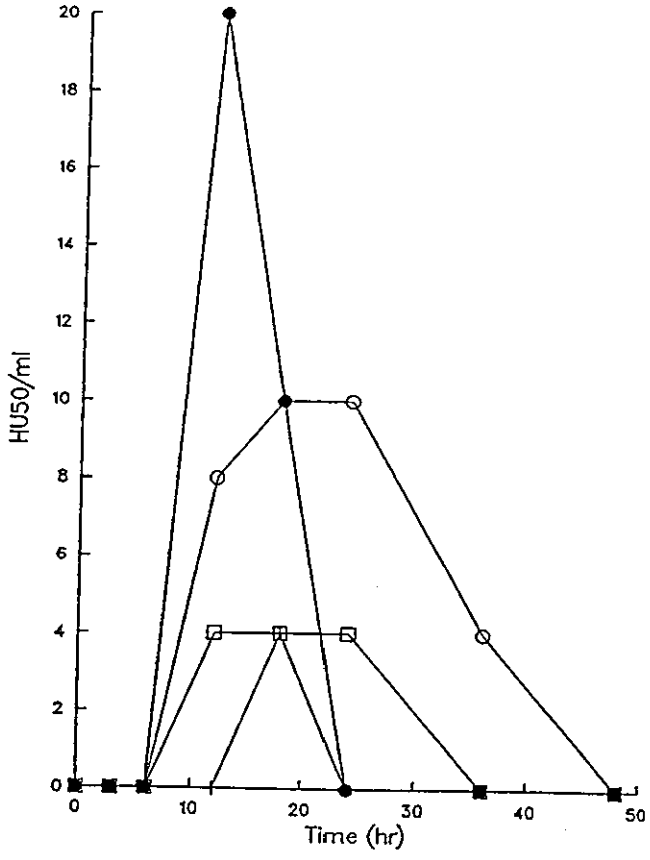


FIGURE 3.15

The addition of 0.1% YE to CDM/5 resulted in luxuriant growth of Staph. aureus NCTC 5655 during the first 12h (Figure 3.14), with a slightly higher alpha-haemolysin titre of 20 HU50/ml after 12h incubation (Figure 3.15).

iii) Staph. aureus NCIB 8625

BHI promoted rapid, good growth of Staph. aureus NCIB 8625 (Figure 3.7), with yields of alpha-lysin reaching 256 HU50/ml after 24h, equivalent to that produced by Staph. aureus NCTC 5655 in BHI (Figure 3.10).

In the chemically defined medium, CDM/2 (containing only three essential and two required amino acids; see Tables 3.5 and 3.6), the growth of Staph. aureus NCIB 8625 occurred at a lower rate, yielding a 5-fold overall increase in optical density within 12h (Figure 3.16). No alpha-haemolysin was detected during the 48h incubation. Increasing the number of amino acids to twelve (CDM/3) gave increases in cellular yields of approximately 52-fold during the first 12h (Figure 3.16). Nevertheless, a low titre, 4 HU50/ml of alpha-haemolysin was detected after 12h incubation (Figure 3.17).

The amino acid isoleucine was required for the growth of Staph. aureus NCIB 8625 (Table 3.5). However, omitting isoleucine from CDM/3 and replacing it with the two amino acid tryptophan and lysine (both showed no absolute requirement for the growth of this strain; Table 3.5) as presented in CDM/A (thirteen amino acid medium; Table 3.6), resulted in a poor growth rate (Figure 3.16), with almost similar growth patterns to that of CDM/2 (containing five amino acids). Overall, small titres (4 HU50/ml) of alpha-lysin, equivalent to that seen in CDM/3, were produced after 36h (Figure 3.17).

In the medium contaning 0.1% YE in PBS (YEBS) the growth increased approximately eight fold during the first 6h incubation (Figure 3.16) giving cellular yields similar to that gained in CDM/3. However, not much difference was detected in the quantity of cells

Fig. 3.16: Growth curve (O.D. at 540 nm) in shaken cultures (90 rev/min) at 37°C, for Staph. aureus NCIB 8625 grown in various amino acid containing chemically defined media and with media supplemented with 0.1% (w/v) YE: △ , CDM/2; O, CDM/3; ● , CDM/3 plus YE; +, CDM/A. YEBS, □ .

Fig. 3.17: Alpha-haemolysin titre (HU50/ml) of supernatant liquids from cultures of Staph. aureus NCIB 8625 grown at 37 °C in shaken culture (90 rev/min) in various amino acid containing chemically defined media and with media supplemented with 0.1% (w/v) YE. Titre estimated after 1h contact in PBS at 37 °C using rabbit erythrocytes: O, CDM/3; ●, CDM/3 plus YE; +, CDM/A. YEBS, □ .

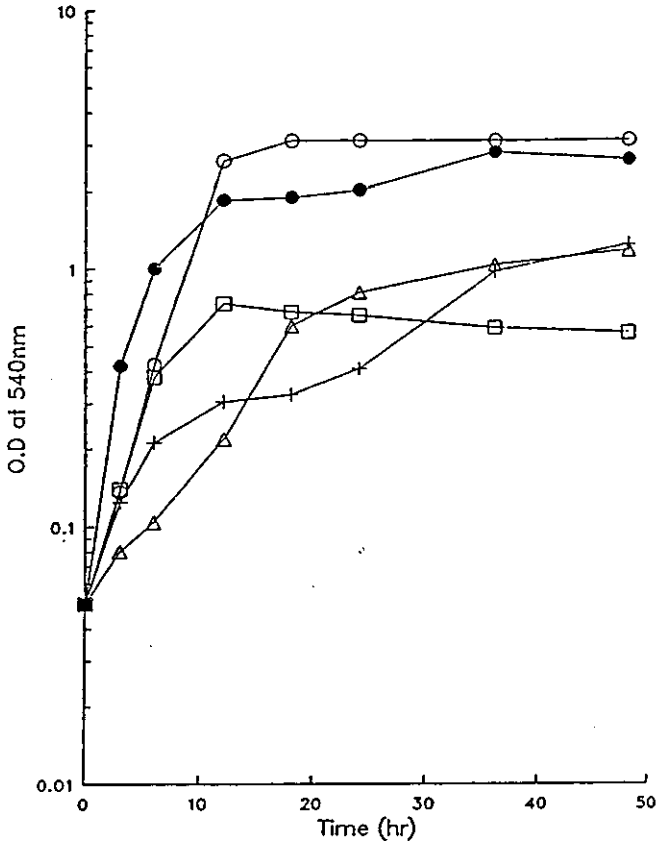


FIGURE 3.16

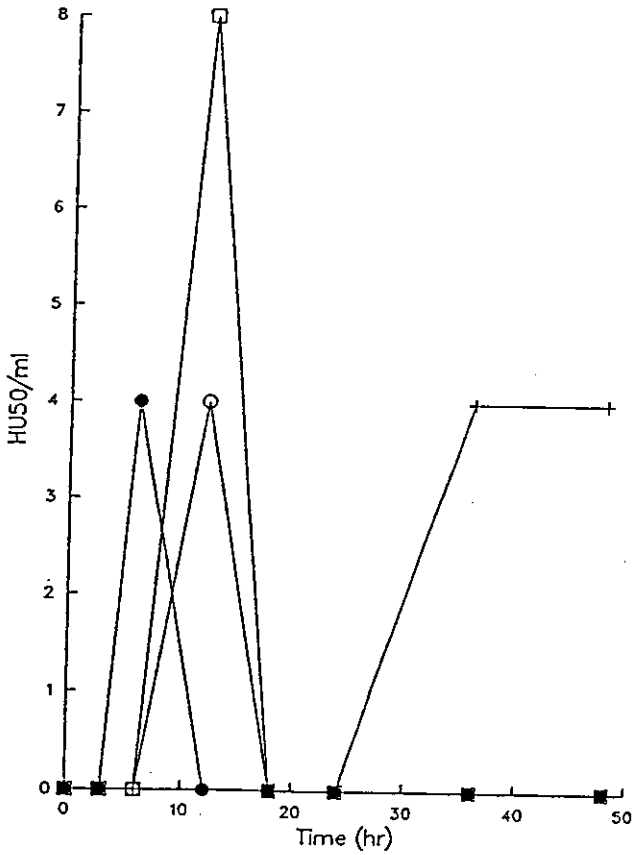


FIGURE 3.17

after 18h to that of CDM/2 and CDM/A, though a small titre 8 HU50/ml of alpha-lysin was formed at 12h (Figure 3.17). On the other hand, in medium CDM/3 supplemented with 0.1% YE resulted in the faster growth rate and a twenty fold increase in cell mass during the first 6h, and after 12h incubation the growth was almost similar to that observed in CDM/3 (Figure 3.16). The addition of 0.1% YE to CDM/3 overall, influenced only the early appearance (at 6h) of alpha-haemolysin, giving the same titre of 4 HU50/ml to that gained with CDM/3 or CDM/A (Figure 3.17).

iv) Staph. aureus NCTC 7428

Staph. aureus NCTC 7428 showed good growth in BHI Figure 3.7. The titre of beta-haemolysin produced in BHI, was 160 HU50/ml after 24h incubation, rising to 1280 HU50/ml after 48h (Figure 3.10).

Little or no growth occurred in the chemically defined medium CDM/B (Table 3.6) containing only the 5 essential amino acids (see Tables 2.2 and 3.5), during the 48h incubation (Figure 3.18). However, preliminary experiments had shown that the addition of three required amino acids (leucine, methionine, and phenylalanine; see Tables 2.2 and 3.5) to medium CDM/B (Table 3.6), gave a mean generation of approximately nine hours. Increasing the number of amino acids to thirteen (CDM/A, Table 3.6), by the addition of five supplementary amino acids to the five essential and three required amino acids (see Table 3.5) increased the growth of Staph. aureus NCTC 7428 giving at least a five fold greater optical density during the first 6h (with a generation time of approximately three hours), and reaching the maximum cell yield at 24h incubation (Figure 3.18). Beta-haemolysin was detected after 6h in CDM/A and continued to increase reaching a maximum titre of 20 HU50/ml after 24h incubation (Figure 3.19). Further increase in the number of amino acids in chemically defined medium to 18 (as in medium CDM; see Table 2.1) resulted in an improved cellular yield of Staph. aureus NCTC 7428 (Figure 3.8), but overall it did not influence the yield of beta-lysin, compared to that produced in CDM/A (Figure 3.19).

Fig. 3.18: Growth curve (O.D. at 540 nm), in shaken cultures (90 rev/min) at 37°C, for Staph. aureus NCTC 7428 grown in various amino acid containing chemically defined media and with media supplemented with 0.1% (w/v) YE: △ , CDM/B; +, CDM/B plus YE; ● , CDM/A; O, CDM/A plus YE. YEBS, □.

Fig. 3.19: Beta-haemolysin titre (HU50/ml) of supernatant liquids from cultures of Staph. aureus NCTC 7428 grown at 37°C in shaken culture (90 rev/min) in various amino acid containing chemically defined media and with media supplemented with 0.1% (w/v) YE. Titre estimated after contact for 1h at 37°C and then 1h at 4°C in PBS supplemented with 0.002 mol/L magnesium sulphate using sheep erythrocytes: △, CDM/B; +, CDM/B plus YE; ●, CDM/A; O, CDM/A plus YE; ▲, CDM. YEBS, □.

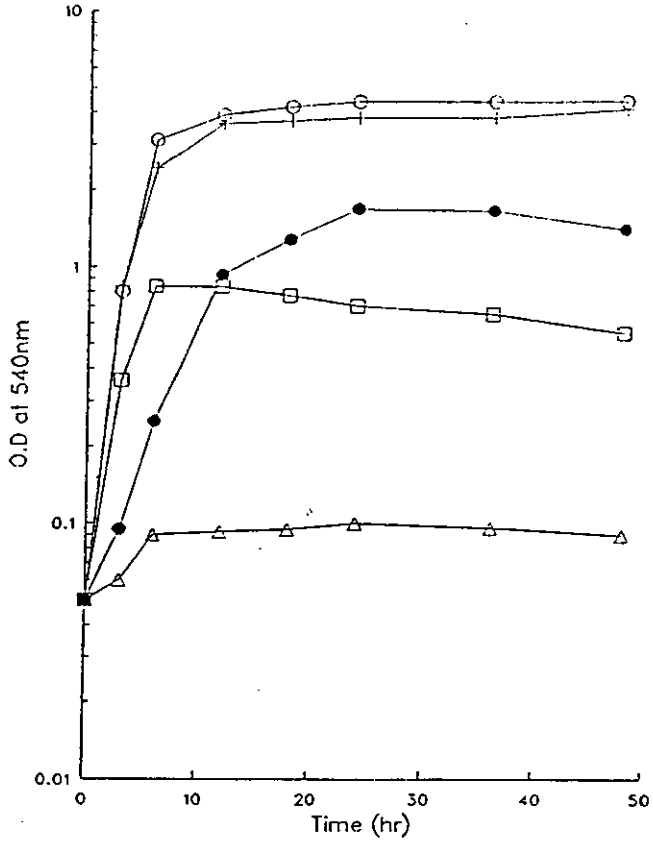


FIGURE 3.18

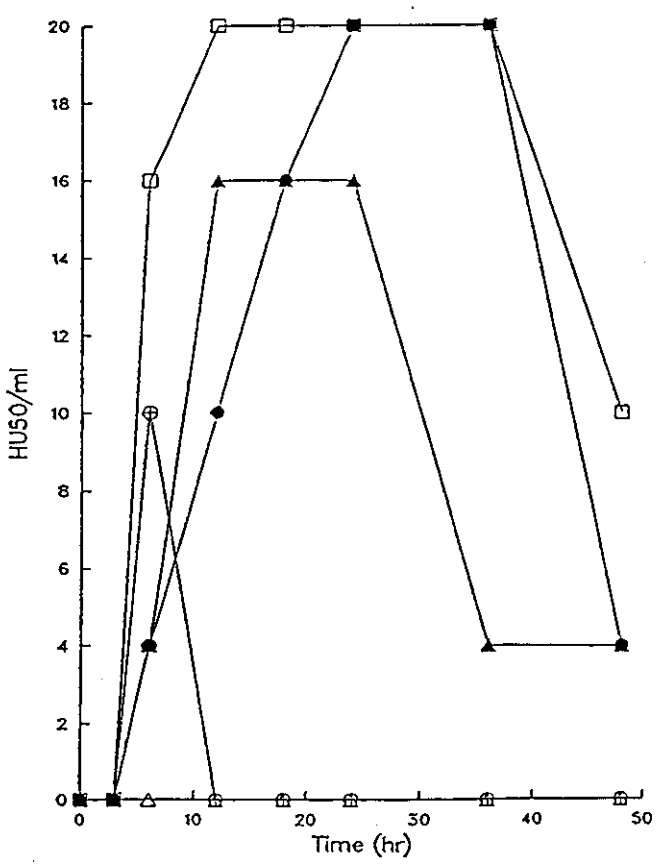


FIGURE 3.19

In the study of the growth of Staph. aureus NCTC 7428 in 0.1% YE in PBS (YEBS; Figure 3.18) rapid growth occurred during the first 6h, giving at least a seventeen fold increase in optical density. Thereafter, the optical density gradually decreased. Beta haemolysin was produced after 6h, and reached the maximum level of 20 HU50/ml after 12h (Figure 3.19).

The growth of Staph. aureus NCTC 7428 in five essential amino acid medium (CDM/B), and CDM/A (Table 3.6) both supplemented with 0.1% YE, was enhanced (Figure 3.18), although low titre (10 HU50/ml) of beta-haemolysin were detected after 6h growth in both media (Figure 3.19).

v) Staph. aureus NCTC 8532

In BHI, adequate growth of Staph. aureus NCTC 8532 occurred (Figure 3.7). Haemolsin was not produced during the first 12h incubation. However, after 18h in BHI haemolysin was detected (4 HU50/ml) increasing to the maximum level of 10 HU50/ml (detected with 2% sheep erythrocytes suspended in PBS at 37^{o}C) after 36h incubation (Figures 3.10 and 3.21). The haemolytic activity was not enhanced by subsequent cold-incubation (4^{o}C), and did not lyse 2% rabbit erythrocytes suspended in PBS.

Preliminary experiments had shown that the six amino acid medium, which contains the three essential (arginine, cysteine and valine) and the three required (isoleucine, phenylalanine and threonine) amino acids (see Tables 2.2 and 3.5) only supported poor growth with generation time of ca. 9h.

Increasing the number of amino acids to twelve (CDM/1; Table 3.6), by the addition of the six supplementary amino acids (see Table 3.5) to the six amino acid medium mentioned above, improved growth profiles with at least seven fold overall increase in optical density of the culture within 12h (Figure 3.20).

Fig. 3.20: Growth curve (O.D. at 540 nm), in shaken cultures (90 rev/min) at 37°C, for Staph. aureus NCTC 8532 grown in various amino acid containing chemically defined media: O, CDM/1 and +, CDM/A.

Fig. 3.21: Haemolysin titre (HU50/ml) of supernatant liquid from cultures of Staph. aureus NCTC 8532 grown at 37°C in shaken cultures (90 rev/min) in different media. Titre estimated after 1h contact in PBS at 37°C using sheep erythrocytes: +, CDM/A and ■, BHI.

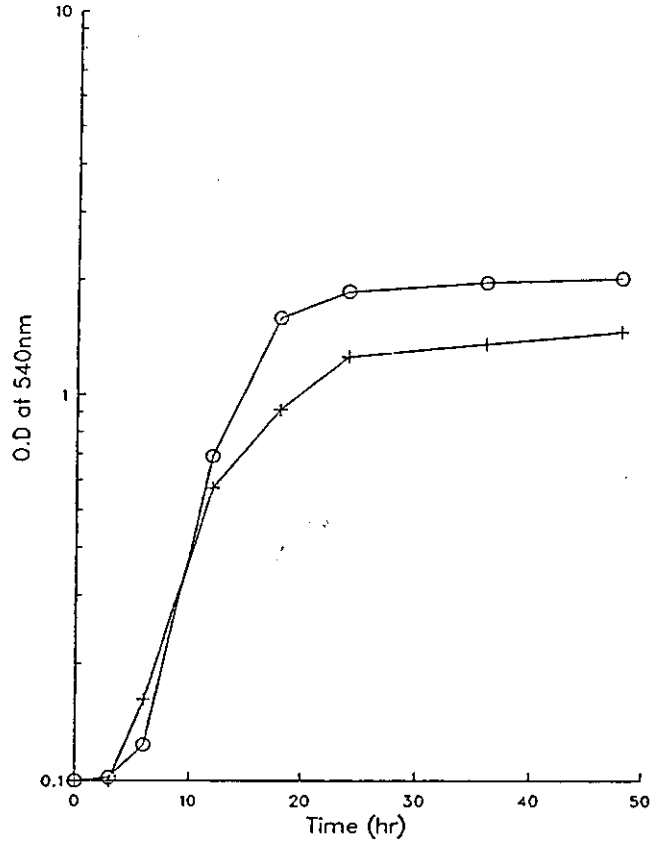


FIGURE 3.20

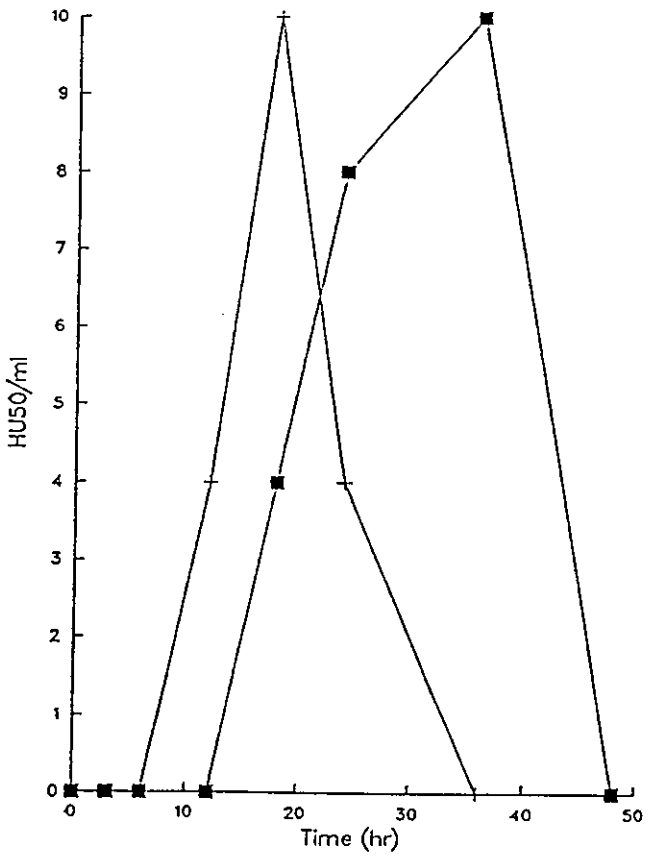


FIGURE 3.21

In CDM/A Staph. aureus NCTC 8532 grew well and increased about six fold in optical density during the first 12h (Figure 3.20). In general, the growth patterns of Staph. aureus NCTC 8532 in both chemically defined media (CDM/1 and CDM/A) were similar (Figure 3.20). However, the cells were also found to adhere to the surface of the glass culture vessels and to clump giving only small "clots" of cells. Overall, this was less than that seen with Staph. aureus Wood 46, in chemically defined media.

Furthermore, it was noted that in both CDM/1 and CDM/A the golden pigment formation of Staph. aureus NCTC 8532, after 12h incubation, was enhanced. In contrast to BHI weak pigment (creamy) was produced during the 48h incubation (see also Table 3.1).

The production of the haemolysin by Staph. aureus NCTC 8532 was investigated in CDM/A. Figure 3.21 showed that Staph. aureus NCTC 8532 produced a haemolysin in CDM/A after 12h, and the level increased to reach the same maximum titre after 18h (10 HU50/ml) of that formed in BHI. Also, the haemolysis titration was detected with only sheep erythrocytes and did not require further cold-incubation. However, the brown colouration (see 3.1.3) was not observed during the quantitative analysis of haemolysin titre.

No haemolysin was detected with Staph. aureus NCTC 8532 growing in YEBS.

vi) Staph. epidermidis NCTC 7944

Staph. epidermidis NCTC 7944 grew well in BHI (Figure 3.7), there was a rapid growth with at least an eighty fold increase in optical density within 6h, after which there was a gradual increase till 48h.

In the study of the amino acid requirements of six staphylococci strains (Table 3.5) glutamic acid was found to be required only for the growth of Staph. epidermidis NCTC 7944. Increasing the concentration of glutamic acid to 14 mg/100 ml in CDM/11 (Table 3.6)

increased the growth rate of Staph. epidermidis NCTC 7944. Therefore, glutamic acid was used in both CDM/10 and CDM/11 (Table 3.6) at 14 mg/100 ml with this organism.

To investigate the biotin requirements for the growth of Staph. epidermidis NCTC 7944, medium CDM/11 was supplemented with different concentrations of biotin: no biotin added, 0.0003 mg/100 ml, and 0.2 mg/100 ml. The results presented in Figure 3.22 show that Staph. epidermidis NCTC 7944 grew in biotin-deprived CDM/11 and that growth was virtually unaffected by the addition of different amounts of biotin.

Studies with Staph. epidermidis NCTC 7944 in a simplified chemically defined medium containing two essential and three required amino acid (CDM/10; see Tables 3.5 and 3.6), gave adequate growth profiles, with at least a four fold increase in optical density within 6h (Figure 3.23). Increasing the number of amino acids to twelve (CDM/11, Table 3.6) by the addition of seven supplementary amino acids, however, stimulated the growth of Staph. epidermidis NCTC 7944. At least a two fold increase in both cellular yield and growth rate to that when grown in CDM/10 (Figure 3.23). A marked decrease in the growth rate of Staph. epidermidis NCTC 7944 was observed when glutamic acid and glycine (required and supplementary amino acids respectively, see Table 3.5) were replaced with three amino acids (tryptophan, serine and tyrosine) for which the organism had no absolute requirement (see Table 3.5) to give the thirteen amino acid medium, CDM/A (Table 3.6). The maximum yield was similar to that obtained in CDM/10 (Figure 3.23).

The haemolytic activity of Staph. epidermidis NCTC 7944 was studied and the results revealed that there was no appreciable level or minimum haemolytic activity of haemolysin produced in either BHI, CDM/11 and CDM/A detected with 2% rabbit erythrocytes suspended in PBS at 37°C.

Fig. 3.22: Growth curves (O.D. at 540 nm), in shaken cultures (90 rev/min) at 37 °C, for Staph. epidermidis NCTC 7944 grown in CDM/11 supplemented with various amounts of biotin: △, biotin-deprived CDM/11; □, CDM/11 plus 0.0003 mg/100 ml biotin; and O, CDM/11 plus 0.2 mg/100 ml biotin.

Fig. 3.23: Growth curve (O.D. at 540 nm), in shaken cultures (90 rev/min) at 37 °C, for Staph. epidermidis NCTC 7944 grown in various amino acid containing chemically defined media: △, CDM/10; ●, CDM/11; and □, CDM/A.

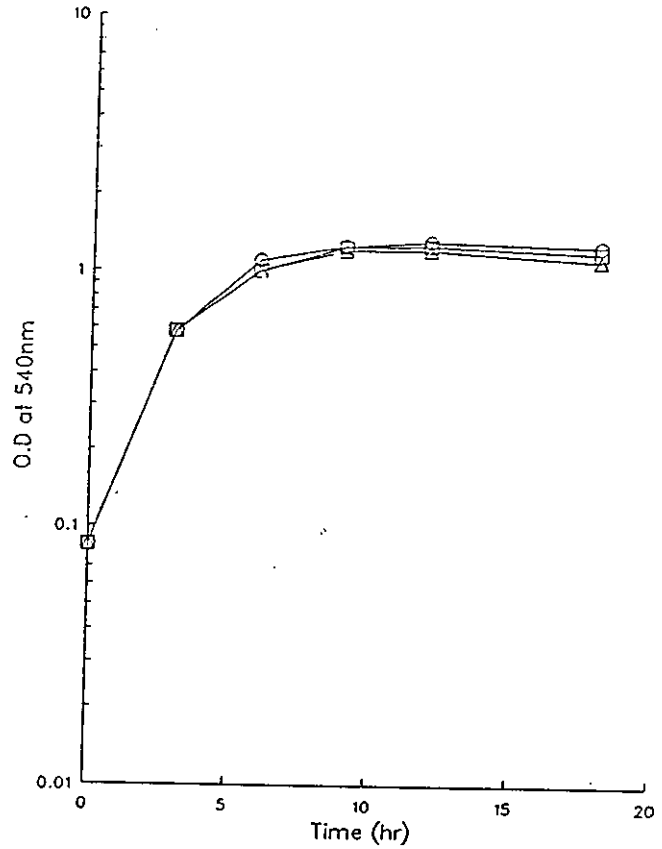


FIGURE 3.22

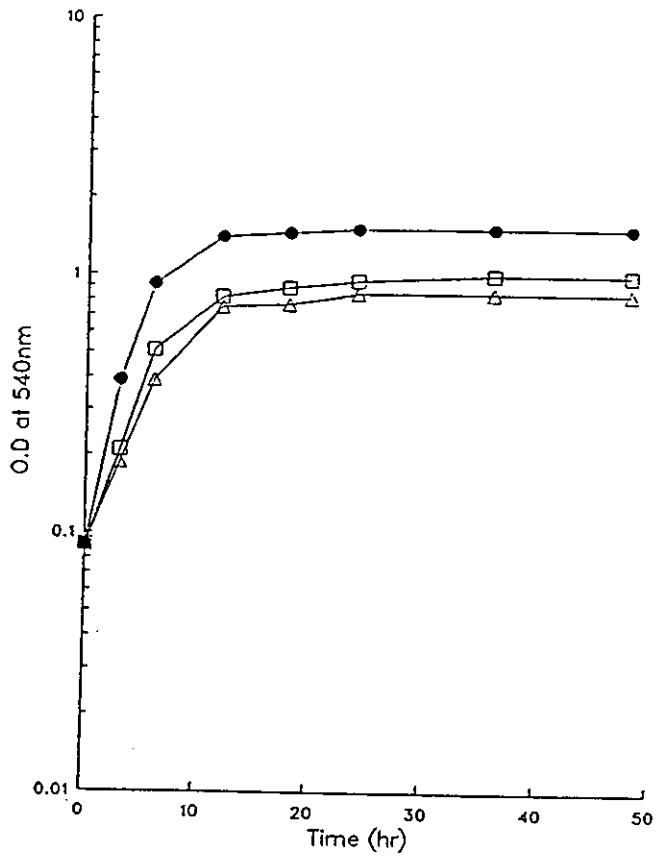


FIGURE 3.23

3.3 A COMPARATIVE STUDY OF GROWTH AND HAEMOLYSINS PRODUCTION BY SOME STAPHYLOCOCCI IN MEDIA BASE ON FISH-WASTE EXTRACT (FE)

For preparation of media based on a simple fish-waste extract (FE) see Appendix 1. The freeze-dried product (see Appendix 1) was used in this study with all fish-extract media.

The growth of the six staphylococci strains in Fish Infusion Broth (FIB; medium 2 see Appendix 1) is illustrated in Figure 3.24. All strains grew abundantly with a heavy but similar turbidity, and the growth was comparable with that obtained in Brain Heart Infusion broth (BHI; see Figure 3.7).

The haemolytic activity of Staph. aureus strains on 7% rabbit's or sheep's blood enriched agar plates of FIB was clearly demonstrated with the zones of haemolysis characteristic of the strain studied being obtained and were comparable with those obtained from blood agars based on BHI (see Table 3.3).

All strains of Staph. aureus (Wood 46, NCIB 8625, NCTC 5655 and NCTC 7428) grew abundantly in the various fish extract based media with a heavy but uniform turbidity (illustrative examples are given in Figures 3.25a and b). Small differences in maximum growth rate and overall yields were, however, detectable in media consisting of 3.7% w/v extracts (beef-extract, BE or fish-extract, FE) or 3.7% w/v peptones (peptone or proteose peptone) (see Appendix 1).

There was no significant difference in the level of alpha-lysin produced by Staph. aureus Wood 46 in either FIB or BHI, where the highest titre of alpha-haemolysin in both media gave 2560 HU50/ml after 24h incubation (Figure 3.26a).

Similar alpha-lysin production patterns were observed, though at much lower levels (HU50/ml) with strain NCIB 8625 in BHI and FIB media. Strain NCTC 5655, however, produced more alpha-lysin in BHI (Figure 3.26a).

Fig. 3.24: Growth curves (O.D. at 540 nm), in shaken cultures (90 rev/min) at 37°C, for Staphylococci six strains in FIB: Staph. aureus strains; ▽, Wood 46; O, NCTC 5655; □, NCIB 8625; ●, NCIC 7428; ■, NCTC 8532; and +, Staph. epidermidis NCTC 7944.

Fig. 3.25a: Growth curves (O.D. at 540 nm), in shaken cultures (90 rev/min) at 37°C for Staph. aureus (i) Wood 46 in: ■, FIB; △, BHI; ●, 3.7% beef-extract (BE); □ , 3.7% peptone; O, 3.7% fish-extract (FE); and +, 3.7% proteose peptone; (ii) NCTC 5655 in: ▲, FIB; and ▽, BHI; (iii) NCIB 8625 in: ▼, FIB; and x, BHI.

Fig. 3.25b: Growth curves (O.D. at 540 nm), in shaken cultures (90 rev/min) at 37°C, for Staph. aureus NCTC 7428 in: ■, FIB; △, BHI; ●, 3.7% beef-extract (BE); □ , 3.7% peptone; O, 3.7% fish-extract (FE); and +, 3.7% proteose peptone.

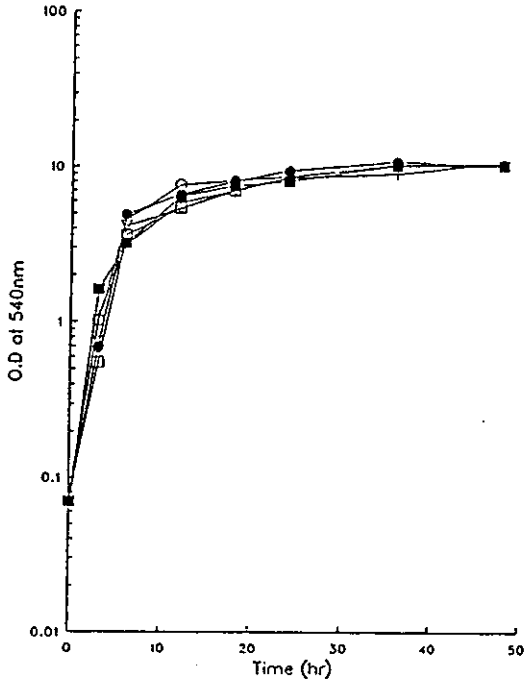


FIGURE 3.24

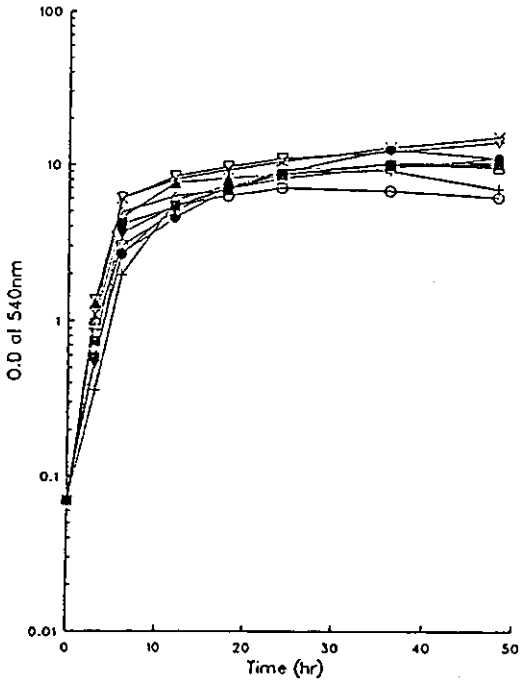


FIGURE 3.25a

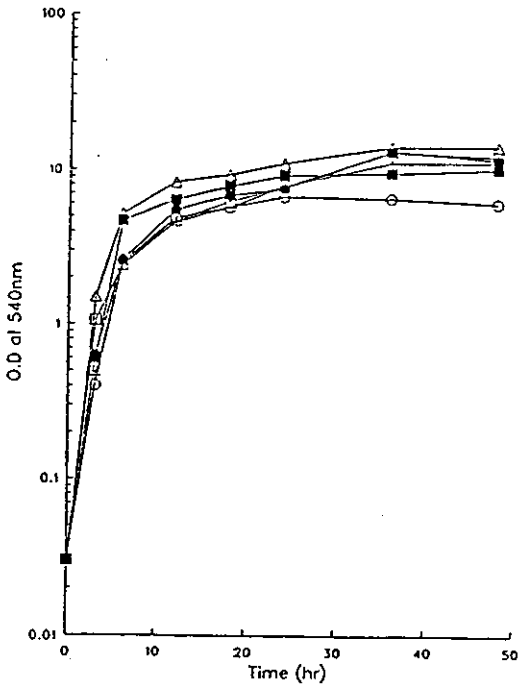


FIGURE 3.25b

Fig. 3.26a: Alpha-haemolysin titre (HU50/ml), of the supernatant liquid from cultures of Staph. aureus estimated after 1h contact in PBS at 37 °C using rabbit erythrocytes: (i) Staph. aureus Wood 46 in: ■, FIB; △, BHI; ●, 3.7% beef-extract (BE); □, 3.7% peptone; O, 3.7% fish-extract (FE); and +, 3.7% proteose peptone; (ii) Staph. aureus NCIC 5655 in: ▲, FIB; and ▽, BHI; (iii) Staph. aureus NCIB 8625 in: ▼, FIB; and x, BHI.

Fig. 3.26b: Beta-haemolysin titre (HU50/ml), of the supernatant liquid from cultures of Staph. aureus NCTC 7428, estimated using sheep erythrocytes suspended in PBS, supplemented with 0.002 mol/L magnesium sulphate, after 1h at 37 °C and then 1h at 4 °C; ■, FIB; △, BHI; ●, 3.7% beef-extract (BE); □, 3.7% peptone; O, 3.7% fish-extract (FE); and +, 3.7% proteose peptone.

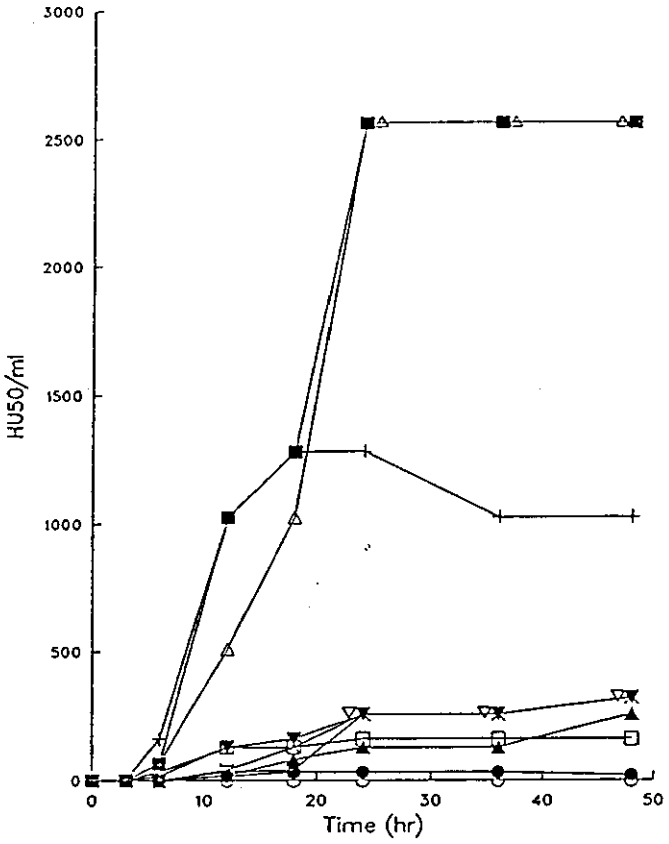


FIGURE 3.26a

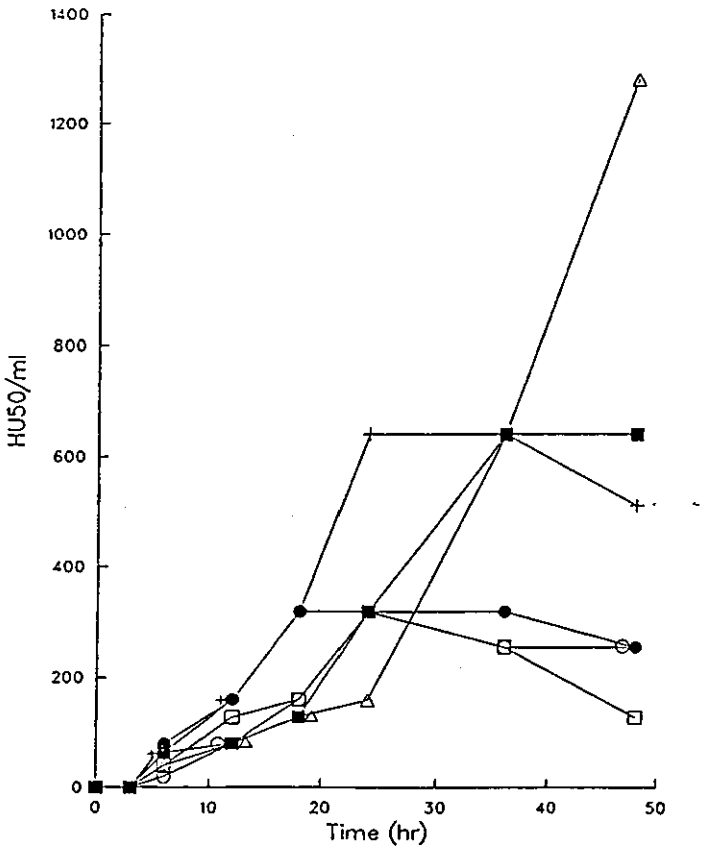


FIGURE 3.26b

When Staph. aureus Wood 46 was grown in media containing 3.7% w/v aqueous solutions of beef extract (BE) or peptone alone, alpha-haemolysin production was markedly depressed, with maximum HU50/ml values of 32 and 160 for beef-extract (BE) and peptone respectively. Growth in 3.7% w/v proteose peptone solution gave a maximum HU50/ml in excess of 1000 after 12h. Alpha-lysin production was, however, completely abolished in 3.7% w/v fish-extract (FE; Figure 3.26a).

Beta-haemolysin was produced by Staph. aureus NCTC 7428 in all media used (Figure 3.26b). Highest titres were achieved in BHI, although beta-lysin production was most rapid in a medium consisting of 3.7% w/v proteose peptone. The HU50/ml values were equivalent in media FIB, BHI and 3.7% w/v proteose peptone (approximately 650 units) after 36h. The maximum titre of beta-lysin was significantly lower in media comprising 3.7% w/v of fish/beef extract or peptone alone (media Nos 7, 5 and 6 respectively; see Appendix 1).

3.4 INFLUENCE OF MEDIUM COMPOSITION ON THE in vitro ACTION OF PHAGOCYTOSIS, PMN's CRUDE EXTRACT (PMNCE) AND SERUM BACTERICIDES OF STAPHYLOCOCCI

3.4.1 Preliminary Studies: Determination of the Test Experimental Ratio of Rabbit Polymorphonuclear (PMN)-to-Staphylococci

The results of incubating 1-2 x 10^7 PMN's/ml (see 2.13) with various concentrations of an 18h culture of Staph. aureus NCTC 8532 grown in BHI (1-2 x 10^7 to 1-2 x 10^9/ml) (see 2.16) in gel-HBSS (see 2.18), in the absence of 10% rabbit normal serum (10% RNS; included in other experiments) are given in Figure 3.27. For PMN-to-bacteria ratios of 1:100 about 81 and 75% of the bacteria were ingested during the 30 and 120 min contact, respectively. At a PMN-to-bacteria ratio of 1:10 about 86 and 93% of the bacteria were ingested during the same respective time periods. The incubation of PMN-to-bacteria in a ratio of 1:1 showed that during the 30 min about 94% of the bacteria were ingested and during the two hour period about 98% of the bacteria were not available for viable counting. Phagocytosis experiments were thus carried out over a

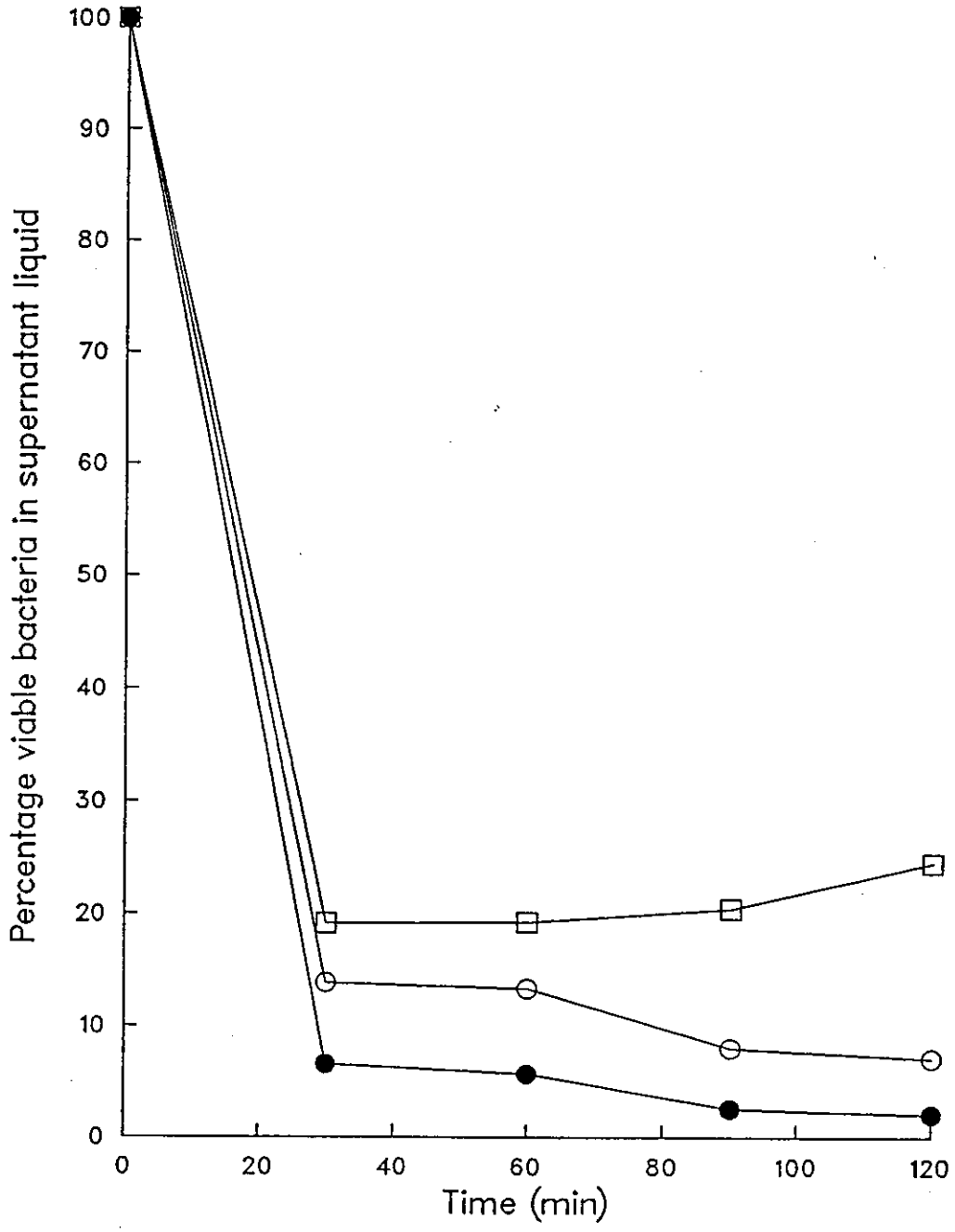


Fig. 3.27: Phagocytosis of Staph. aureus NCTC 8532 (grown in BHI) estimated with rabbit polymorphonuclear leucocytes suspended in gel-HBSS in the absence of 10% RNS (non-opsonized) at 37°C and 4 rev/ min, at various cells-to-bacteria ratios: ● , 1:1; ○ , 1:10; and □, 1:100.

period of 120 min, using a ratio of 1 cell of PMN to 1 bacterium to give the most reliable information about the rate of ingestion. This ratio 1:1 is applied for all phagocytosis studies.

However, preliminary experiments in which the staphylococci grown in BHI were incubated in the HBSS alone at 37°C, and 4 rev/min, revealed a striking bactericidal effect. The addition of 0.1% gelatin to HBSS stabilized the bacteria but did not support significant multiplication. Gelatin as a constituent of solution gel-HBSS was therefore used in all subsequent studies.

3.4.2 Sensitivity of Staphylococci (Six Strains) Grown in Various Cultivation Media to Phagocytosis, 10% RNS, and the Bactericides of PMN's and Serum

i) Staph. aureus NCTC 8532

The phagocytosis of Staph. aureus NCTC 8532 grown in BHI, FIB and chemically defined media CDM/1 and CDM/A (Table 3.6) by rabbit PMN's in the presence of 10% RNS are presented in Figure 3.28a. Staph. aureus NCTC 8532 from BHI was rapidly ingested by rabbit PMN's, 95% loss of detectable viability was achieved in 30 min. Phagocytosis of this strain when grown in the other media (FIB, CDM/1 and CDM/A) still occurred to a considerable extent; only about 85% viability loss was achieved in 30 min and similar patterns of phagocytosis were obtained after which little further phagocytosis occurred. The percentage of phagocytosis given in Figure 3.28a was obtained according to the formula No 2 (see 2.18). This gives a corrected phagocytic index, namely,the effect of phagocyte cells only. Figure 3.28b shows uncorrected phagocytosis (showing effects of both PMN's and 10% RNS), obtained from formula No 1 (see 2.18). However, the results from formulas No 2 and 1 (Figure 3.28a and b, respectively) shows only small differences in the phagocytosis rate.

Therefore, to study the effect of each factor (PMN's and 10% serum) individually on staphylococci strains, formula No 2 was used in all subsequent studies in phagocytosis, since it gives the corrected phagocytosis index.

Fig. 3.28a: Phagocytosis (corrected) by rabbit PMN's suspended in gel-HBSS with 10% RNS present at 37 °C and 4 rev/min of Staph. aureus NCTC 8532 from: ●, CDM/1; △ , CDM/A; □, FIB; and ■, BHI.

Fig. 3.28b: Phagocytosis (uncorrected) by rabbit PMN's suspended in gel-HBSS with 10% RNS present at 37 °C and 4 rev/min of Staph. aureus NCTC 8532 from: ●, CDM/1; △ , CDM/A; □, FIB; and ■, BHI.

Fig. 3.28c: Effect of 10% RNS in gel-HBSS at 37 °C and 4 rev/min on the survival of Staph. aureus NCTC 8532 from: ● , CDM/1; △, CDM/A; □, FIB; and ■, BHI.

Fig. 3.28d: Effect of bactericidal substances from supernatant bacteria-free, and PMN's-free at 37 °C and 4 rev/min on the survival of Staph. aureus NCTC 8532 from: ●, CDM/1; △, CDM/A; □, FIB; and ■, BHI.

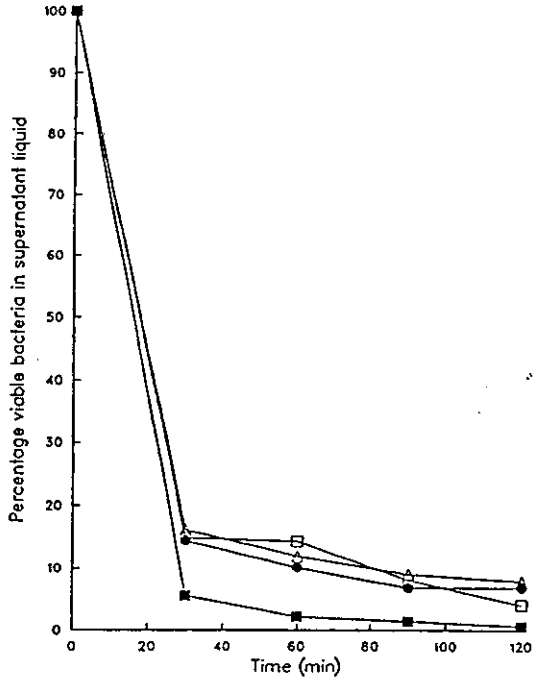


FIGURE 3.28a

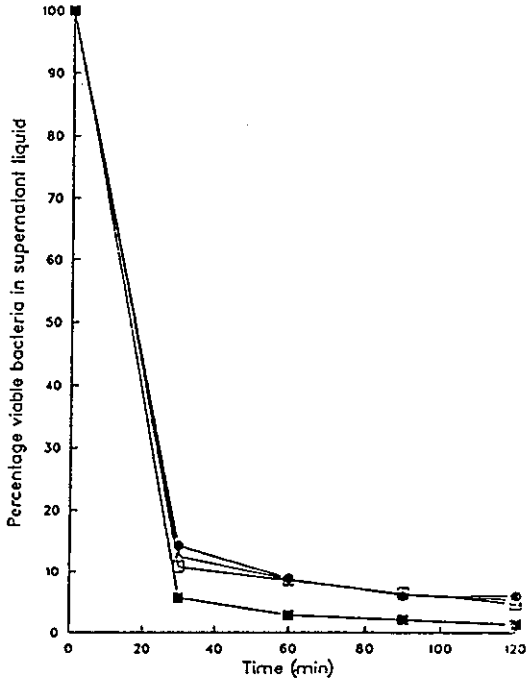


FIGURE 3.28b

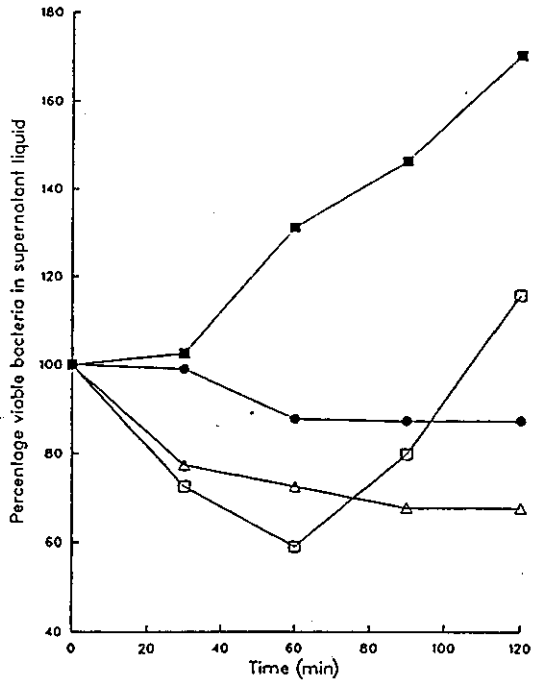


FIGURE 3.28c

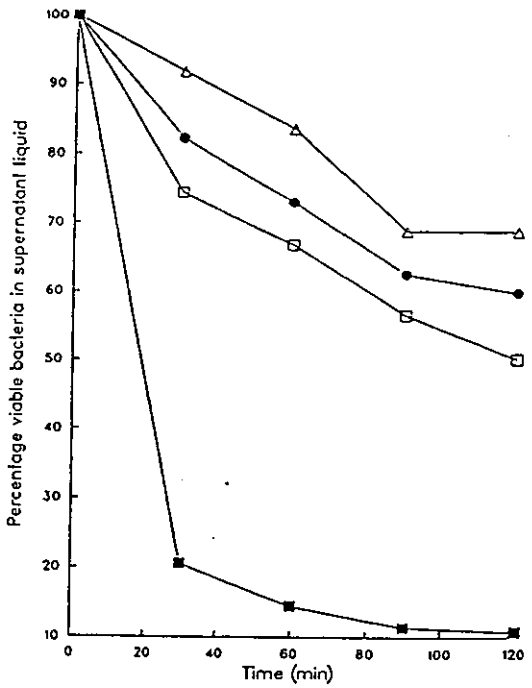


FIGURE 3.28d

The effect of 10% (v/v) rabbit normal serum (10% RNS) on Staph. aureus NCTC 8532 grown in different media was studied. It was found that in the presence of 10% RNS alone in gel-HBSS the number of viable bacteria increased in the case of NCTC 8532 from BHI after 30 min. Cells from FIB showed a substantial initial decline in viability (up to 60 min) followed by a steady rise in viability (60 to 120 min; Figure 3.28c). In the case of NCTC 8532 from chemically defined media, the number of viable bacteria was reduced slightly and cells of strain NCTC 8532 from CDM/A were more susceptible to killing by 10% RNS than those from CDM/1.

Furthermore, studies on the bactericidal effect from both PMN's and 10% RNS obtained from previously processed phagocytosis (bacteria-free, and PMN's-free supernatants after 120 min of phagocytosis; see 2.18) on Staph. aureus NCTC 8532 from different media, revealed again that NCTC 8532 from BHI was most sensitive and greatly reduced the viable bacteria (Figure 3.28d); in comparison cells of strain NCTC 8532 were less susceptible to killing by bactericidal substances when grown in chemically defined media (CDM/A and CDM/1). Medium FIB provided cells of strain NCTC 8532 that were less susceptible than NCTC 8532 from BHI but more sensitive than cells of the same strain grown in chemically defined media (Figure 3.28d).

ii) Staph. aureus NCTC 7428

Staph. aureus NCTC 7428 grown in different media (BHI, FIB and CDM/A) was incubated with rabbit PMN's at a cell ratio of 1:1 in the presence of 10% RNS to study the effect of various media on phagocytosis. The data shows (Figure 3.29a) that cells of strain NCTC 7428 cultivated in BHI were more sensitive to ingestion by PMN's, by which about 86% of the bacteria were phagocytised during the first 30 min. In contrast, cultivation in both FIB and CDM/A gave cells with similar sensitivity (78 $\sim$ 80% in 30 min) to phagocytes, and less ingestion than with cells grown in BHI.

Fig. 3.29a: Phagocytosis by rabbit PMN's suspended in gel-HBSS with 10% RNS present at 37°C and 4 rev/min of Staph. aureus NCTC 7428 from: △ , CDM/A; □ , FIB; and ■ , BHI.

Fig. 3.29b: Effect of 10% RNS in gel-HBSS at 37°C and 4 rev/min on the survival of Staph. aureus NCTC 7428 from: △ , CDM/A; □ , FIB; and ■ , BHI.

Fig. 3.29c: Effect of bactericidal substances from supernatant bacteria-free, and PMN's-free at 37°C and 4 rev/min on the survival of Staph. aureus NCTC 7428 from: △ , CDM/A; □ , FIB; and ■ , BHI.

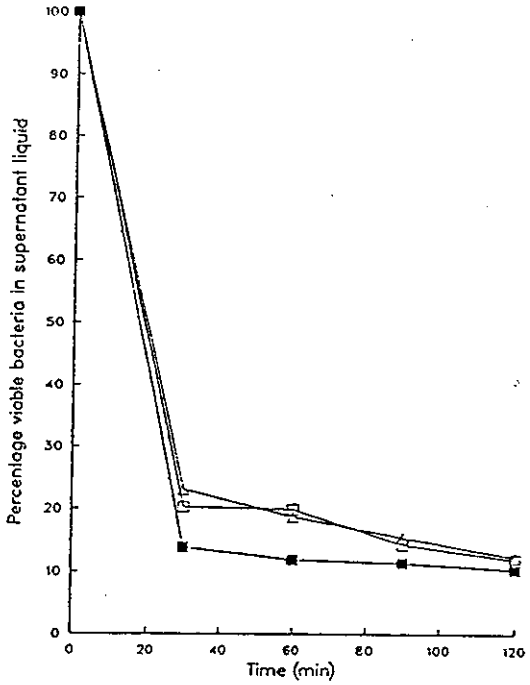


FIGURE 3.29a

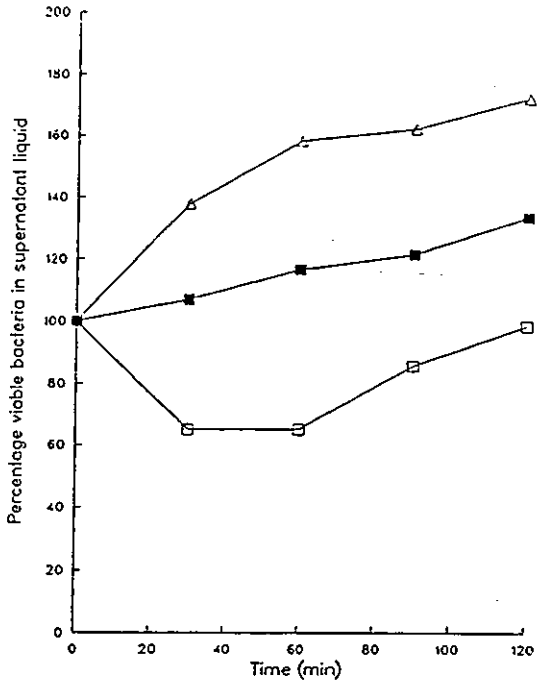


FIGURE 3.29b

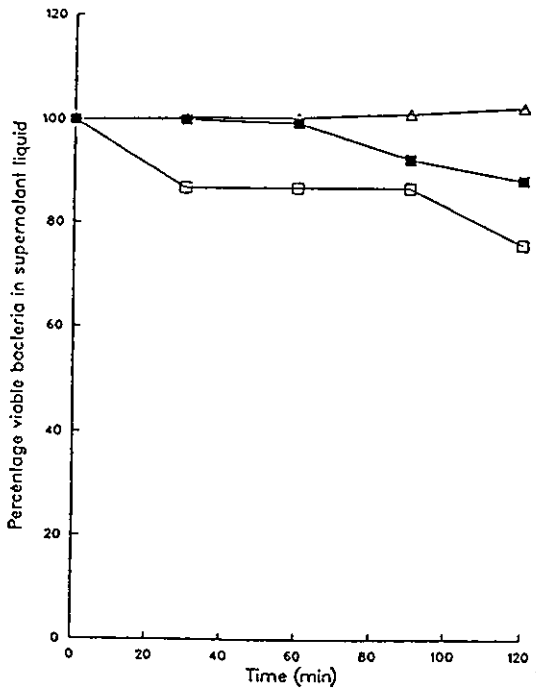


FIGURE 3.29c

After 120 min, however, cells of strain NCTC 7428 grown in FIB and CDM/A had been ingested to almost the same extent as that obtained with cells grown in BHI (90%).

The survival of Staph. aureus NCTC 7428, from different media, in gel-HBSS containing 10% RNS is presented in Figure 3.29b. It was found that cells of strain NCTC 7428 from CDM/A increased in percentage viability (to approximately 170%) over the 120 min incubation.

In contrast cells from BHI were relatively unaffected (plus 30%), and cells incubated originally in FIB medium were reduced in percentage viability during the first 60 min; thereafter they gradually increased in viability to almost the initial viable number by 120 min.

The bactericidal effect of the bacteria-free and PMN's-free supernatant liquid (obtained after 120 min of phagocytosis) on cells of NCTC 7428 was investigated, and the results are presented in Figure 3.29c. During the 120 min cells initially from CDM/A were unaffected. Cells cultivated in BHI were initially unaffected (up to 60 min), but by 120 min viability had fallen by approximately 12%. Overall, growth in FIB gave cells which were more susceptible to the bactericidal effect from PMN's and 10% RNS, viability eventually falling (after 120 min) by some 24%.

iii) Staph. aureus NCIB 8625

There was considerable variation in the extracellular survival of cells of Staph. aureus NCIB 8625 originally grown in different media, when incubated in the presence of rabbit PMN's in gel-HBSS containing 10% RNS. Figure 3.30a illustrates some representative results. Cells cultivated in BHI were cleared rapidly (94%) during the first 30 min of incubation and viability eventually (120 min) decreased by about 98%. Variations in survival profiles were obtained in other media also, in which the number of extracellular bacteria declined to about 40, 83, 84 and 87% of the initial number

Fig. 3.30a: Phagocytosis by rabbit PMN's suspended in gel-HBSS with 10% RNS present at 37°C and 4 rev/min of Staph. aureus NCIB 8625 from: ○, CDM/2; ●, CDM/3; △, CDM/A; □, FIB; and ■, BHI.

Fig. 3 .30b: Effect of 10% RNS in gel-HBSS at 37°C and 4 rev/min on the survival of Staph. aureus NCIB 8625 from: ○, CDM/2; ●, CDM/3; △, CDM/A; □, FIB; and ■, BHI.

Fig. 3.30c: Effect of bactericidal substances from supernatant bacteria-free, and PMN's-free at 37°C and 4 rev/min on the survival of Staph. aureus NCIB 8625 from: ○, CDM/2; ●, CDM/3; △, CDM/A; □, FIB; and ■, BHI.

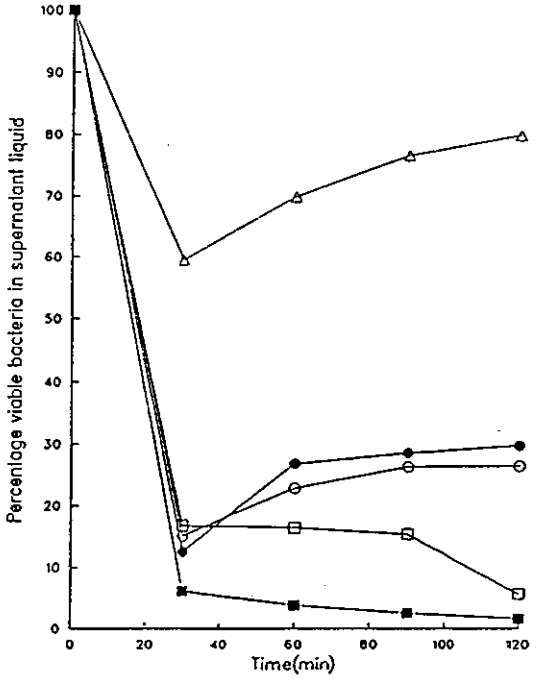


FIGURE 3.30a

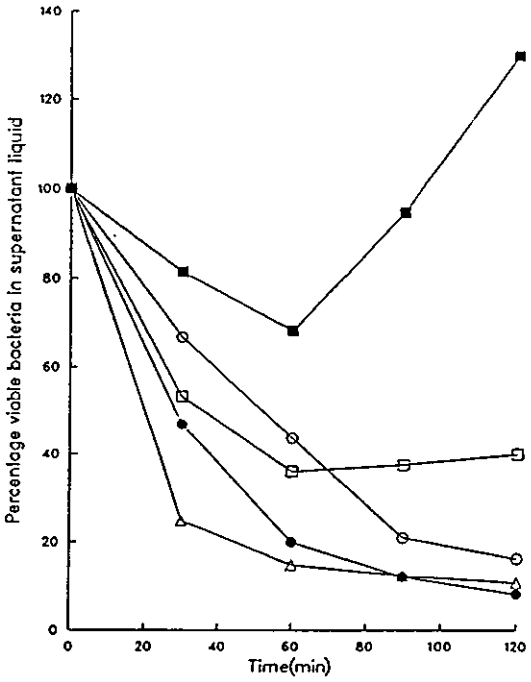


FIGURE 3.30b

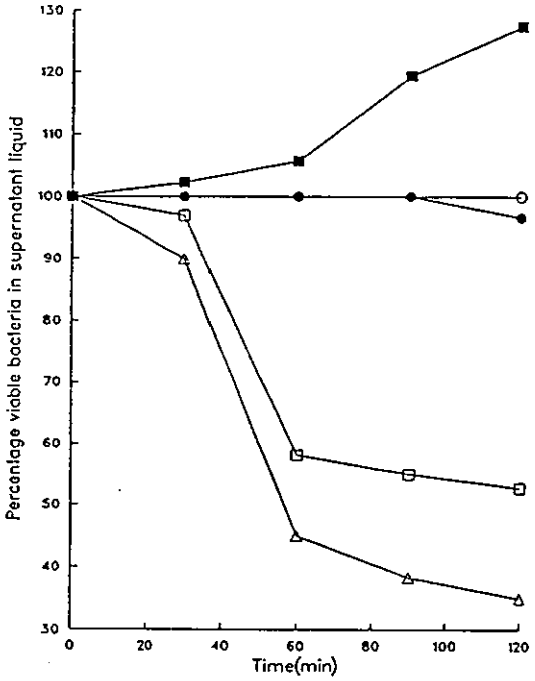


FIGURE 3.30c

whether from media CDM/A, FIB, CDM/2 or CDM/3 (see Table 3.6) respectively. In each of the chemically defined media variants the number of extracellular bacteria increased gradually after the first 30 min. Cells from FIB, however, showed a further decline in the extracellular number until 120 min (Figure 3.30a).

On the other hand, results obtained for the survival of strain NCIB 8625 (grown initially in different media) in 10% RNS without PMN's in gel-HBSS (Figure 3.30b) showed that the viability of bacteria from BHI and FIB declined during the first hour, after which cells from BHI recovered and increased in number, whilst those from FIB remained virtually unchanged. The viability of cells pre-incubated in the various chemically defined media decreased steadily during the overall incubation period. Cells from CDM/A (containing thirteen amino acids) were most sensitive with those from CDM/2 (containing five amino acids) reducing in viability at a lower rate. Eventually, however, reductions of over 80% in viability were achieved (120 min) with cells from chemically defined media.

The effect of bactericidal substances from the cell free supernatant obtained after 120 min of phagocytosis of strain NCIB 8625, on the survival of this organism in gel-HBSS is presented in Figure 3.30c. The number of cells pre-incubated in CDM/2 and CDM/3 remained almost the same throughout the 120 min incubation. The number of cells from BHI increased slowly during the 120 min (to 127% approximately). In comparison the results obtained with cells from FIB and CDM/A showed a sharp decline in viability and about 47 and 65% of cells were killed during the overall incubation period (120 min) respectively.

iv) Staph. aureus NCTC 5655

A series of experiments were performed with rabbit PMNs in gel-HBSS in the presence of 10% RNS to compare the rate of phagocytosis of Staph. aureus NCTC 5655 grown in different media. Results presented in Figure 3.31a showed that during the first 30 min cells of Staph. aureus NCTC 5655 were reduced in viability by about 45, 86, 91, 93

Fig. 3.31a: Phagocytosis by rabbit PMN's suspended in gel-HBSS with 10% RNS present at 37°C and 4 rev/min of Staph. aureus NCTC 5655 from: ○, CDM/4; ●, CDM/5; △, CDM/A; □, FIB; and ■, BHI

Fig. 3.31b: Effect of 10% RNS in gel-HBSS at 37°C and 4 rev/min on the survival of Staph. aureus NCTC 5655 from: ○, CDM/4; ●, CDM/5; △, CDM/A; □, FIB; and ■, BHI

Fig. 3.31c: Effect of bactericidal substances from supernatant bacteria-free, and PMN's-free at 37°C and 4 rev/min on the survival of Staph. aureus NCTC 5655 from: ○, CDM/4; ●, CDM/5; △, CDM/A; □, FIB; and ■, BHI

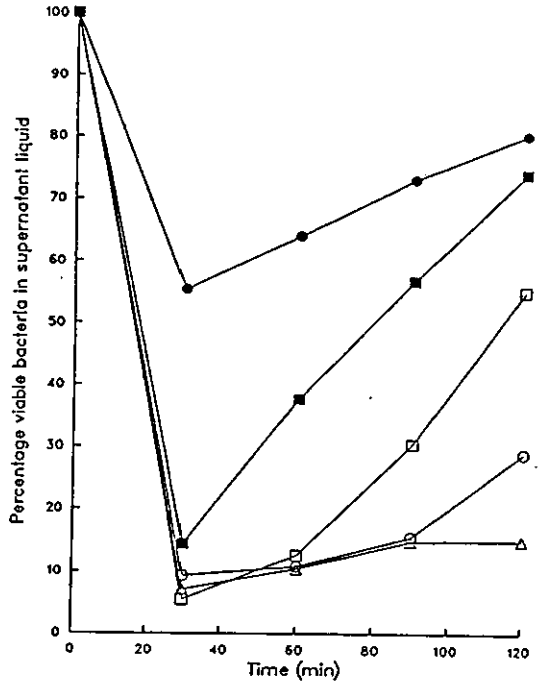


FIGURE 3.31a

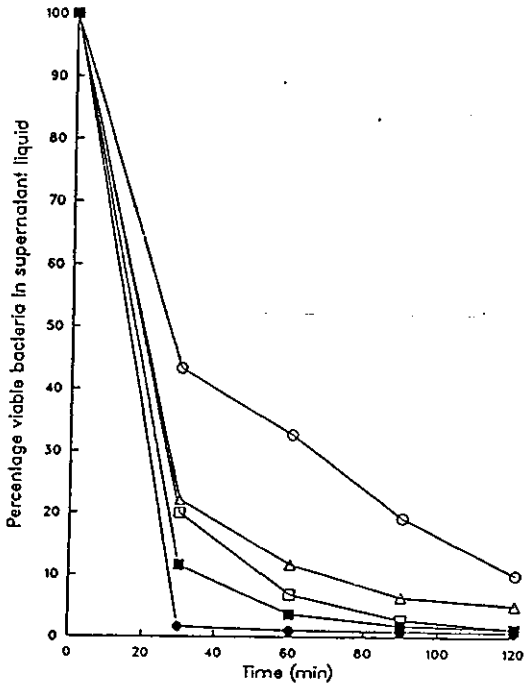


FIGURE 3.31b

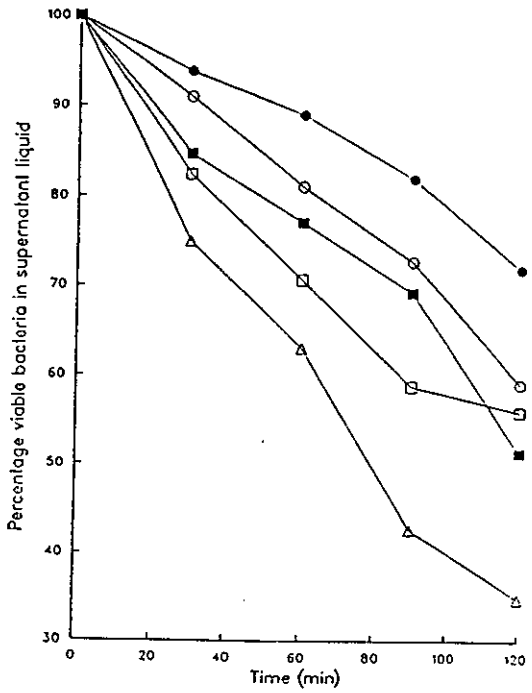


FIGURE 3.31c

and 95% for cells pre-incubated in CDM/5, BHI, CDM/4, CDM/A and FIB respectively. Afterwards the percentage viability increased (though less so for cells from CDM/A). However, the viability of Staph. aureus NCTC 5655 from CDM/5 was generally less affected by PMN's in comparison to the same strain grown in the other media which showed falls in viability of about 20, 26, 45, 71 and 85% from CDM/5, BHI, FIB, CDM/4 and CDM/A, respectively after 120 min.

The effect of 10% RNS in gel-HBSS on the survival of Staph. aureus NCTC 5655 during the incubation period (120 min) is shown in Figure 3.31b. The viability was reduced sharply during the first 30 min by about 57, 78, 80, 88 and 98% of Staph. aureus NCTC 5655 grown in CDM/4, CDM/A, FIB, BHI and CDM/5 respectively. The decline in viable bacteria gradually continued until 120 min, but still the NCTC 5655 from CDM/4 (the five amino acid medium) was the less sensitive to the 10% RNS in gel-HBSS than the same strain grown in other media (CDM/A, FIB, BHI and CDM/5).

The inhibitor substances from bacteria-free and PMN's-free supernatant liquids (which obtained after incubation of bacteria with PMN's in gel-HBSS containing 10% RNS for 120 min) were studied (Figure 3.31c), and it was found that Staph. aureus NCTC 5655 from CDM/5 was less susceptible to the bactericidal action than the same strain grown in the other media. Furthermore, CDM/A produced NCTC 5655 was more sensitive to the inhibitor substances than the other media.

v) Staph. epidermidis NCTC 7944

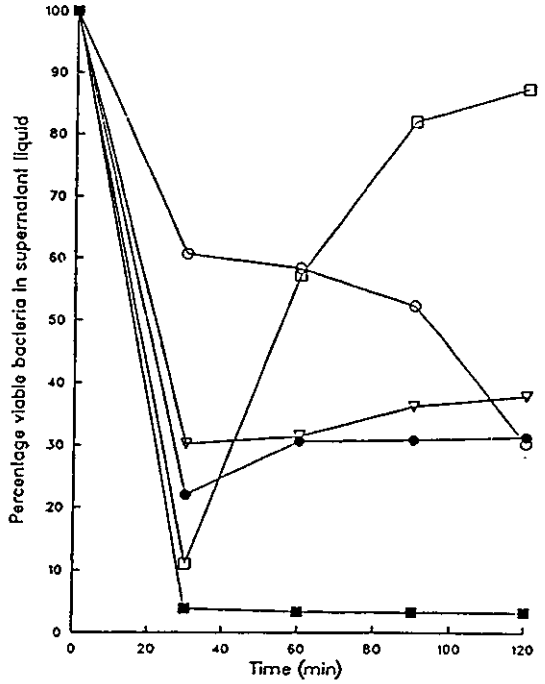
The phagocytosis of Staph. epidermidis NCTC 7944 cultivated in different media was varied and depended on the constitution of the initial cultivation media (Figure 3.32a). Extracellular viable counts of cells raised in BHI fell rapidly during the first 30 min of incubation by some 96% and remained at this low figure for 120 min. In contrast cells from FIB medium though initially (30 min) were reduced by about 89%, they then recovered to about 87% of initial number by the end of the incubation time (120 min). In

Fig. 3.32a: Phagocytosis by rabbit PMN's suspended in gel-HBSS with 10% RNS present at 37°C and 4 rev/min of Staph. epidermidis NCTC 7944 from: O, CDM/10; ●, CDM/11; ▽, CDM/A; □, FIB; and ■, BHI

Fig. 3.32b: Effect of 10% RNS in gel-HBSS at 37°C and 4 rev/min on the survival of Staph. epidermidis NCTC 7944 from: O, CDM/10; ●, CDM/11; ▽, CDM/A; □, FIB; and ■, BHI

Fig. 3.32c: Effect of bactericidal substances from supernatant bacteria-free, and PMN's-free at 37°C and 4 rev/min on the survival of Staph. epidermidis NCTC 7944 from: O, CDM/10; ●, CDM/11; ▽, CDM/A; □, FIB; and ■, BHI

Fig. 3.32d: Effect of carbohydrates in CDM/A on the survival of Staph. epidermidis NCTC 7944: (i) 1% glucose on: ▽, phagocytosis; O, effect of 10% RNS in gel-HBSS; □, the bactericidal substances of bacteria- and PMN's-free; (ii) 1% mannitol on: △, phagocytosis; ●, 10% RNS in gel-HBSS; and ■, bactericidal substances of bacteria-free and PMN's-free supernatant fluids following bacteria-PMN interaction



FIFURE 3.32a

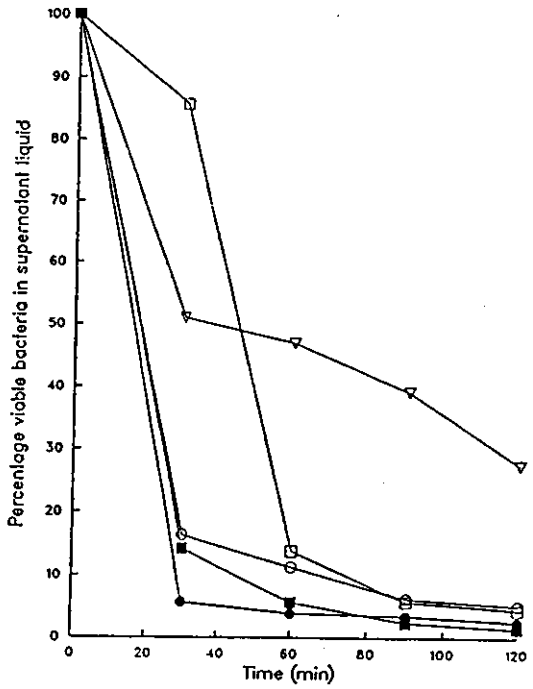


FIGURE 3.32b

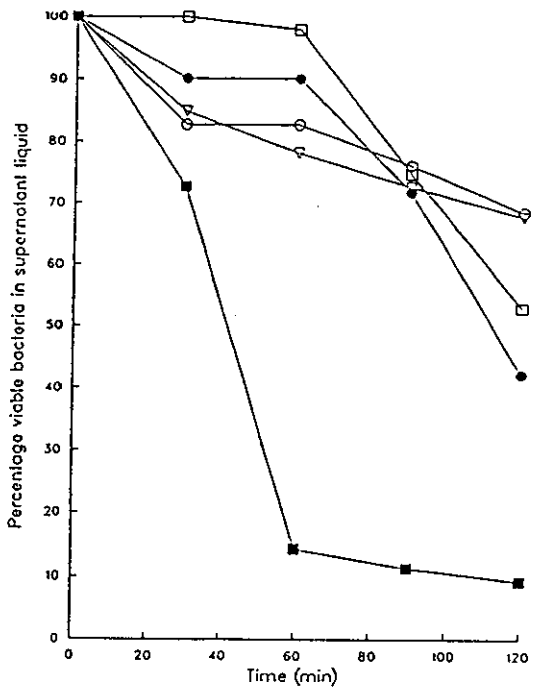


FIGURE 3.32c

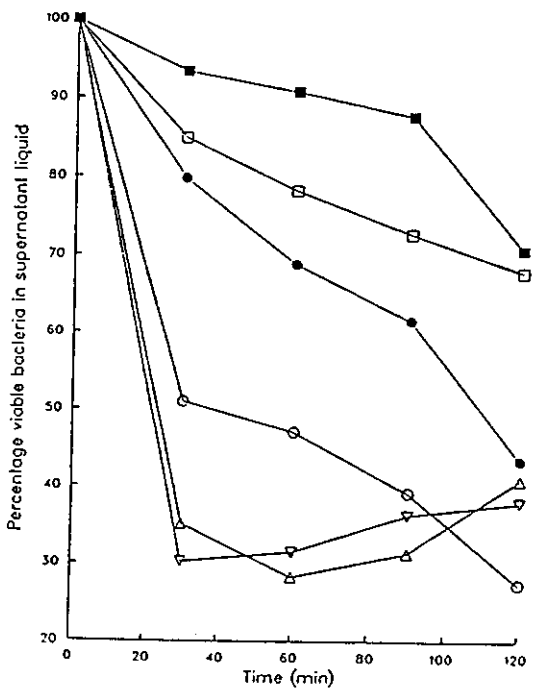


FIGURE 3.32d

chemically defined media both CDM/11 and CDM/A (see Table 3.6) viability profiles were, for CDM/A and CDM/11, similar to that obtained with cells from BHI but with less loss of viability (70 and 78% respectively). Cells from medium CDM/10 (containing five amino acids, see Table 3.6) were more resistant to PMN's than the same strain obtained from other media (BHI, CDM/11 and CDM/A). Overall, however, the extracellular number of NCTC 7944 from CDM/10 was eventually reduced in the presence of 10% RNS in gel-HBSS over the 120 min of incubation (Figure 3.32a) to reach a similar (final) phagocytosis index to that of CDM/11 and CDM/A.

Ten per cent RNS in gel-HBSS reduced the viability of Staph. epidermidis NCTC 7944 (obtained from different media) after 30 min of incubation by 94, 86, 84, 49 and 14% from CDM/11, BHI, CDM/10, CDM/A and FIB respectively (Figure 3.32b). Afterwards the killing continued but at a slower rate, except that there was a sharp decline in the viable numbers of Staph. epidermidis NCTC 7944 from FIB to reach an almost similar number to that from CDM/10, CDM/11 and BHI at 120 min.

Results presented in Figure 3.32c show a sharp reduction in the total viable numbers of Staph. epidermidis NCTC 7944 from BHI during the first 60 min of contact with the cell free supernatant liquids collected after phagocytosis of bacterial cells. About 86% of the bacteria were killed, whilst over the same incubation period cells cultivated in FIB were unaffected. Over the next hour, however, about a 47% reduction in viable count occurred. Cells initially grown in medium CDM/11 gave a similar survival profile to those from FIB. The viability of population of Staph. epidermidis NCTC 7944 raised in both medium CDM/10 and CDM/A was only slightly reduced (by approximately 30%) over the 120 min incubation period.

In all experiments above with Staph. epidermidis NCTC 7944, glucose was used as a carbon source in the basal chemically defined media (see 3.2.2 and Table 2.1). Changing the sugar carbon source from 1% of mannitol to 1% glucose in CDM/A only had a small effect on cell

survival (Figure 3.32d) except for serum killing and extracellular bactericides (supernatant liquids) which were more effective when glucose was used.

vi) Staph. aureus Wood 46

The phagocytosis in gel-HBSS by PMN's in the presence of 10% RNS of Staph. aureus Wood 46 obtained after cultivation for 18h in various media was determined (Figure 3.33a). Results after 30 min of incubation showed that 97, 90, 80, 65 and 41% of extracellular numbers were cleared when the cells were cultivated in BHI, FIB, CDM/8, CDM/7 and CDM/A, respectively. Subsequently in each case, except for CDM/A, the extracellular numbers remained at approximately the same level until 120 min. Cells cultivated initially in medium CDM/A were able to survive better and recovered to numbers approaching initial inoculum levels after 120 min of incubation.

The effect of 10% RNS on the survival of cells of Staph. aureus Wood 46 (initially grown in different media) in gel-HBSS was studied, and the results are represented in Figure 3.33b. During the first 30 min of incubation there was a sharp decrease in the bacterial growth, to 1, 16, 21, 40 and 49% of initial numbers for cells from CDM/8, FIB, CDM/A, CDM/7 and BHI respectively. However, cells from medium CDM/8 were virtually undetectable after 120 min contact. For cells from other media about 3, 6, 21 and 25% remained viable for 120 min (CDM/A, FIB, CDM/7 and BHI, respectively).

The effect of bactericidal substances from bacteria- and PMN's-free supernatant liquids (obtained after 120 min incubation of 1:1 bacteria-to-PMN's in the presence of 10% RNS in gel-HBSS), on the growth of Staph. aureus Wood 46 from different media is illustrated in Figure 3.33c. In general, cells from CDM/8 were rapidly destroyed during the first 30 min in which about 95% of the cells were killed. In contrast during the same period the total number decreased about 47 and 40% of Wood 46 from BHI and CDM/A respectively. Whilst, small, similar decreases in viability occurred

Fig. 3.33a: Phagocytosis by rabbit PMN's suspended in gel-HBSS with 10% RNS present at 37°C and 4 rev/min of Staph. aureus Wood 46 from: ○, CDM/7; ●, CDM/8; △, CDM/A; □, FIB; and ■, BHI.

Fig. 3.33b: Effect of 10% RNS in gel-HBSS at 37°C and 4 rev/min on the survival of Staph. aureus Wood 46 from: ○, CDM/7; ●, CDM/8; △, CDM/A; □, FIB; and ■, BHI.

Fig. 3.33c: Effect of bactericidal substances from supernatant bacteria-free, and PMN's-free at 37°C and 4 rev/min on the survival of Staph. aureus Wood 46 from: ○, CDM/7; ●, CDM/8; △, CDM/A; □, FIB; and ■, BHI.

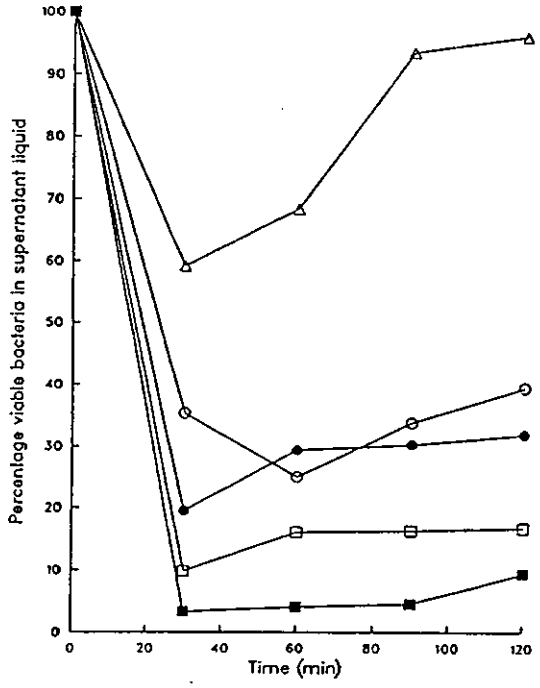


FIGURE 3.33a

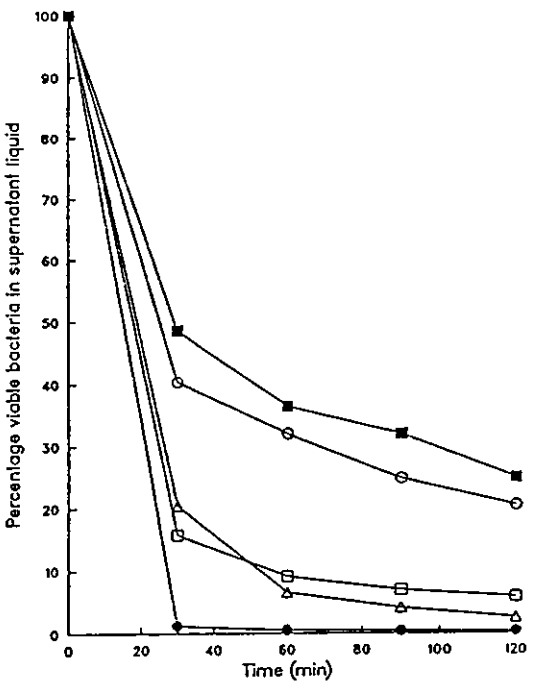


FIGURE 3.33b

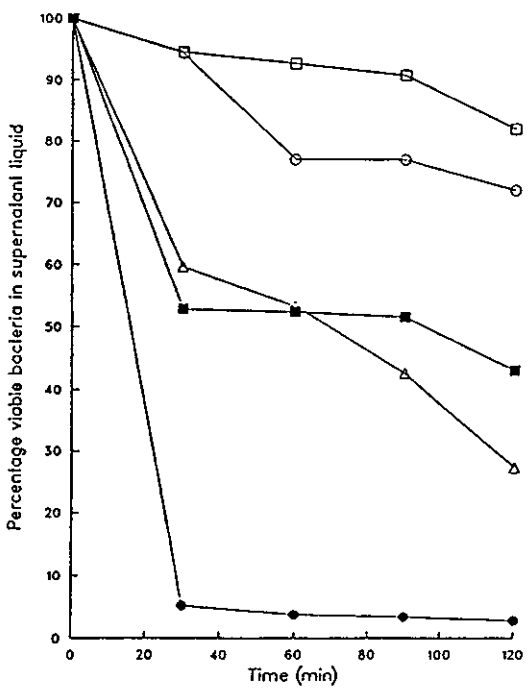


FIGURE 3.33c

with cells from CDM/7 and FIB during the first 30 min. Consequently killing by bactericidal substances on Staph. aureus Wood 46 continued gradually till 120 min, after which 97, 73, 57, 28 and 18% of strain Wood 46 cells from CDM/8, CDM/A, BHI, CDM/7 and FIB were destroyed respectively.

3.4.3 Additional Studies on Phagocytosis of Strain Wood 46

i) Amino acid concentration

The concentrations of the twelve amino acids present in CDM/8 were varied to give CDM/9 (see Table 3.6), and cells of Staph. aureus Wood 46 cultivated in both CDM/8 and CDM/9 were of similar sensitivity to 10% RNS in gel-HBSS (Figure 3.34) and only small variations in the phagocytosis index of Wood 46 from CDM/8 and CDM/9 were observed. However, cells incubated in CDM/9 were less susceptible to the effect of bactericidal substances (supernatant liquid from bacteria- and PMN's-free) than cells from CDM/8.

ii) Effects of normal (heated, unheated) and immune sera

The nature of the serum factors on the killing of staphylococci or supporting the phagocytosis was examined by subjecting normal serum to heating treatment, at 56°C for 30 min, before incorporating it in the assay. The phagocytosis of Staph. aureus Wood 46 from media CDM/7, CDM/8 and BHI was observed in the presence of 10% RNS or 10% rabbit heated serum (10% RHS) in gel-HBSS.

The ability of 10% rabbit serum to support the phagocytosis of cells from medium CDM/7 and CDM/8 was reduced markedly by heating and though phagocytosis took place it was followed (up to 120 min) by viability increases (Figure 3.35a). Whilst with cells of strain Wood 46 from BHI, only very small differences in the phagocytosis were observed with 10% RHS in comparison to that gained with 10% RNS.

Ten per cent RHS in gel-HBSS had less effect on the viability of Staph. aureus Wood 46 from all media (Figure 3.35b) in particular Wood 46 from CDM/8, in comparison to 10% RNS.

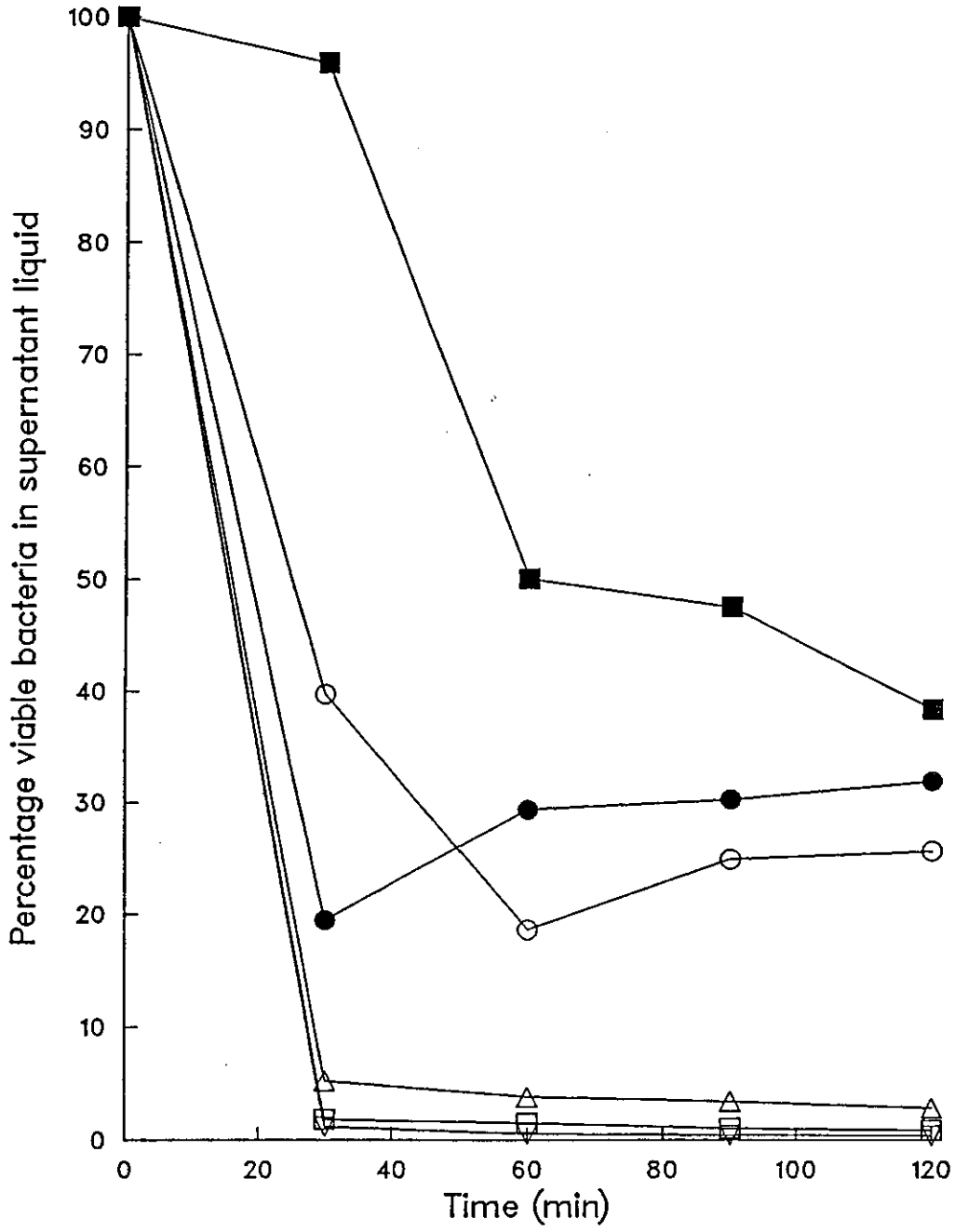


Fig. 3.34: Effect of varying amounts of 12-amino acids (CDM/8; CDM/9) used for the cultivation of Staph. aureus Wood 46; (i) CDM/8, on the: ●, phagocytosis; ▽, 10% RNS in gel-HBSS; △, bactericidal substances from bacteria-free and PMN's-free; (ii) CDM/9, on the O, phagocytosis; □, 10% RNS in gel-HBSS; and ■, the bactericidal substancesof bacteria-free and PMN's-free supernatant fluid following bacteria-PMN interaction

Fig. 3.35a: Phagocytosis by rabbit PMN's suspended in gel-HBSS at 37 °C and 4 rev/min in the presence of (i) 10% RNS; Staph. aureus Wood 46 from: O, CDM/7; ●, CDM/8; and ■, BHI; (ii) 10% RHS; Staph. aureus Wood 46 from: ▽, CDM/7; △, CDM/8; and □, BHI.

Fig. 3.35b: Effect of (i) 10% RNS in gel-HBSS at 37 °C and 4 rev/min on the survival of Staph. aureus Wood 46 from: O, CDM/7; ●, CDM/8; and ■, BHI; (ii) 10% RHS in gel-HBSS at 37 °C and 4 rev/min on the survival of Staph. aureus Wood 46 from: ▽, CDM/7; △ , CDM/8; and □, BHI.

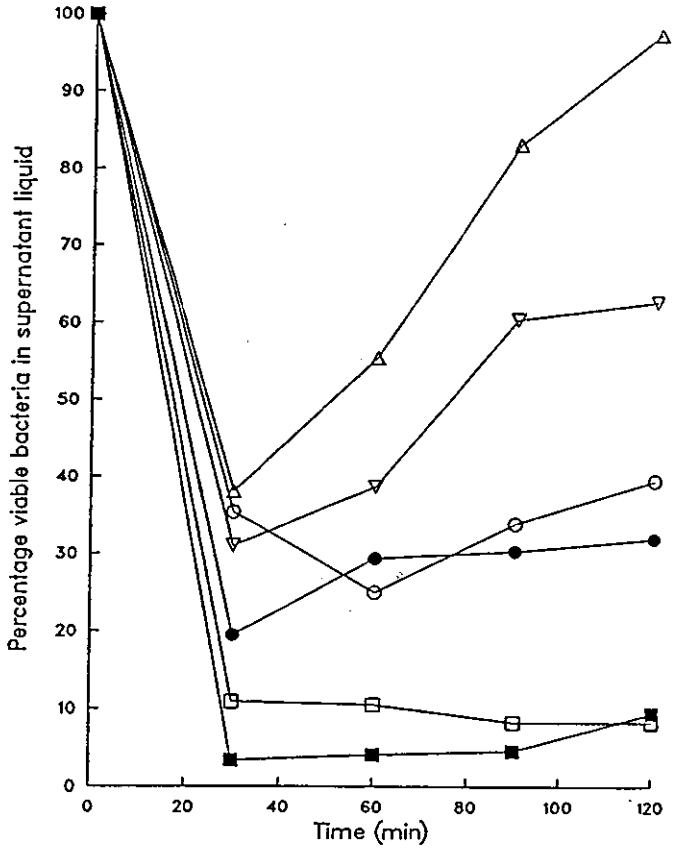


FIGURE 3.35a

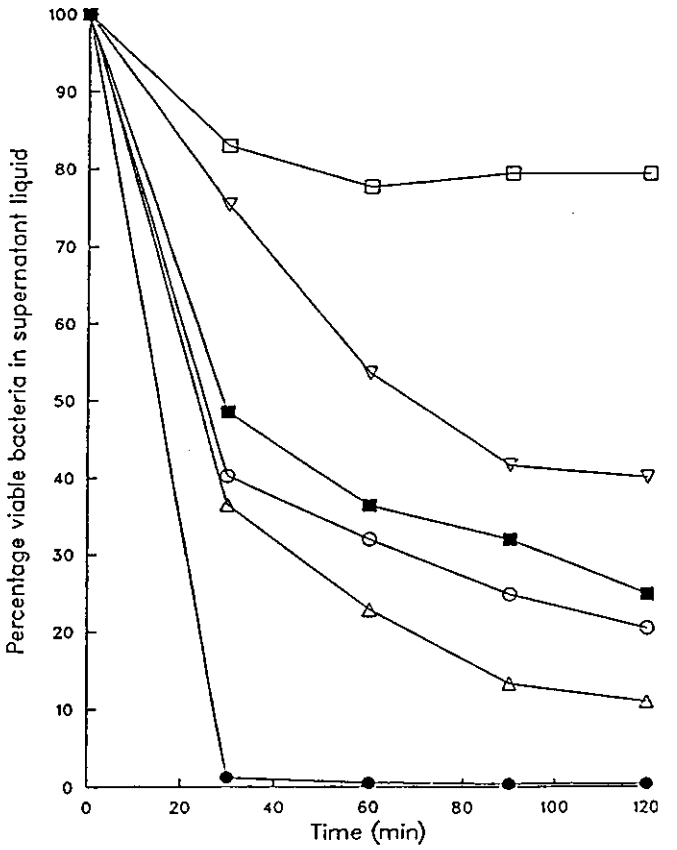


FIGURE 3.35b

Furthermore, data from preliminary experiments showed that immune rabbit sera of Staph. aureus Wood 46 (obtained by immunized rabbits with formalized non-encapsulated Staph. aureus Wood 46 grown in CDM/7, CDM/8 and BHI; see 2.11) was not significantly different from normal serum in supporting phagocytosis. Cells of strain Wood 46 from CDM/8 was, however, more sensitive to 10% immune sera than cells from CDM/7 and BHI.

iii) The Intracellular Killing Susceptibility to Rabbit's PMN

The intracellular killing by rabbit PMN's in the presence of 10% RNS in gel-HBSS of Staph. aureus Wood 46 grown for 18h in CDM/7, CDM/8 and BHI was studied. Results are illustrated in Figure 3.36 and show that the number of viable cells of Staph. aureus Wood 46 from different media found within PMN's decreased rapidly (about 62, 67 and 73% during the first 30 min of incubation from CDM/8, CDM/7 and BHI respectively). Afterwards the intracellular killing occurred at a slower rate in all until 120 min. Overall, the highest percentage of intracellular killing was demonstrated with Staph. aureus wood 46 which had been cultivated in BHI.

However, the intracellular killing of Staph. aureus Wood 46 from CDM/7, CDM/8 and BHI did not occur during incubation with rabbit PMN's at 4°C in a 10% RNS-gel-HBSS (as control experiments; Figure 3.36).

iv) Susceptibility to Bactericidal Substances from Rabbit's PMN's - Crude Extracts (PMNCE)

The results presented in Figure 3.37 show that the bactericidal action of PMN's crude extracts (PMNCE) on Staph. aureus Wood 46 from CDM/7, CDM/8 and BHI were varied. Cells from BHI were more sensitive (about 4 ∿ 5 fold) to killing by PMNCE than cells from CDM/7 and CDM/8, respectively, after 30 min of incubation. The killing continued and viable cells from BHI were not detectable completely during the second hour of incubation with PMNCE. For cells from CDM/8 and CDM/7 about 98 and 84% respectively were killed after 120 min.

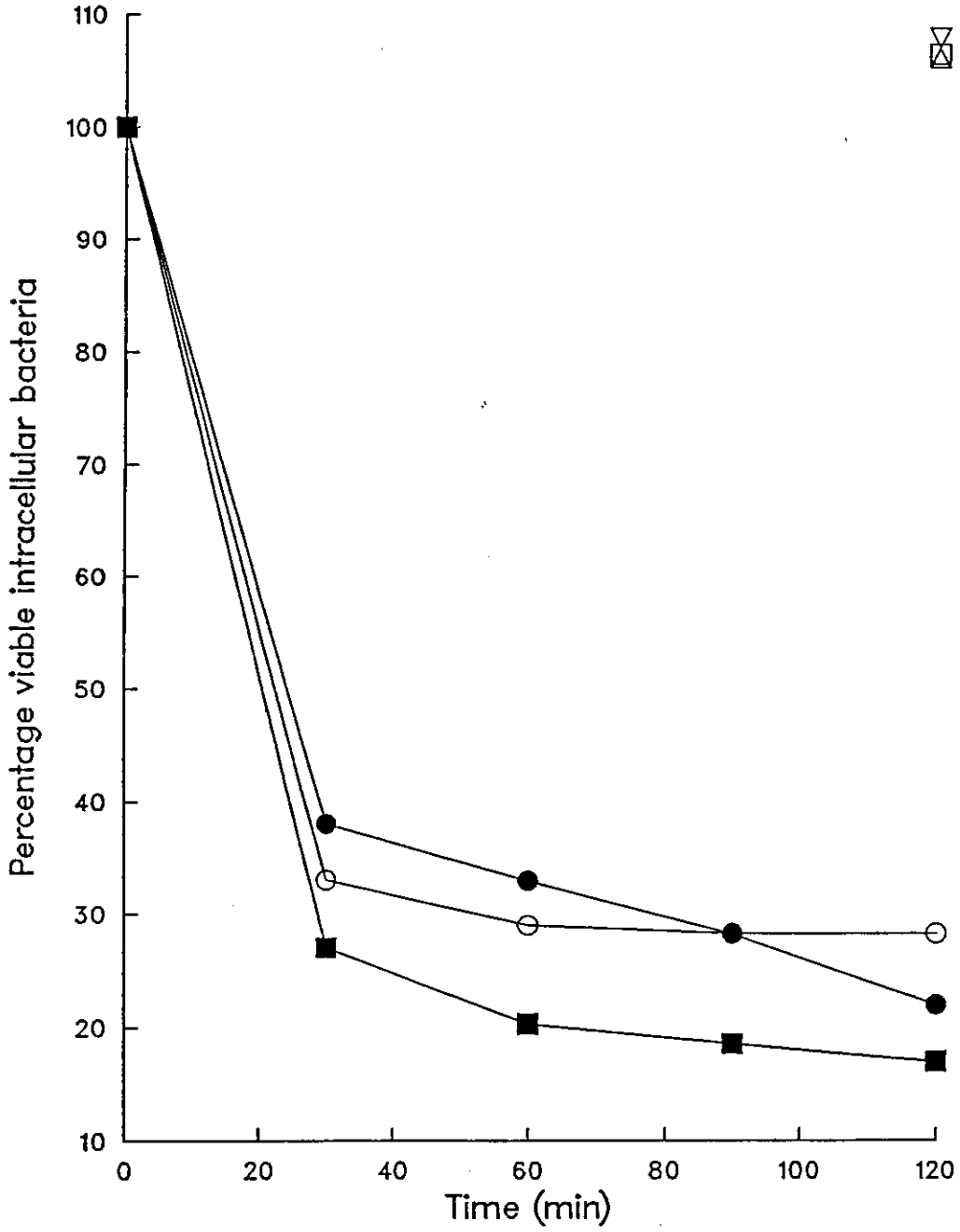


Fig. 3.36: Kinetics of intracellular-killing by rabbit PMN's with 10% RNS present in gel-HBSS at 37°C and 4 rev/min after 15 min phagocytosis, bacteria:cell ratio of 1:1 of Staph. aureus Wood 46 from: ○ , CDM/7; ●, CDM/8; and ■, BHI; or as above, but at 4°C unshaken (as control experiments) for Wood 46 from: ▽, CDM/7; △, CDM/8; and □, BHI.

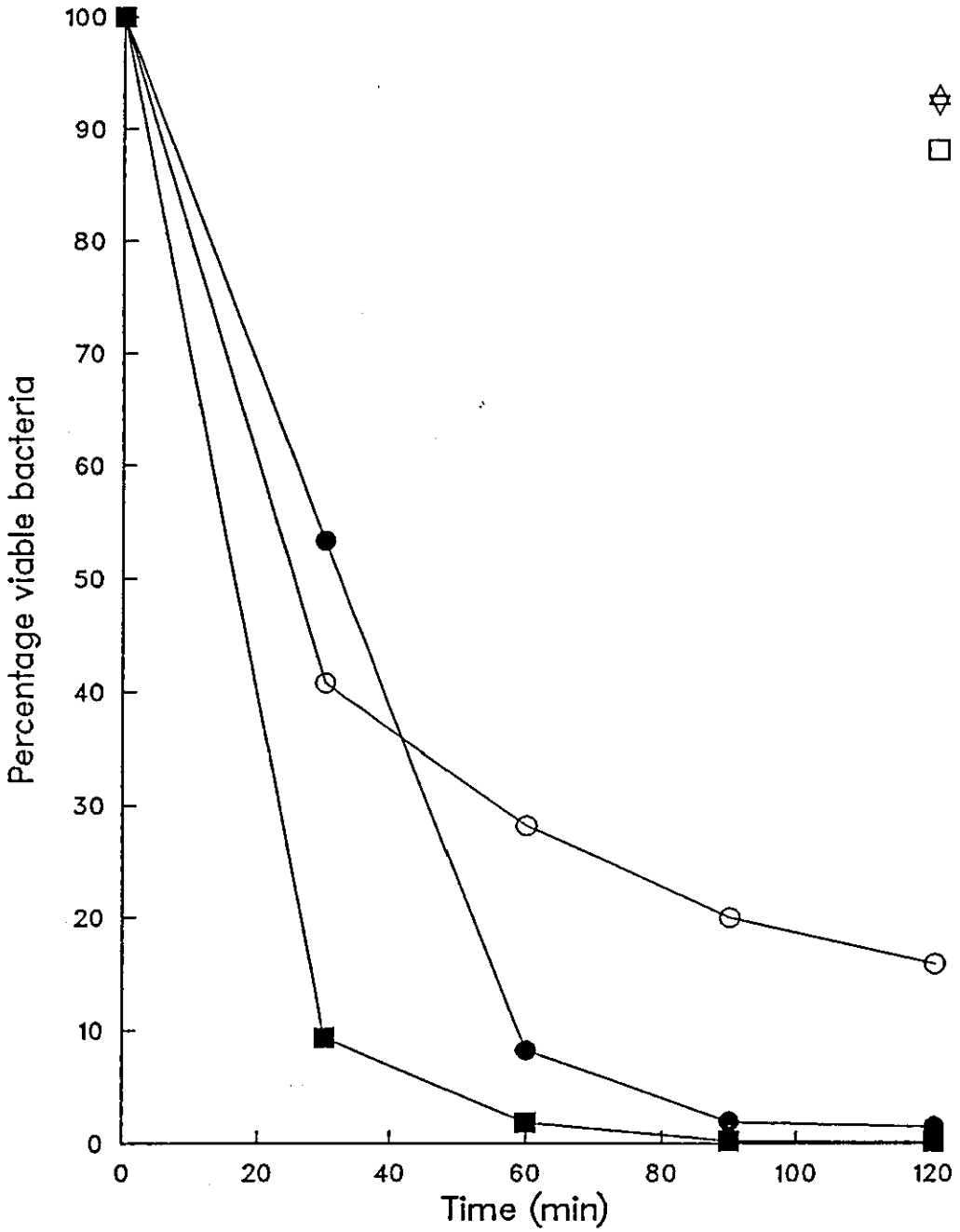


Fig. 3.37: Effect of 1-2 x 10^7/ml rabbit PMN's crude extracts (PMNCE) in gel-HBSS at 37°C and 4 rev/min on the survival of Staph. aureus Wood 46 from: ○ , CDM/7; ● , CDM/8; and ■ , BHI; or effect of gel-HBSS only at 37°C and 4 rev/min (as control experiments) for Wood 46 from: ▽ , CDM/7; △ , CDM/8; and □ , BHI.

Bacterial cells from various media in gel-HBSS only (as control) retained approximately 100% viability over 120 min incubation (Figure 3.37).

3.5 ANTIGENICITY OF Staph. aureus WOOD 46 GROWN IN DIFFERENT MEDIA

The antigenicity of Staph. aureus Wood 46 grown in media CDM/7, CDM/8 (see Table 3.6) and in BHI, was assessed using rabbit's antisera raised in rabbits by inoculation with cells of Staph. aureus Wood 46 cultured in CDM/7, CDM/8 and BHI (see 2.11).

Results obtained from double immunodiffusion in agar (Table 3.7), slide agglutination, and antiserum agar method (ASA; see 2.12), all indicate that Staph. aureus Wood 46 grown in chemically defined media (CDM/7 and CDM/8) or rich media (BHI) were of identical antigenicity (see Figure 3.38). This was supported by the cross-agglutination of the cellular antigens with various immune sera (see Table 3.7). However, different levels of sensitivity were noticed in that the immune serum prepared from Staph. aureus Wood 46 grown in BHI had rather high titres of cross-reacting antibodies with all, whereas, in others the titres were relatively low (Table 3.7).

Normal rabbit serum used as control gives negative reactions with all antigens produced by Staph. aureus Wood 46 in the CDM/7, CDM/8 and BHI (Table 3.7).

In contrast to the methods used above for the detection of antigens, the counter immunoelectrophoresis showed negative results with all.

TABLE 3.7: Cross-reaction of Staph. aureus Wood 46 cultivated in different media on double immunodiffusion in agar

Antigens of Strain Wood 46 from	Dilutions of Normal Serum				Dilutions of antiserum strain Wood 46 from											
					CDM/7				CDM/8				BHI			
	0	1	2	4	0	1	2	4	0	1	2	4	0	1	2	4
CDM/7	N	N	N	N	S	S	S	N	S	S	S	N	S	S	S	W
CDM/8	N	N	N	N	S	S	S	N	S	S	S	N	S	S	S	W
BHI	N	N	N	N	S	S	S	N	S	S	S	N	S	S	S	W

Titres given as reciprocal values; N, no detectable cross-reacting; W, weak cross-reacting; and S, strong cross-reacting

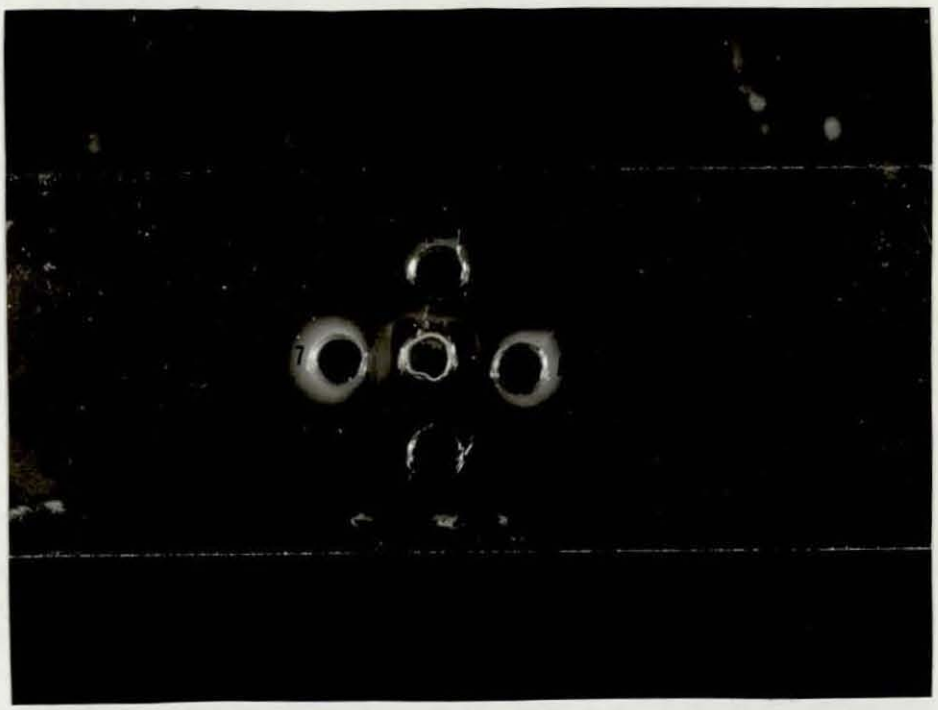


FIGURE 3.38: Agar-double immunodiffusion pattern demonstrating the identical cross-agglutination of antigen (live Staph. aureus Wood 46 from CDM/7) to different antiserum strain Wood 46
Footnote: NS, normal rabbit serum; A, antigen; ANT, antisera for strain Wood 46 grown in CDM/7, CDM/8 and BHI

CHAPTER FOUR
DISCUSSION AND CONCLUSION

4.1 CHARACTERISTICS OF SIX STAPHYLOCOCCI STRAINS

Colonies of Staph. aureus are usually golden-yellow but may be pale yellow or fawn, or even completely white as with Staph. aureus Wood 46 (see Table 3.1) with which there is no pigment produced. Staph. aureus NCTC 8532 grown in chemically defined media (CDM/1 and CDM/A) produced a golden-yellow pigment, whilst it gave a white to pale yellow pigment on BHI-agar. This shows that pigment formation in some Staph. aureus strains is media dependent.

Both coagulase positive and negative staphylococci do not produce acid from xylose (Table 3.1). This supports the recent edition of Bergey's Manual of Systematic Bacteriology (Kloos and Schleifer, 1986; see Table 1.2). Staph. epidermidis NCTC 7944, however, produced weak acid from mannitol under both aerobic and anaerobic conditions. Thus there is some evidence, as outlined by Fraser (1964) that certain coagulase-negative staphylococci (dog strains) are capable of producing acid from mannitol. Similarly Baird-Parker (1965) reported that Staph. epidermidis type 5 is recognised by its ability to produce acid from mannitol aerobically and that some members of this subgroup will also produce acid from mannitol anaerobically. However, these organisms are well separated from Staph. aureus as neither phosphatase nor coagulase are produced.

Acetoin can be detected in lipid containing media, in particular, tributyrin with Staph. aureus only. This may be due to the ability of Staph. aureus to use glycerol tributyrate to form acetoin as the end products. There is, however, no direct relationship between lipase activity and acetoin production (see Table 3.4). On the other hand there is considerable limitation of lipolytic activity with lipids having a chain length greater than C_8 (Table 3.4). Thus it has been reported that most staphylococci produce lipolytic

enzymes capable of hydrolyzing only simple fats and other esters (Stewart, 1965). Moreover, the growth of Staph. aureus NCIB 8625 was reduced in the presence of lipids triacetin and triolein, whilst Staph. aureus NCTC 7428, C. renale NCTC 7448 and Ps. aeruginosa NCTC 7244 did not grow in the presence of lipid triacetin. The growth of Staph. aureus NCTC 7428 was abolished by triolein (Table 3.4). Differences in the susceptibility of lipids to different strains of Staph. aureus were seen and there were considerable differences in lipase activity and acetoin production in the presence of the different lipids.

Lipase production may also be medium dependent since Staph. aureus NCTC 7428 produced lipase in tributyrin-agar (Oxoid) but not in CDM/A (see Tables 3.1 and 3.2). Selective lipolytic activity and concurrent acetoin production, however, may be a more reliable property of staphylococci than clumping factor, pigment formation, caseolysis, or haemolysin production (see Tables 3.1 and 3.3). Concurrent lipolysis and acetoin production from lipids should, therefore, be considered for inclusion in part of the classification of staphylococci.

Not all of the five strains of Staph. aureus formed "clumping-factor" (also called bound-coagulase or plasma-agglutination-factor), but all produced "free-coagulase". This suggests that the "free-coagulase" test may be more reliable than the "clumping-factor" tests. The slide test for coagulase is fairly reliable, although false negative reactions occasionally occur.

Staph. aureus NCTC 7428 (beta-haemolysin producer strain) and Staph. epidermidis NCTC 7944 were both egg-yolk negative (see Table 3.1), regardless of whether 1% glucose was present or not. Not all Staph. aureus that produced beta-lysin are lecithinase positive (Gillespie and Alder, 1952), the staphylococcal phospholipase present in the beta-haemolysin fraction acting only on sphingomyelin and not on lecithin (Gulasekharam, Cheyne, et al, 1963; see Figure 1.3). However, it is of interest to note that the distribution of the

opacity-producing property (lecithinase enzyme) in some staphylococci strains (Staph. aureus NCTC 7428 and Staph. epidermidis NCTC 7944) did not correspond with their production of lipase enzyme (Table 3.1). Thus the lipase enzyme may not be implicated in this phenomenon. This agrees with Owens (1974), and Owens and John (1975), who showed that general lipolytic enzymes were not involved in the egg-yolk phenomenon. Therefore, it may be concluded that the egg-yolk reaction should refer only to the hydrolytic degradation of phosphatidyl choline "lecithin" (Owens, 1974).

On the other hand, in the present study the presence of 1% glucose in the egg-yolk agar reduced the opaque zones (lecithinase activity) around the region of Staph. aureus strains (Table 3.1), whilst Gillespie and Alder (1952), and Owens and John (1975) reported that 1% glucose enhanced the turbidity due to the growth of Staph. aureus in egg-yolk broth media. This could of course simply reflect the properties of the different staphylococci strains used and variation in culture conditions.

However, it was found that all strains of lecithinase-positive Staph. aureus grown on egg-yolk agar without adding 1% glucose produced distinctive colonies with a "star-like shape" (see Figure 3.3). This phenomenon was not seen in the presence of 1% glucose, or with lecithinase-negative strains of staphylococci.

In the present study casein was used as an indicator of proteolytic activity and the results have shown (Table 3.1) that only Staph. aureus strains Wood 46 and NCTC 5655 and Staph. epidermidis NCTC 7944 were caseolases positive, whilst all six strains hydrolysed gelatin. This would tend to support the suggestion that the possession of a gelatinase is not a true indication of proteolytic activity (Cowan and Steel, 1974).

Both coagulase-positive and negative staphylococci produced heat-labile DNase (see Table 3.1) at various temperatures, though only small zones of DNase activity were detected with Staph. epidermidis NCTC 7944 in comparison with those produced by the Staph. aureus strains. Only Staph. aureus strains were shown to produce a thermostable nuclease (TNase; see Table 3.1), supporting the observation that TNase are produced by all coagulase-positive strains but less frequently by coagulase-negative (Gemmell, 1983; Table 1.2).

Furthermore, the modified method for TNase-assay used in this study gave positive results with all five Staph. aureus strains (see Table 3.1 and Figure 3.2), though negative results were obtained with all five strains of Staph. aureus when the method described by Bennett (1984) was used.

Gladstone (1937) reported that Staph. aureus did not produce coagulase in chemically defined media. However, in the present study all staphylococci grown in chemically defined medium CDM/A produced lipase (except Staph. aureus NCTC 7428) and catalase, and all Staph. aureus strains produced "free-coagulase" (except for strain NCTC 7428) and phosphatase (Table 3.2). Only Staph. aureus strains Wood 46 and NCTC 5655 and Staph. epidermidis NCTC 7944 produced proteinases. However, DNase activity was abolished and acetoin production from tributyrin was reduced. Finally acetoin may be produced from mannitol (Table 3.2) in CDM/A.

Methicillin-sensitive (MS) Staph. aureus strains had MIC values from 1 to 4 µg/ml at 37°C (Lukaszkiewicz and Chojnowski, 1985). All six staphylococci grown in BHI-agar in the present study had a degree of methicillin-resistant (MR), with MIC values ranging from 7.62 µg/ml for Staph. epidermidis NCTC 7944 to 38.30 µg/ml for Staph. aureus Wood 46. Staphylococci grown in CDM/A-agar were, however, more sensitive to methicillin than in BHI-agar. This may reflect a shift in extracellular enzyme production in the different media.

Overall it seems that the biochemical activities of the staphylococci are strain and medium dependent, though acetoin production (from glucose, mannitol and tributyrin), free-coagulase, phosphatase and TNase are fairly reliable tests for the characteristic differentiation between Staph. aureus and Staph. epidermidis.

Haemolysin, DNase, proteinase and lipase enzymes are produced by coagulase-negative Staph. epidermidis NCTC 7944. These exoproteins of coaglase-negative staphylococci have been taken by their possible pathogenicity in infections of man and animals (Gemmell and Schumacher-Perdreau, 1986).

4.2 GROWTH AND HAEMOLYSIN PRODUCTION OF STAPHYLOCOCCI

4.2.1 General Growth Requirements

All six staphylococci grew with good yield in chemically defined medium containing 18 amino acids (CDM). The method used to develop the chemically defined medium described in this study gives the basic amino acids required for the growth of each of the six strains. Single deletion from CDM showed that these organisms are auxotrophic for cysteine supporting the observations of Giehl, et al, (1987) for Staph. aureus (M and ENDO strains). Cysteine gave similar growth of Staph. aureus to cystine (Figure 3.9a), agreeing with the observations of Fildes and Richardson (1937).

The growth of all six strains was stimulated by the inclusion of methionine (Table 3.5). It was reported that methionine, as a potential source of -SH group, only poorly encourages the growth of Staph. aureus, but has an accelerating influence on growth in media containing cystine (Fildes and Richardson, 1937). These results indicate that the growth of staphylococci require an organic source of sulphur and that although cysteine could not be substituted by methionine, the reverse was possible. Valine was essential for the five strains of Staph. aureus with a less widespread dependency on proline (Sharma and Haque, 1973; Guirard, 1974). According to the

results in Table 3.5, it appears that strain (as well as species) specific differences in amino acid requirements occur amongst the six staphylococci supporting the results of Gladstone (1937) with strains of Staph. aureus.

Though media supplemented with only 5 or 6 amino acids were developed for some strains, these media were specific to individual organisms. Generally, the simple combination of those amino acids defined as essential or required, using the single deletion approach from a medium containing large numbers of amino acids, did not automatically give rise to nutritionally adequate media. More fastidious staphylococci still required supplementation and the only defined medium giving adequate growth, facilitating comparison of all six staphylococci, containing 13 amino acids (CDM/A) were derived from Staph. aureus NCTC 7428.

Nine amino acids (cysteine, proline, valine, arginine, threonine, phenylalanine, methionine, histidine and leucine) were generally present in the range of 10 to 13 amino acid containing media (see Table 3.6) used for all six staphylococci strains, though histidine and leucine were not included in the 12 amino acid medium, CDM/1 for Staph. aureus NCTC 8532. Therefore it appears that the six staphylococci strains differed significantly in their media requirements for only 8 amino acids. They are: isoleucine, aspartic acid, tryptophan, glycine, lysine, serine, glutamic acid and tyrosine (see Table 3.6). None of the six strains of staphylococci had a requirement for alanine to be included in their media.

Disputes over the amino acid requirements of staphylococci are common in the literature and Gladstone (1937) reported that the eight most important kinds of amino acid for rapid growth of Staph. aureus strains were cystine, proline, valine, arginine, phenylalanine, leucine, glycine and aspartic acid, while Yamaguchi and Kurokawa (1972) found that all staphylococcal strains required only five different amino acids, namely, cysteine, proline, valine, glycine and aspartic acid. On the other hand, Tschape (1973) has

shown that only cysteine, proline and arginine are essential for all Staph. aureus strains to grow in minimal media, in addition to which valine, histidine, glycine and isoleucine enhanced the growth of different Staph. aureus strains.

In the present work, only the growth of coagulase-negative Staph. epidermidis NCTC 7494 had a requirement for glutamic acid (see Table 3.5), and increasing the concentration of glutamic acid to 14 mg/100 ml in chemically defined media (CDM/10 and CDM/11; see Table 3.6) enhanced the overall growth rate. A similar observation has been made for the growth of Staph. aureus S-6 on glutamic acid as the main carbon source in a defined medium (Mah, et al, 1967).

It would appear that as a collective group, the staphylococci have a few essential amino acids in common. Beyond this, other requirements are species and strain dependent and may also be affected by other media constituents.

The basal chemically defined media contains several salts which were included (see Table 2.1). Magnesium and iron are required for the best yields of alpha-haemolysin production (Gladstone, 1938) and small amounts of potassium ions may be necessary for haemolysin appearance (Walbum, 1922). Ammonia was used as a basic source of nitrogen (Gladstone, 1937). The vitamins (thiamine, nicotinic acid and biotin) are essential for the growth of staphylococci (Cove, et al, 1980), although in the present study the growth of Staph. epidermidis NCTC 7944 showed complete biotin-independence (see Figure 3.22). This is not, however, a general characteristic of other coagulase-negative strains of Staph. epidermidis which have been shown to be separable from strains of Staph. aureus by their biotin-requirement (Gretler, Mucciolo, et al, 1955; Cove, et al, 1980).

The carbohydrates (glucose or mannitol) were used as primary carbon sources in the basal medium. One per cent glucose gives slightly better growth of Staph. epidermidis NCTC 7944 (see Figure 3.9b) than

1% mannitol, whilst the growth rate of coagulase-positive Staph. aureus NCIB 8625 was shown to be almost the same in the presence of 1% mannitol or glucose (see Figure 3.9a).

4.2.2 Haemolytic Activity

All six staphylococci studied had inherent haemolytic activity. However, the type of haemolysin produced was strain dependent and the ability to demonstrate its presence, depended on both medium composition and the species origin of the blood used. Even coagulase-negative Staph. epidermidis NCTC 7944 can produce medium dependent, non-species specific haemolysis of the delta type (BHI; see Table 3.3). The production of a haemolysin by Staph. epidermidis in a chemically defined medium has not been reported before and in the present study was only observed as small, sharp zones with rabbit blood agars (see Table 3.3). For all staphylococci on solid media, the largest zones were obtained in BHI based blood agars (Table 3.3).

Rabbit erythrocytes were extremely sensitive to alpha-haemolysin and sheep blood gave better detection of beta-haemolysin than any other type of blood used. Human blood was particularly insensitive to the haemolysins produced by all strains except for NCIB 8625 (BHI base; Table 3.3) which is probably not of human origin (Cowan et al, 1954). Guinea pig blood was insensitive to the haemolysins of Staph. aureus strain NCTC 7428 which, though normally considered to be a pure beta haemolysin producer, haemolysed horse blood cells on BHI-agar after 24 hr at 37^{o}C. Overall, the results depicted in Table 3.3 support the general use of sheep blood/BHI-agars in the general detcetion of staphylococcal haemolysins, if only blood from a single species is to be used.

Small batch to batch variations in the sensitivity of bloods to haemolysins were also noted as reported by Bernheimer (1965). EVen prewashing blood cells free from serum prior to inclusion in media may influence detection sensitivity. The maintenance of organisms on blood rich media contributes to the titre. However, since

haemolysins are still produced in liquid media containing no blood, it seems that once induced, haemolysin formation capability is not quickly lost (Gladstone, 1938). Pronounced variation has been observed among different strains of Staph. aureus in the ability to produce haemolysin in different media. Strain Wood 46 was superior to the other strains in the production of alpha-haemolysin. Different strains of the same bacterial species vary in their ability to produce toxin in vitro (Arbuthnott, 1978).

Highest yields of cells and haemolysins were produced in BHI despite the presence of glucose, which has been shown to suppress the production of alpha-haemolysin and other exoproteins in defined media (Duncan and Cho, 1972; Totake, 1979; Coleman, 1983; Taylor and Holland, 1988). Yields of beta-lysin in a heart infusion broth were reported by Sharma and Haque (1973) to be less than those obtained in various defined media. This was, however, after 24h incubation. In the present study, beta-lysin production in BHI was at least 8-fold greater than that detected in media CDM and CDM/A at 24h, after which it increased, eventually exceeding defined media levels by some 1000 HU50/ml (see Figures 3.10 and 3.19). The inclusion of proteose peptone in BHI may be significant in haemolysin production (Jassim, et al, 1988). The high levels of haemolysin, held in close proximity to the growing colonies on solid media, may account for the more efficient detection of staphylococcal haemolysis on BHI based blood agars.

In chemically defined media, the replacement of glucose with mannitol did not significantly interfere with overall cellular yields of Staph. aureus (see Figure 3.9a) but permitted haemolysin production at detectable levels, even for coagulase-negative Staph. epidermidis NCTC 7944 (Table 3.3). In a comparative study of toxin production in media based on glucose with those containing other carbon sources, Duncan and Cho (1972) reported that higher haemolysin yields were obtained in the presence of lactose, suggesting that haemolysin production involves secondary metabolism.

Taylor and Holland (1988) considered that another exoprotein, toxic shock syndrome toxin 1 (TSST-1), was synthesised during the secondary phase of diauxic growth, and Abbas-Ali and Coleman (1977) and Coleman (1981) have presented evidence of a common regulatory mechanism for extracellular protein formation. Mathieu, de Repentigny, et al, (1968) however, have reported that in a thymine requiring mutant of Staph. aureus Wood 46 alpha-toxin production required normal DNA replication, although other toxins were still produced during thymineless death.

Similarly Duncan and Cho (1972) showed that most extracellular proteins continue to be synthesised during the transient repression of alpha-haemolysin. Certain haemolysin production generally became detectable and achieved high titres during the later stages of exponential growth (see for example, Figures 3.7 and 3.10; 3.8, 3.12 and 3.13; 3.18 and 3.19), as reported by Turner (1978b) and Mollby (1983) for delta-lysin; Low and Freer (1977a,b) for beta-lysin; or Duncan and Cho (1971) and McNiven and Arbuthnott (1972) for alpha-lysin. However, the synthesis of most extracellular proteins is induced when the bacterial growth becomes nutrient limited at the end of the exponential growth phase and the reason for alpha-lysin preferential synthesis during the nutrient limiting conditions is less obvious (Arvidson, 1983). Though prolonged incubation, however, into the stationary phase reduced yields in chemically defined media. Similar losses of haemolysin were noted by Gladstone (1938) in agitated cultures (heavily shaken or gas sparged), and may relate to extracellular proteolytic activity. However, in this context, it is interesting to note that strain Wood 46 (caseinase and gelatinase positive; see Tables 3.1 and 3.2) had no direct effect on haemoglobin except in the presence of (initially) intact blood cells.

In this study Staph. aureus has been shown to be capable of producing haemolysin in medium containing only 0.1% yeast extract in PBS (YEBS) in which similar yields to those produced in chemically defined media containing 12 to 18 amino acids were obtained, except

for Staph. aureus Wood 46 which gives high titre of alpha-haemolysin in CDM, CDM/8 and CDM/9 (Figure 3.13), in comparison to that produced in 0.1% YE in PBS. It has been reported that yeast extract is required for both high yields of cells and for the production of extracellular proteins of Staph. aureus (Arvidson, et al, 1971).

Yeast extract has been employed to increase alpha-toxin yields (Dalen 1973a). This appears to be the case with alpha-lysin production (Figures 3.13 and 3.15), but beta haemolysin production was suppressed (Figure 3.19). A similar suppression by a yeast extract diffusate has been reported for delta haemolysin (Turner 1978b). These observations clearly indicate that the conditions for the beta-haemolysin production are different and it appears that 0.1% YE supplemented in chemically defined media enhances Staph. aureus alpha-haemolysin production but is antagonistic for the production of beta-haemolysin.

Dalen (1973b,c) suggested a role for histidine in the action of yeast extract in alpha-lysin production and, in the present study, using Staph. aureus Wood 46, the removal of histidine from a 5 amino acid containing chemically defined medium (CDM/6; see Table 3.6), though still permitting growth (see Figure 3.11), inhibited alpha-haemolysin production. However, increasing the concentration of histidine four-fold (14 mg/100 ml) in CDM/7 (five amino acid medium) did not influence the growth rate or the level of alpha-haemolysin (see Figures 3.12 and 3.13). This agreed with the observation of Gladstone (1938) and Miller and Fung (1973) that increasing the concentration of an amino acid has no influence on increasing of the alpha-haemolysin or enterotoxin B production of Staph. aureus in chemically defined media.

Leboeuf-Trudeau, de Repentigny, et al, (1969) suggested that tryptophan was required for alpha-haemolysin production by Staph. aureus Wood 46 in media based on casamino acids. In the present study, this was not the case in a 5 amino acid medium (CDM/7; Table

3.6) in which alpha-lysin was detected in the absence of tryptophan (Figure 3.13). In more complex chemically defined media (containing 12 to 13 amino acid) variations in amino acid composition and concentration (including histidine) varied maximum alpha-haemolysin titre. Complex feedback control mechanisms will operate in media containing a wide range of amino acids and the significance of such control in haemolysin production requires further investigation (Coleman, 1981).

In general the results from this study have shown that the addition of amino acid not required for the growth of Staph. aureus strains to chemically defined media reduced the haemolysin formation. For example, alpha-lysin production of Staph. aureus Wood 46 was reduced more than six fold (Figure 3.13) when unrequired amino acids (alanine, aspartic acid and lysine; see Tables 3.5 and 3.6) were used in Dalen-CDM. Similarly, the addition of the three unrequired amino acids (lysine, serine and tyrosine; see Table 3.5) for the growth of Staph. aureus NCTC 5655 to ten amino acid medium (CDM/5) as presented in CDM/A (see Table 3.6) resulted in a similar growth pattern (see Figure 3.14), but reduced the alpha-lysin production markedly (Figure 3.15). This supports the observations of Arbuthnott (1970) that it is possible to obtain good growth of normally toxigenic staphylococci with little or no alpha-lysin formation. Similarly Gladstone (1938) has reported that alpha-haemolysin production is sensitive to alterations in the composition of the chemically defined media and to the conditions of the growth.

In general, the results indicate that the production of staphylococcal haemolysins is largely dependent on the range of amino acids required for the growth of each individual strain and that this must be determined for each individual strain if maximal yields are to be obtained. As the number of amino acids was reduced from 18 to 5 there were overall reductions in the growth rate, as well as alpha-haemolysin production of Staph. aureus. This indicates that even though the Staph. aureus may be able to synthesise the omitted supplemental amino acid, the biosynthetic processes were

slow and became the rate-limiting factor in growth and alpha-haemolysin production.

Haemolysin formation, however, is not necessarily related directly to the growth rate and this, in general, agrees with many previous studies (Duncan and Cho, 1971 and 1972; Abbas-Ali and Coleman, 1977; Coleman and Abbas-Ali, 1977; Totake and Ichikawa, 1983) in which an inverse correlation was observed between the differential rates of haemolysin production and growth rate of Staph. aureus.

The combination of haemolytic activity with the brown colouration observed with Staph. aureus NCTC 8532 growing on blood agar based on the chemically defined medium (CDM/A; see Table 3.3) has not been reported previously for staphylococci. This strain has been designated an alpha-haemolysin producer (Cowan, et al, 1954) but the relative insensitivity of rabbit compared to sheep blood would not appear to support this (Table 3.3). Overall, it is not zeta-haemolysin described by Fraser (1964) since zeta-lysin produced "hot-cold" effects on ruminant and rabbit blood. Nor is it the "new haemolysin" reported by Turner and Pickard (1980) since no opaque zone of lysis occurred on horse blood agar with Staph. aureus NCTC 8532. These observations reported here may, therefore, be indicative of a new, medium dependent, type of haemolytic activity.

The production of a brown colouration around colonies has been observed with Clostridium botulinum growing on horse blood agar (Wilson and Miles, 1975). Similar, though smaller, brown areas (acid haemin, haematin, or other iron complexes) can be produced at extreme pH values (e.g. <4 or >9) on blood agars in the absence of organisms, but in liquid culture, Staph. aureus NCTC 8532 remained within the pH range 7.4 to 5.0 throughout incubation. Strain Wood 46, which often achieved pH 4 (Figure 3.11) following prolonged incubation always produced haemolysis of the classic alpha lysin-type and, unlike strain NCTC 8532, did not cause colour shifts in haemoglobin plates.

A comparison of the spectrum for a standard solution of methaemoglobin with that for the material extracted from the agar surrounding colonies of Staph. aureus NCTC 8532 on blood agar plates (Figure 3.5) suggests that the cause of this colour shift is the conversion of haemoglobin to methaemoglobin, Fe(II) to Fe(III), in which the iron is still loosely attached to the "haem pocket". This is supported by the degree of reversibility (sodium dithionate) of the colour change. Methaemoglobin has been noted as appearing in the blood in increased amounts in cases of severe sepsis (Carter, Coxon, et al, 1959). In the present study, however, this effect was not observed in human blood agar plates nor was any brown colouration detected during the quantitative evaluation of haemolysin titre.

It has been reported that Staph. aureus Wood 46 is a hydrophilic strain (Reifsteck, et al, 1987). In the present study, in chemically defined media containing minimal numbers of amino acids are being converted Staph. aureus Wood 46, and to a lesser extent Staph. aureus NCTC 8532 from hydrophilic to hydrophobic (bacterial cells adhered to the surface of the glass culture vessels and clumped together making large clots). It has been suggested that protein A is not a major determinant of hydrophobicity (Reifsteck, et al, 1987), and Staph. aureus Wood 46 is protein A negative (Srinivasakumar and Rao, 1986). On the other hand, the results using both safranine staining on agar plates and Indian ink indicated that both Staph. aureus Wood 46 and NCTC 8532 grown in either BHI or CDM/A were unencapsulated strains, suggesting that the absence of capsules is not an important determinant of hydrophobicity.

Reifsteck, et al, (1987) reported, however, that hydrophobicity is due to a protein or protein-associated molecule localised at the cell surface of the bacteria as a component of either the cell wall, cell membrane, or both, although they also showed that neither trypsin nor pepsin altered the hydrophilicity of Staph. aureus Wood 46. The study of Nesbitt, et al, (1982) showed that the cell walls of Streptococcus sanguis tended to be hydrophobic and they concluded

that hydrophobic amino acids associated with the cell wall contributed to the observed hydrophobicity of intact cells. Similarly, strains of Staph. aureus from bovine mastitis are capable of changing their cell surface hydrophobicity when grown in different media (Mamo, Rozgonyi, et al, 1987). Therefore, it may suggest that the hydrophobicity-hydrophilicity of staphylococci are not a constant characteristic, and may be altered by cultivation in different media.

4.2.3 Growth and Haemolysin Production in Media Based on Fish-Waste Extract

Dependency of growth and haemolysin production on medium composition is further illustrated by studies of microbial growth and haemolysin production in media based on simple extracts of beef and fish as follows: a range of media based on fish extract supported bacterial and fungal growth (see Appendix 1). Fish Infusion Broth (FIB; Medium No 2, see Appendix 1) encouraged luxuriant growth of a variety of organisms (see 2.1) including staphylococci and gave good titres of both alpha- and beta-staphylococcal haemolysins.

In contrast to the effects of yeast extract on haemolysin production reported previously, it was found that media 5 (3.7% beef-extract) and 6 (3.7% peptone) diminished the titre of alpha-lysin more than beta-lysin. Medium 7 (3.7% fish-extract, FE), however, completely abolished the production of alpha-lysin whilst permitting beta-lysin to be produced at a similar level to that obtained in media 5 and 6 (see Figures 3.26a,b).

The combination of proteose peptone with fish-extract (medium 2) was synergistic in the production of alpha-lysin and others have found that proteose peptone/serum combinations influence the production of diphtheria toxin (King, et al, 1949). For beta-haemolysin titre, however, the combination of FE with proteose peptone appears additive rather than synergistic. Both the synergistic and additive effects of proteose peptone on staphylococcal haemolysin titre may justify its inclusion in media developed to enhance haemolysin production.

It would appear that the simple extract of digested, readily available, fish waste (Appendix 1) has distinct possibilities as an inexpensive, major component of routine laboratory media for both organism maintenance and differentiation.

4.3 INTERACTIONS BETWEEN STAPHYLOCOCCI AND PMN's IN SERUM

The serum requirements for efficient phagocytosis and intracellular-killing of coagulase-positive staphylococci have been demonstrated in rabbit blood leucocytes and monocytes (Shayegani, et al, 1964; Shayegani and Mudd, 1966). However, Staph. aureus NCTC 8532 investigated in this study showed no difference in their basic opsonic requirements to phagocytosis, since NCTC 8532 from BHI was engulfed to similar rates in the absence or presence of 10% rabbit normal serum (10% RNS) (see Figures 3.27 and 3.28a, respectively). Others have shown that the phagocytosis of Staph. aureus by mice macrophages was not affected by the absence of serum (Whaley and Singh, 1973) and Hof, Repine, et al, (1980) and Hoidal, et al, (1981) have shown that human alveolar macrophages were able to ingest and kill staphylococci in the absence of opsonins. Peterson, Verhoef, et al (1976) demonstrated that in vitro opsonization and phagocytosis of Staph. aureus strains differed noticeably from strain to strain as a result of the variations in cell surface components. Therefore, it may be concluded that phagocytosis of some staphylococci strains may still occur in the absence of serum in the system. Overall this is dependent on the bacterial strain, as well as, the experimental conditions (for example, the ratio of PMN's to bacteria).

In contrast to the pronounced differences between the effect of fresh and heated serum on the phagocytosis of Staph. aureus it was revealed that in the presence of 10% rabbit heated serum (10% RHS) a reduction in the phagocytosis rate occurred with Staph. aureus Wood 46 grown in chemically defined media (Figure 3.35a) when compared with that observed in the presence of 10% rabbit normal serum (10% RNS). Similar results were reported by Li, et al (1963); Shayegani

and Mudd (1966) and Wheat et al (1974) for both rabbit and human serum. Therefore, it may be concluded that the reduction in the phagocytosis rate of Staph. aureus in the presence of heated serum is due to the absence of heat-labile components "complement" (Li, et al, 1963).

The present study using Staph. aureus wood 46 showed that the immune rabbit sera (obtained by immunized rabbits with cells of Wood 46) failed to enhance more phagocytosis than normal rabbit serum. These results are supported by many authors (Shayegani, et al, 1964; Shayegani and Mudd, 1966; Shayegani, 1970). Shayegani (1970) and Avtalion and Shahrabani (1975) concluded that the presence of specific antibody appears to play little or no role in phagocytosis of Staph. aureus. Therefore, it appears that the presence of specific antibodies has less effect than the complement cascade in serum. Complement is largely involved in enhancing the effects of phagocytes and serum bactericides on Wood 46, since heating of normal serum reduced the phagocytosis (Figure 3.35a) and the bactericidal action of serum (Figure 3.35b).

The composition of the original medium of incubation greatly influenced the phagocytosis and intracellular-killing of the six staphylococci. The highest phagocytosis rate occurred with all staphylococci from BHI, except for strain NCTC 5655 which recovered shortly after it had apparently been ingested (see Figures 3.28a, 3.29a, 3.30a, 3.31a, 3.32a and 3.33a). Fish Infusion Broth (FIB; see Appendix 1) was used as another rich medium to make comparisons on phagocytosis for all strains with those grown in BHI. Generally, all staphylococci grown in this medium showed similar phagocytosis susceptibility and were comparable to BHI except that the coagulase-negative Staph. epidermidis NCTC 7944 (Figure 3.32a) and Staph. aureus strain NCTC 5655 (Figure 3.31a) were re-detected in increasing numbers in the medium after 30 min of apparent ingestion.

In general, the results indicate that less phagocytosis occurs with all staphylococci grown in the chemically defined media (see Figures

3.28a, 3.29a, 3.30a, 3.31a, 3.32a and 3.33a), although strains NCTC 8532 and NCTC 7428 gave similar phagocytised to that from FIB (Figures 3.28a, 3.29a). Variations in medium amino acid composition (Figures 3.30a, 3.31a, 3.32a and 3.33a), or in the amount of amino acid (Figure 3.34) affected staphylococci susceptibility to phagocytosis. However, Staph. aureus strain NCIB 8625 and Wood 46 (Figures 3.30a and 3.33a) from medium CDM/A and NCTC 5655 from medium CDM/5 (Figure 3.31a) were less phagocytised and bacterial cells were liberated into the medium during the later stages of the experiments. Species variation was, however, also apparent and the coagulase-negative Staph. epidermidis NCTC 7944 harvested from 5 amino acid supplement chemically defined medium was less susceptible to phagocytosis (Figure 3.32a) and had a greater survival rate than the coagulase-positive Staph. aureus strains NCTC 8532, NCTC 7428 and NCTC 5655 when they were grown into the same medium (CDM/A, see Figures 3.28a, 3.29a, 3.31a, 3.32d), or than all the Staph. aureus five strains when initially cultivated in FIB. This is in contrast to reports that Staph. epidermidis (formerly albus) is killed by rabbit and human polymorphonuclear leucocytes, and that the pathogenic Staph. aureus strains were able to survive phagocytosis (Rogers and Tompsett, 1952; Kapral and Shayegani, 1959). Lyons (1937) suggested that pathogenic strains were not killed by the process of ingestion and that the bacterial production of leucocidin within the phagocytes resulted in the destruction of the leucocytes and the escaping of viable staphylococci. However, in the present study the leucocidin production may be discounted from the protection since samples of cells after harvesting from media were washed free of associated leucocidin and the trypan blue test showed after 120 min of incubation bacteria and PMN's in gel-HBSS about 90% of PMN's were viable. Similarly, the coagulase-negative Staph. epidermidis NCTC 7944 is assumed to be leucocidin negative (since no publications were found confirming that leucocidin may be produced by Staph. epidermidis). However, even if some haemolysins were carried over to the phagocytosis experiments, it is still reasonable to assume under the same propositions that one may discount the effect of haemolysin on phagocytosis. More haemolysins are produced

in BHI than in chemically defined media but still bacteria from chemically defined media were more resistant to phagocytosis and intracellular killing than ones cultivated in BHI.

Moreover, Staph. epidermidis NCTC 7944 cultivated overnight in FIB, CDM/10, CDM/11 and CDM/A, rather than BHI was less sensitive to phagocytosis (Figure 3.32a). Similarly, Clark and Easmon (1986) have shown that the phagocytosis of Staph. epidermidis grown in peritoneal-dialysis effluent was lower than cells grown in BHI. The difference in phagocytosis may be explained from the observations of Williams et al, (1988) who showed that Staph. epidermidis grown in human peritoneal dialysate had differences in the structure of the cell surface compared with bacteria grown in nutrient broth.

The present study showed also that the intracellular behaviour of Staph. aureus Wood 46 (used as a model strain in this experiment) is medium dependent. Cells from BHI were more amenable to intracellular killing by rabbit PMN's than cells grown in chemically defined media (Figure 3.36), similarly with extracellular killing (Figure 3.33a). Varying the numbers of amino acids in the chemically defined media (CDM/7 and CDM/8; see Table 3.6) gave no significant differences in the rate of both phagocytosis and intracellular killing of Staph. aureus Wood 46 (see Figures 3.33a and 3.36). These observations, therefore, may indicate that Staph. aureus Wood 46 from chemically defined media as opposed to rich media such as BHI and FIB, will be more likely to survive in the conditions within phagocytic cells. This is probably the most important aspect of the pathogenicity of these bacteria, and pathogenic staphylococci are generally more resistant than non-pathogenic ones to intracellular killing (Kapral and Shayegani, 1959; Melly, et al, 1960; Rogers and Melly, 1960; Koenig, et al, 1962).

These results are consistent with those of other authors (Stinebring, 1962; Smith and Fitzgeorge, 1964) who found virulent Brucella abortus obtained from infected animals had an increased

ability to survive intracellularly following phagocytosis, compared with the same strain grown in laboratory media. Similarly, Kolawole (1983) observed *in vitro* and *in vivo* the differences in the reductions in phagocytic killing and showed that *Staph. aureus* grown *in vivo* (or in plasma) was markedly more resistant to the intracellular killing than the same strain grown *in vitro* (nutrient broth No 2).

The bactericidal action of the crude extracts from PMN's (PMNCE) was evaluated in gel-HBSS with cells of *Staph. aureus* Wood 46 harvested from BHI, CDM/7 and CDM/8. The results showed that cells grown in BHI were more sensitive to PMNCE than those from chemically defined media (Figure 3.37), supporting the previous observation from this study that more phagocytosis and intracellular killing occured with cells of Wood 46 grown in rich media (Figures 3.33a and 3.36). Kolawole (1983) has also shown that staphylococci were resistant to polymorph bactericidins after growth in plasma and susceptible after growth in nutrient broth, BHI and modified 110 broths. Similarly, Gladstone *et al* (1974) reported that *Staph. aureus* grown in a chemically defined medium were more resistant to the granular extract (cationic proteins), whereas, those grown in BHI or proteose peptone were susceptible. However, they also reported that simply varying medium composition by omitting one or more amino acids from a chemically defined medium supplement with 17 amino acids had no effect on the resistance of *Staph. aureus* to the granular extract. In the present study, this was not the case with the very minimal, 5 amino acid based chemically defined medium (CDM/7) from which cells of Wood 46 were less susceptible to PMNCE than those from 12 amino acid based chemically defined medium (CDM/8) (Figure 3.37).

It thus seems reasonable to conclude that the variations in the susceptibility of *Staph. aureus* Wood 46 to phagocytosis, intracellular killing and PMNCE are medium dependent, particularly since the PMN's and serum were from single species (rabbit) and individual sources.

Gladstone and Walton (1970) were unable to demonstrate a bactericidal or bacteriostatic action of normal rabbit serum on Staph. aureus and Staph. epidermidis. Ehrenkranz, et al (1971) studied the susceptibility of different staphylococci strains to human serum bacteriostasis and found that the growth of Staph. aureus Wood 46 was unaffected by 10% serum. They concluded that the lack of susceptibility of Staph. aureus Wood 46 may simply be due to a deficiency in some cellular attribute as a result of long-term laboratory propagation which may cause lack of other substances necessary for susceptibility to serum bacteriostasis.

The present study, however, has shown that the six staphylococci tested, whether coagulase-positive or negative strains, were susceptible to 10% RNS under test conditions, and that the degree of suceptibility was dependent on the medium of inoculum development (see Figures 3.28c, 3.29b, 3.30b, 3.31b, 3.32b and 3.33b). In chemically defined media, generally, the more minimal the medium, the more resistant the cells. For example, staphylococci from 5 amino acid media were phenotypically more resistant to 10% RNS than those grown in 10 to 12 or even 13 amino acid media (see Figures 3.30b, 3.31b, 3.32b and 3.33b). Therefore, it appears that increasing the numbers of amino acids in chemically defined media from 5 to 12 or 13 is accompanied by a decrease in the protection of staphylococci to killing by normal serum. Varying the amount of the 12 amino acids (CDM/8 and CDM/9; see Table 3.6), however, gave no significant differences in the killing of Staph. aureus Wood 46 by 10% RNS (Figure 3.34).

Replacing mannitol with glucose in CDM/A, however, rendered Staph. epidermidis NCTC 7944 more susceptible to serum bactericidins (Figure 3.32d), though from the comparative study of six staphylococci strains grown in CDM/A, it is apparent that the coagulase-negative Staph. epidermidis NCTC 7944 was generally less susceptible to the bactericidal factors of 10% RNS than the various strains of the coagulase-positive Staph. aureus (see Figures 3.30b, 3.31b, 3.32b and 3.33b). This was unexpected since there are

numerous reports which indicate that the coagulase-positive Staph. aureus are more resistant to serum bactericidins in vitro than the coagulase-negative Staph. epidermidis (Tejler, 1937; Spink and Paine, 1940; Yotis and Ekstedt, 1959; Fletcher, 1962; Borowski and Tybusz, 1963; Cybulska and Jeljaszewicz, 1966a; Ehrenkranz et al, 1971). These results, therefore, may indicate that there is no relationship between the coagulase formation and the ability of the organisms to survive the presence of serum (Cybulska and Jeljaszewicz, 1966a and b) and, for example, the coagulase-negative Staph. epidermidis NCTC 7944 and Staph. aureus NCTC 7428 (coagulase-negative in CDM/A; see Table 3.2), both showed a degree of resistance to the serum (Figures 3.29b, 3.32b) when they were grown in CDM/A. The destroying inhibitor substances in serum do not, therefore, appear related to coagulase production as suggested by Fletcher (1962).

Gladstone (1973) reported that if serum-beta-lysins are excluded from serum by removing cells and platelets from plasma before allowing it to clot, both Staph. aureus and Staph. epidermidis grow well in rabbit serum, but the results reported in this study were obtained in whole serum since the cells and platelets were not removed before allowing the serum to clot (see 2.10). However, heating the serum affected the efficiency of serum bactericides (see Figure 3.35b).

These results, therefore, suggest that part of the killing of staphylococci may be due to serum and it again shows that the staphylococcal-serum susceptibility is media dependent, and may be a phenotypic adaptation occurring during the incubation of the staphylococci in the different media. Hence the different values of interaction between the serum-bactericidins and staphylococci strains could be reflecting the staphylococcal envelope.

The six staphylococci grown in different media show various susceptibilities to the bactericidal power of bacteria-free and PMN's-free supernatant liquids obtained from the interaction of

PMN's and bacteria in gel-HBSS in the presence of 10% RNS (see 2.18; and Figures 3.28d, 3.29c, 3.30c, 3.31c, 3.32c and 3.33c). Therefore, it appears that the inhibitor substances of both PMN's and 10% RNS in bacteria-free and PMN's-free supernatant were not abolished completely by the previous growth of staphylococci in the phagocytosis process. These bactericidal substances were released from cells either during ingestion or as a result of cell death and may kill organisms extracellularly (Zurier, Weissmann, et al, 1974). Gladstone and Walton (1971) emphasise that the extracellular killing of staphylococci cannot be related to bactericidal factors (beta-lysins) from 20% rabbit plasma or serum. They presumed that cationic proteins from the leucocytes were liberated during the course of their experiments into the medium either by disruption of some of the leucocytes or by extrusion of their lysosomal contents. The results reported in this study, however, showed clearly that in addition to the bactericidal action of PMNCE (Figure 3.37), 10% RNS in gel-HBSS also has a bactericidal action on the growth of staphylococci (for example, see Figures 3.28c, 3.29b, 3.30b, 3.31b, 3.32b and 3.33b).

In general, it appears that growth in the various chemically defined media and to a lesser extent FIB, encourages intraleucocytic survival for staphylococci including Staph. epidermidis (Figures 3.28a, 3.29a, 3.30a, 3.31a, 3.32a and 3.33a). These media may well enhance the ability of staphylococci to produce a mucoid, slime layer or capsules which may protect the organisms against the phagocytosis. The presence of capsules has been shown to affect the phagocytosis of staphylococci (Morse, 1960; Yoshida and Takeuchi, 1970; Melly, Duke, et al, 1974). A high percentage of Staph. aureus strains produce slime more readily than capsules in response to nutritional conditions (Wilkinson, 1983). The results from safranine staining on agar plates and a microscopic staining examination of cell samples with Indian ink, showed that only Staph. aureus NCTC 7428 and NCIB 8625 may produce dense slime in both media BHI and CDM/A. Negative results were obtained with other strains though, in comparison, cells of NCTC 7428 from BHI and CDM/A and of NCIB 8625

from BHI were still phagocytised rapidly (Figures 3.29a and 3.30a). On the other hand, the comparative study on the surface antigenic properties of Staph. aureus Wood 46 cultured in CDM/7, CDM/8 and BHI (see Table 3.7 and Figure 3.38) were similar. Gladstone, et al (1974) also showed no differences in the surface antigenic properties of Staph. aureus grown under different nutrient conditions. Therefore, the developed resistance of staphylococci grown in different media to phagocytosis, intracellular killing and to the bactericidal materials in PMNCE and serum is unlikely to be due to the presence of a slime layer and capsules.

Two very similar, alternative explanations assume specific medium dependent cell surface changes (Ellwood and Tempest, 1972; Schleifer, et al, 1976), that may markedly affect the surface properties of the bacteria. One possibility is that such surface changes would encourage clumping of staphylococci making engulfment more difficult and protecting central cells from bactericides. Clumping certainly occurred with Staph. aureus strains Wood 46 and NCTC 8532 when they were grown in chemically defined media, although surprisingly this could be minimised by using plastic vessels. Segal, Harper, et al, (1982) also reported that clumped Staph. aureus cells appeared to be more resistant to killing than single cells.

The other possibility is that such variations may have quite differents chemical present at their surfaces than previous generations, and which give rise to cells which, therefore, would not facilitate the primary responses which lead to the complement cascade etc.

Smith (1964) suggested that such phenotypes may lack one or more virulence determinants or may even possess apparent virulence factors never found in vivo. Phagocyte cells are attracted to organisms by chemotaxis (see Figure 1.4), which is involved in the antibody and C3b-mediated adherence of opsonized bacteria (Verhoef, et al, 1977b). The opsonic activity of complement residue C3b is

generated by enzymatic cleavage from native C3 (Stossel, Field, et al, 1975; see Figure 1.4) and it is deposited on the bacterial cell surface (Gigli and Nelson, 1968; Stossel, Field, et al, 1975). The bacterial cell surface of Staph. aureus is reported to be the most important factor for the mechanisms of chemotactic activity (Adlam and Easmon, 1983) and the complement in normal serum is activated by all major cell surface components of Staph. aureus (Wilkinson, Kim et al, 1978; Verbrugh, Van Dijk, et al, 1979; Verbrugh, Van Dijk, et al, 1980) and this may contribute to staphylococcal opsonization with C3b. Clearly then the reduction in the phagocytosis rate, or the increase in the intraleucocytic survival of staphylococci (Li, et al, 1963) may be due to an inhibition of the mechanisms activating the complement cascade because of phenotypic variation of the staphylococcal cell surface. Further investigations (see Appendix II) are, however, needed to confirm this.

The present work has demonstrated that the phagocytic uptake and intracellular-killing of staphylococci and the interaction of staphylococci with serum and with the bactericidal substances leaked from PMN's or PMNCE can be variable and is at least partly dependent on the composition of the medium in which the bacteria were initially cultivated.

Many workers have suggested that staphylococcal virulence and pathogenicity are closely associated with the production of alpha- and beta-haemolysin and coagulase (Christie, et al, 1946; Marks, 1952; Taubler, et al, 1963; Foster, 1967; Freeman, 1979). However, the present study has shown that the production of Staph. aureus alpha- and beta-haemolysins was markedly reduced in the various chemically defined media, in comparison with BHI and FIB. However, bacterial cells harvested from chemically defined media (free of associated haemolysin) are, generally, more resistant to phagocytosis, intracellular-killing or bactericidal substances from PMN's and serum than those from FIB and BHI. This may indicate an increase in staphylococcal virulence when grown in chemically defined media even though the formation of haemolysin (or coagulase)

is reduced. Of course staphylococci produce many other extracellular and cellular materials (see Table 1.3, Figure 1.5) and these may be affected by medium composition and will have a part to play in phagocytosis.

These observations may lead to a better understanding of the mechanisms of staphylococcal infections, and may explain how the different nutritional conditions found in different hosts and tissues could determine different levels of bacterial persistence. In vitro the studies of phagocytosis and indeed of haemolysis must use bacterial cells of known and consistent history of cultivation. The use of simple, common chemically defined medium is to be encouraged to enable interspecies/strain in vitro comparisons and the development of more realistic animal models which may explain, in part, the pathogenicity and virulence of staphylococci when grown in vivo.

The effects of medium composition on staphylococcal growth and their biological activities are complex.

APPENDIX 1

THE USE OF FISH WASTE IN BACTERIOLOGICAL CULTURE MEDIA

1.A INTRODUCTION

The development of suitable media for growing the organisms of medical importance e.g. bacteria, yeasts and moulds, is still a subject of considerable study. Such organisms which cause diseases often require complex media. In many cases, bacteria will not grow unless supplied with specific protein, vitamins, minerals, and carbohydrates. Media made from mixed or isolated animal proteins have been found satisfactory and occasionally blood, simple aqueous extracts of meat or extract of autolyzed yeasts have been added to supply specific vitamins, or nutrients (Cruickshank, 1970).

Extracts prepared from fish have been used in bacteriological culture media. Such extracts were prepared from the autodigestion of stomach walls (Hach and Polulach, 1936), or from press water (Drangsholt, 1948), or digestion of fish flesh (Tarr and Deas, 1948). The products were all able to support bacterial growth, including fastidious organisms, but enzymatic hydrolysis of the material gave media with optimum growth, whilst acid or alkaline hydrolysis gave a product inferior to normal peptone media (Tarr and Deas, 1949). The techniques adopted were often lengthy, taking up to several weeks (Tarr and Deas, 1948), or using special equipment (Anderson and Fellers, 1949). A detailed examination of the potential of growth media formulated from catfish waste was carried out by Beuchat (1974) who showed this material to be a suitable source of peptone.

The aim of this aspect of the current study was to use extracts of enzymatically digested fish waste (whole heads, fins, skeletal debris) as a substitute for beef extract in laboratory media. In addition to studying the growth of a wide range of microorganisms in such media, the production of staphylococcal haemolysis would also be investigated.

1.B MATERIALS AND METHODS

1.Bi) Preparation of Digest of Fish Waste

Fish waste, including heads, bones, and fins was chopped and soaked overnight in 0.4g/dl trypsin (BDH) at 37°C, pH 7.0 to 8.0. The solid material was removed by centrifuging at 5000g for 20 min at 4°C (MSE; Mistral 6L). The supernatant fluid was transferred to a separating funnel and the aqueous layer separated from the lipid. Activated charcoal (2g/dl) was added to the aqueous layer which was then heated for 5 min at 60°C, to reduce odour, and then filtered through celite. Preliminary experiments indicated that this material could be used (with added peptone and salt) as the basis of a routine culture medium. However, to give a more stable, easy to store product a concentrate was prepared: samples of the filtrate were either freeze dried to constant weight or oven dried (60°C) to a viscous paste. Typical yields of solids were 7 to 9% w/v of original mass. Distilled water was then added to produce a concentrated solution (approximately 35% w/v) which was stored frozen at -20°C.

The lipid obtained during the initial extraction was clarified with 5 volumes of acetone. The acetone solution was decanted and the acetone removed by evaporation. The remaining liquid (FL; mainly neutral lipids) was retained.

1.Bii) Preparation of Media

Various media were prepared to facilitate comparison. Individually they comprised (g/L): Medium 1 - fish extract, 10; peptone, 10; NaCl, 5; Medium 2, Fish Infusion broth (FIB) - fish extract, 17.5; proteose peptone, 10; NaCl, 5; glucose, 2; Medium 3 - beef extract, 10; peptone, 10; NaCl, 5; Medium 4 - brain heart infusion (BHI; see 2.2); Medium 5 - beef extract (Oxoid L29), 37; Medium 6 - peptone (Oxoid, L37) 37; Medium 7 - fish extract, 37; Medium 8 - proteose peptone (Oxoid L46) 37. Media 5, 6, 7 and 8 were intended to be equivalent (in concentration terms) to the total nutrient content of Medium 4, but using a single constituent. Medium 3 was

used mainly for culture maintenance and inoculum development for solid media for other organisms other than Staphylococci strain (see 2.1).

Where appropriate, media were solidified by the addition of agar (see 2.2) and in all cases the pH of the medium was adjusted to 7.4 prior to autoclaving at 121°C for 15 min. Both freeze-dried and oven-dried products were used in solid media. All fish extract broths were based on the freeze-dried material. When a blood-based solid medium was required, media 2 (freeze-dried extract; FIB) and 4 (BHI) were enriched by the addition of 7% v/v defibrinated sheep blood (see 2.3 and 2.5c). For studies of lipolytic activity 1 ml of the lipid extract (FL) was dispersed in 100 ml of medium 1 (plus agar) (unless otherwise stated, see 2.5c) and compared, after inoculation and incubation, with similar plates containing 1% tributyrin.

1.Biii) Growth Conditions and Haemolysin Productions

Solid Media: Media were inoculated as spread plates using 12-18h broth cultures (Medium 3) except for S. henetus and N. opaca which had been incubated for 72h. Cultures were incubated at 37°C for 36-48h for bacterial cultures (25°C for 72h for S. henetus and N. opaca) and 30°C for fungi.

Liquid Media: Staph. aureus was chosen as the test organism to quantify growth studies because of its wide range of nutritional requirements. Strains were selected to represent various origins and haemolysin production capabilities.

The standard inocula and growth assessment were carried out as above (see 2.6). The haemolytic assay, of the supernatant liquid from a sample of the growth broth culture (media 2, 4, 5, 6, 7 and 8) of Staph. aureus Wood 46, NCIB 8625, NCTC 5655 and NCTC 7428 was estimated on the basis of 50% haemolytic unit (HU50) of a standard erythrocyte suspension (see 2.9).

1.C RESULTS

Fish extract (FE) produced by both drying methods was of low odour and readily soluble in water to give a clear, pale straw-coloured solution in the case of the freeze-dried product, and a light brown solution with the oven-dried material. Incompatibility problems were not encountered with the medium additives used in this study.

All microorganisms (see 2.1) studied grew on the solid versions of all media, irrespective of the drying technique used in the preparation of FE. Colony morphology was similar for the same organism grown on the different media. In general terms, the amount of growth observable on media based on fish waste extracts (media 1, 2, and 7) was after fixed incubation times never less than that found on nutrient agar (Medium 3) plates. In one case (Medium 2; FIB-agar) which is a fish extract medium, further enriched with proteose peptone, the amount of the growth of all microorganisms (see 2.1) was luxuriant.

For the study on the growth and haemolysin production of staphylococci strains in liquid media, including diluted extracts and peptone solutions, see 3.3.

Lipase activity was clearly demonstrated on medium 1 based on fish extract containing the fish oil (FL) and was comparable with that obtained with tributyrin agar (see also 3.1.5).

The methods and results mentioned in this Appendix have been published (Jassim, et al, 1988).

APPENDIX II

Suggested further work based on the results of this investigation are:

1. Study the chemical composition and biological activity of alpha- and beta-haemolysins produced by Staph. aureus in chemically defined media.

2. Biochemical and biological study on the novel haemolytic profile of Staph. aureus NCTC 8532 (effects on haemoglobin?).

3. Study the effect of defined medium composition on the chemical nature of the cell surfaces of staphylococci, and its influence on the mechanisms activating the complement cascade.

4. Investigate amino acids profile of wound exudates to assist the development of meaningful defined media to enable comparisons of growth, haemolysin production and phagocytosis of staphylococci to further understand wound pathogenicity.

Letters in Applied Microbiology 1988, 6, 139–143 MS/079

The preparation and use of media based on a simple fish waste extract

S. JASSIM, W.G. SALT* & R.J. STRETTON *Microbiology Unit, Chemistry Department, University of Technology, Loughborough* LE11 3TU, UK

Received 24 March 1988 and accepted 29 March 1988

JASSIM, S., SALT, W.G. & STRETTON, R.J. 1988. The preparation and use of media based on a simple fish waste extract. *Letters in Applied Microbiology* 6, 139–143.

An aqueous extract of trypsin-degraded fish waste is a good substitute for beef extract in culture media. With beef extract and proteose peptone, it promoted luxuriant growth and staphylococcal toxin production. Though growth occurred in dilute solutions of the extract alone, alpha-toxin production was suppressed. It was synergistic with proteose peptone in the production of alpha-toxin. Separated fish lipids may be used to demonstrate lipase activity.

Extracts prepared from fish have been used in bacteriological culture media. Such extracts were prepared by the chemical or enzymatic digestion of fish flesh or stomach walls (Hach & Polulach 1936; Tarr & Deas 1948, 1949; Beuchat 1974) or from the liquid expressed from fish flesh under high pressure (Drangsholt 1948). All products supported bacterial growth; enzymatic hydrolysis of fish flesh gave media with optimum growth (Tarr & Deas 1949). The techniques adopted were often lengthy or required special equipment (Anderson & Fellers 1949).

The use of extracts of enzymatically digested fish waste (whole heads, fins, skeletal debris) as a substitute for beef extract in laboratory media is now reported.

Materials and Methods

MICRO-ORGANISMS

The organisms used were: *Aspergillus nidulans* CMI 16643, *Aspergillus niger* CMI 31821, *Bacillus subtilis* NCIB 10073 and NCIB 3610, *Byssochlamys fulva* CMI 40021, *Candida albicans* A39, *Escherichia coli* NCIB 10243, *Klebsiella pneumoniae* NCIB 8267, *Nocardia opaca* NCIB 9409, *Pseudomonas aeruginosa* NCTC 7244, *Saccharomyces cerevisiae* NCYC 975 and NCYC 620, *Schizophyllum commune* FPRL 9, *Serratia marcescens* NCIB 11879, *Staphylococcus aureus* NCIB 8625, NCTC 5655, NCTC 7428, NCTC 8532 and Wood 46, *Staphylococcus epidermidis* NCTC 7944 and *Streptomyces henetus* NCIB 11003.

* Corresponding author.

PREPARATION OF DIGEST

Fish waste, including heads, bones and fins was chopped and soaked overnight in 0·4 g/dl trypsin (BDH) at 37°C, pH 7·0–8·0. The solid material was removed by centrifuging at 5000 g for 20 min at 4°C (MSE Mistral 6L) and the supernatant transferred to a separating funnel and the aqueous layer separated from the lipid. Activated charcoal (2 g/dl) was added to the aqueous layer which was heated for 5 min at 60°C to reduce odour and it was then filtered through Celite.

Preliminary experiments indicated that this material could be used, with added peptone and salt, as the basis of a routine culture medium. However, to give a more stable, easy to store product, a concentrate was prepared: samples of the filtrate were either freeze-dried to constant weight or oven-dried (60°C) to a viscous paste.

Typical yields of solids were 7–9% w/w of original mass. Distilled water was then added to produce a concentrated solution (approximately 35% w/v) which was stored frozen at −20°C.

The lipid obtained during the initial extraction was clarified with 5 volumes of acetone. The acetone solution was decanted and the acetone removed by evaporation. The remaining liquid (FL; mainly neutral lipids) was retained.

MEDIA

Various media were prepared to facilitate comparisons. Individually they comprised (g/l): medium 1—fish extract, 10; peptone, 10; NaCl, 5; medium 2—fish extract, 17·5; proteose peptone, 10; NaCl, 5; glucose, 2; medium 3—beef extract, 10; peptone, 10; NaCl, 5; medium 4—brain heart infusion (Oxoid CM225); medium 5—beef extract, 37; medium 6—peptone, 37; medium 7—fish extract, 37; medium 8—proteose peptone (Oxoid L46) 37. Media 5. 6. 7 and 8 were formulated to be equivalent in concentration terms to the total nutrient content of medium 4 but with a single constituent. Medium 3 was used mainly for culture maintenance and inoculum development for solid media.

Where appropriate, media were solidified by the addition of agar (1·5%; Oxoid L13) and in all cases the pH of the medium was adjusted to 7·4 before autoclaving at 121°C for 15 min. Both freeze-dried and oven-dried concentrates were used in solid media. All fish extract broths were based on the freeze-dried material.

When a blood-based solid medium was required, media 2 (freeze-dried extract) and 4 were enriched by the addition of 7% v/v defibrinated sheep blood (Oxoid, SR51). For studies of lipolytic activity 1 ml of the lipid extract (FL) was dispersed in 100 ml of medium 1 (plus agar) and compared, after inoculation and incubation, with similar plates containing 1% tributyrin.

GR

Solu

Media were inoculated as spread plates with 12–18 h broth cultures (medium 3) except for *S. heneius* and *N. opaca* which had been incubated for 72 h. Cultures were incubated at 37°C for 36–48 h for bacterial cultures (25°C for 72 h for *S. henetus* and *N. opaca*) and 30°C for fungi.

Liquid media

Staphylococcus aureus was chosen as the test organism to quantify growth studies because of its wide range of nutritional requirements. Strains were selected to represent various origins and haemolysin production capabilities. Before haemolysis studies in solid and liquid media, *Staph. aureus* was maintained on blood-enriched plates of medium 4. Standard inocula were prepared from 18 h shaken cultures (medium 4) as follows: cells from 100 ml samples were isolated by centrifugation, washed three times with, and finally resuspended in, one-quarter-strength Ringer's solution to a final volume of 100 ml.

Samples (100 ml) of liquid media in 250 ml Erlenmeyer flasks with cotton wool plugs, were inoculated with 1 ml of the standard inoculum of the required strain of *Staph. aureus*. All cultures were incubated at 37°C in an orbital incubator at 90 rev/min. Growth (O.D. 540 nm; 1 cm path) was assessed at time intervals with a Unicam SP500 spectrophotometer. Dense cultures were diluted with fresh medium immediately before growth measurement.

STAPHYLOCOCCAL HAEMOLYSIN PRODUCTION

The haemolysin titre (50% haemolysis) of the supernatant liquid (4000 g, 20 min) from a sample of the growing broth culture (media 2, 4, 5, 6, 7 and 8) of a selected strain, was determined against a standard erythrocyte suspension prepared as follows. Rabbit or sheep erythrocytes were isolated from defibrinated whole blood by centrifuging at 3500 g for 10 min at 4°C, washed three times with and finally resuspended in phosphate buffered saline (PBS: 0·01 mol/l disodium hydrogen phosphate and 0·145 mol/l sodium chloride pH 7·0) to 2% v/v.

Equal volumes (1 ml) of the standard suspension, rabbit erythrocytes for alpha-toxin and sheep erythrocytes for beta-toxin, were added to test tubes each containing 1 ml of a two-fold serial dilution (PBS) of the culture supernatant to which 2 ml PBS had been added. The mixtures were incubated at 37°C for 1 h and then centrifuged (3500 g, 10 min at 4°C) to remove

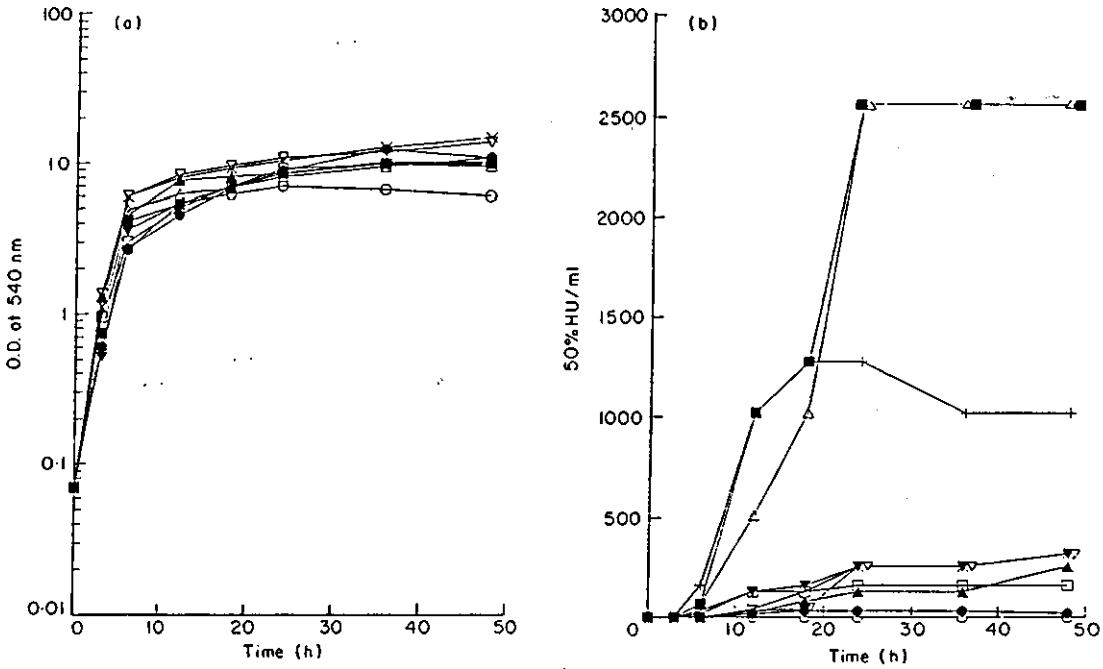


Fig. 1. a. Growth curves (O.D. at 540 nm), in shaken culture (90 rev/min) at 37°C, for *Staphylococcus aureus* (i) Wood 46 in: ■, medium 2; △, medium 4; ● medium 5; □, medium 6 and ○, medium 7; (ii) NCTC 5655 in: ▲, medium 2 and ▽, medium 4; (iii) NCIB 8625 in: ▼, medium 2 and ×, medium 4. b, Alpha-haemolysin activity of supernatant liquid from growing cultures of *Staph. aureus*, estimated after 1 h contact in PBS at 37°C using rabbit erythrocytes (i) Wood 46 in: ■, medium 2; △, medium 4; ●, medium 5; □, medium 6; ○, medium 7 and +, medium 8; (ii) NCTC 5655 in: ▲, medium 2 and ▽, medium 4; (iii) NCIB 8625 in: ▼, medium 2 and ×, medium 4.

intact erythrocytes. The absorbance (545 nm, 1 cm path) of the supernatant liquid was measured and the 50% haemolysis (HU50) endpoint calculated by comparison with the absorbance of the standard erythrocyte suspension after lysis by freezing and thawing. To determine the beta-toxin titre, the PBS was supplemented with 0·002 mol/l magnesium sulphate; assay mixtures were held at 37°C for 1 h and then for 1 h at 4°C before determination of the HU50.

Results and Discussion

Fish extract (FE) produced by both drying methods was of low odour and readily soluble in water to give a clear, pale straw-coloured solution in the case of the freeze-dried product and a light brown solution with the oven-dried material. Incompatibility problems were not encountered with the medium additives used in this study.

All micro-organisms studied grew on the solid versions of all media, irrespective of the drying technique used in the preparation of FE. Colony morphology was similar for the same organism grown on the different media. In general terms, the amount of growth observable on media based on fish waste extracts was similar to that seen on nutrient agar (medium 3). In medium 2, a fish extract medium enriched with proteose peptone, the growth was 'luxuriant'. The growth of *Staph. aureus* on whole blood-enriched agar media 2 and 4 was accompanied by haemolysis zones characteristic of the strain studied. Lipase activity was clearly demonstrated on the plates containing the fish oil (FL) and was comparable with that obtained with tributyrin agar.

In liquid media, including diluted extracts and peptone solutions, all strains of *Staph. aureus* grew abundantly with a heavy but uniform turbidity (Fig. 1a and 2a). Small differences in maximum growth rate and overall

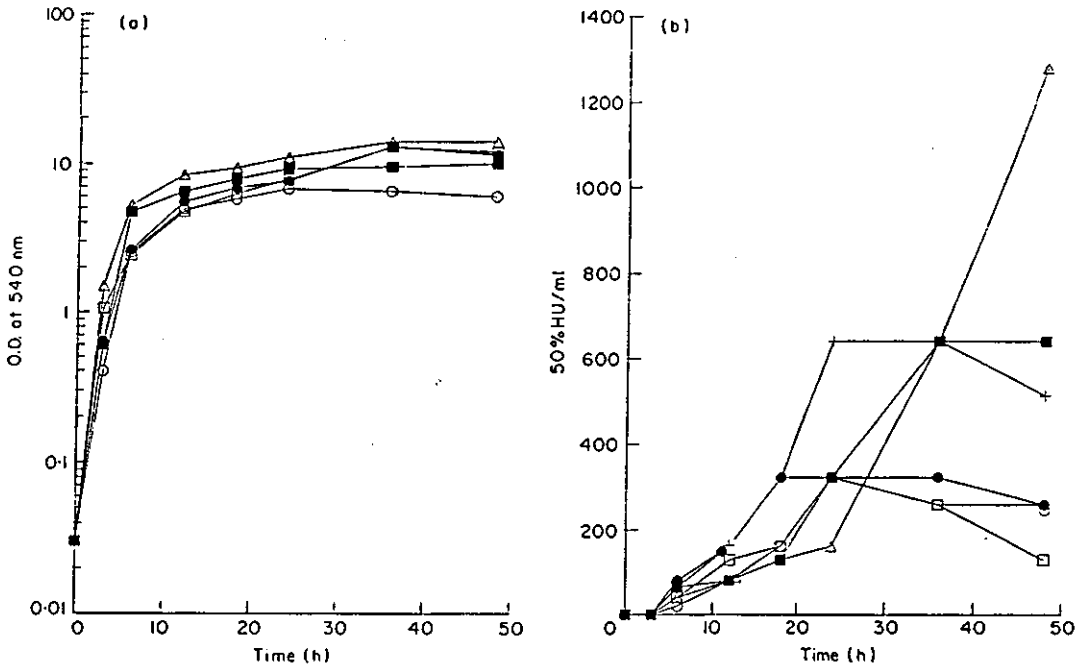


Fig. 2.a, Growth curves (O.D. at 540 nm) of *Staphylococcus aureus* NCTC 7428 at 37°C in shaken culture (90 rev/min) in: ■, medium 2; △, medium 4; ●, medium 5; □, medium 6 and ○, medium 7. b, Beta-toxin activity of the supernatant liquid from growing cultures of *Staph. aureus* NCTC 7428, estimated using sheep erythrocytes suspended in PBS, supplemented with 0·002 mol/l magnesium sulphate, after 1 h at 37°C and then 1 h at 4°C; ■, medium 2; △, medium 4; ●, medium 5; □, medium 6; ○, medium 7 and +, medium 8

yields were, however, detectable in media consisting of extracts or peptone alone. There was no significant difference in the level of alpha-toxin produced by *Staph. aureus* Wood 46, a high titre alpha-toxin producer, in either medium 2 or 4 (Fig. 1b) A similar pattern was observed, though at much lower HU50, with strain NCIB 8625, though strain NCTC 5655 produced more alpha-toxin in medium 4.

When Wood 46 was grown in media containing 3·7 g/dl of beef extract or peptone alone, alpha-toxin production was markedly depressed, with maximum HU50 values of 32 and 160 for beef extract and peptone respectively. Growth in proteose peptone solution (medium 8) gave a maximum HU50 in excess of 1000 after 15 h. Alpha-toxin production was, however, completely abolished in medium 7. Medium 2 was synergistic in the production of alpha-toxin. Proteose peptone/serum combinations have been shown to influence the production of diphtheria toxin (King *et al.* 1949).

Beta-toxin was produced by *Staph. aureus* NCTC 7428 in all media used (Fig. 2b) Highest titres were achieved in medium 4, though toxin production was most rapid in medium 8. The HU50 values were equivalent in media 2, 4 and 8 (approx. 650 units) after 36 h. The maximum titre of beta-toxin was significantly lower in media with 3·7 g/dl of fish/beef extract or peptone alone. In all cases, organisms routinely subcultured on blood agar gave significantly higher titres of toxin. For beta-toxin titres, combination of FE with proteose peptone appears additive rather than synergistic. Both the synergistic and additive effects of proteose peptone on staphylococcal toxin titre may justify its inclusion in media developed to enhance toxin production.

References

ANDERSON, D.W. & FELLERS, C.R. 1949 Preparation of sterile fish-muscle press juice for chemical and bacteriological studies. *Food Technology* 3, 274.

BEUCHAT, L.R. 1974 Preparation and evaluation of a microbiological growth medium formulated from catfish waste peptone. *Journal of Milk and Food Technology* 37, 277–281.

DRANGSHOLT, C. 1948 Preparation of peptone and amino-acid-containing extracts from press water. *British Patent* **641**, 120.

HACH, W. & POLULACH, N. 1936 Nutritive media from fish products. *Archives of Biological Science*, USSR **41**, 163–165.

KING, E.O., FROBISHER, M. & PARSONS, E.I. 1949 *In vitro* test for virulence of *Corynebacterium diphtheriae*. *American Journal of Public Health* **39**, 1314–1320.

TARR, H.L.A. & DEAS, C.P. 1948 Utilisation of fish wastes. Bacteriological peptones from fish. *Progress Reports of the Fisheries Research Board of Canada, Pacific Station* **75**, 43–45.

TARR, H.L.A. & DEAS, C.P. 1949 Bacteriological peptones from fish flesh. *Journal of the Fisheries Research Board of Canada* **7**, 552–560.

BIBLIOGRAPHY

ABBAS-ALI, B. and COLEMAN, G. (1977).
The characteristics of extracellular protein secretion by Staphylococcus aureus (Wood 46) and their relationship to the regulation of alpha-toxin formation. Journal of General Microbiology, 99: 277-282.

ADLAM, C. and EASMON, C.S.F. (1983).
Immunity and hypersensitivity to Staphylococcal infection. In: Staphylococci and Staphylococcal infections (C.S.F. Easmon, and C. Adlam, Eds) Vol. 1, Chap. 9, pp 275-323. Academic Press, London.

ADLAM, C; PEARCE, J.H. and SMITH, H. (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 leukocytes. Journal of Medical Microbiology. 3: 157-163.

ANDERSON, D.W. and FELLERS, C.R. (1949).
Preparation of sterile fish-muscle press juice for chemical and bacteriological studies. Food Technology, 3: 274.

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.

ARBUTHNOTT, J.P. (1970).
Staphylococcal alpha-toxin. In: Microbiol Toxins (T.C. Montie, S. Kadis, and S.J. Ajl, eds), Vol. 111, Bacterial protein toxins, pp 189-232, Academic Press, New York and London.

ARBUTHNOTT, J.P. (1978).
Role of exotoxins in bacterial pathogenicity. Journal of Applied Bacteriology, 44: 329-345.

ARBUTHNOTT, J.P. (1983).
Host damage from bacterial toxins. Philosophical Transactions of the Royal Society, London. B303: 149-165.

ARBUTHNOTT, J.P; FREER, J.H. and BERNHEIMER, A.W. (1967)
Physical states of staphylococcal alpha-toxin. Journal of Bacteriology, 94: 1170-1177.

ARBUTHNOTT, J.P; FREER, J.H. and BILLCLIFFE, B. (1973).
Lipid-induced polymerisation of staphylococcal alpha-toxin. Journal of General Microbiology, 75: 309-319.

ARCHER, G.L. and TENENBAUM, M.J. (1980).
Antibiotic resistant Staphylococcus epidermidis in patients undergoing cardiac surgery. Antimicrobial Agents and Chemotherapy. 17: 269-272.

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 simplified defined media. Biochimica et biophysica Acta 406: 60-67.

ARTENSTEIN, M.S; MADOFF, M.A. and WEINSTEIN, L. (1963).
Studies of the biologic activity of purified staphylococcal alpha-toxin. Yale Journal of Biology and Medicine. 35: 373-389.

ARTZ, C.P. and MONCRIEF, J.A. (1969).
The problem of infection. In: The treatment of burns. W.B. Saunders, (ed). Philadelphia, London, pp 305-328.

ARVIDSON, S.O. (1983).
Extracellular enzymes from Staphylococcus aureus. In: Staphylococci and Staphy-lococcal infections. (C.S.F. Easmon and C. Adlam, Eds). Vol. 2, pp 745-808, Academic Press, London.

ARVIDSON, S; HOLME, T.and WADSTROM, T. (1971).
Influence of cultivation conditions on the production of extracellular proteins by Staphylococcus aureus. Acta Pathologica et Microbiologica Scandinavica. Section B, 79: 399-405.

AVTALION, R.R. and SHAHRABANI, R. (1975).
Studies on phagocytosis in fish: I. In vitro uptake and killing of living Staphyloccocus aureus by peripheral leucocytes of carp (Cyprinus carpio). Immunology 29: 1181-1187.

BAIRD-PARKER, A.C. (1965).
An analysis of the Staphylococcus: Inherent properties of the organism: Staphylococci and their classification. Annals of the New York Academy of Sciences. 128: 4-25.

BAIRD-PARKER, A.C. (1970).
The relationship of cell wall composition to the current classification of staphylococci and micrococci. International Journal of Systematic Bacteriology. 20: 483-490.

BAIRD-PARKER, A.C. (1979).
Methods for identifying staphylococci and micrococci. In: Identification methods for microbiologists, (F.A. Skinner and D.W. Lovelock, Eds), pp 201-210, Academic Press, London,

BAKER, F.J. and SILVERTON, R.E. (1976).
Introduction to medical laboratory technology. 5th ed. Chapter 30, pp 564-597. Butterworths, London, Boston.

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.

BENNETT, R.W. (1984).
Staphylococcus aureus. In: Bacteriological Analytical Manual of the Division of Microbiology Center for Food Safety and Applied Nutrition, US Food and Drug Administration, 6th edition, pp 14.01-14.05. Association of Official Analytical Chemists, USA.

BERGEY'S MANUAL: 1st ed., 1923; 2nd ed., 1925; 3rd ed., 1930; 4th ed., 1934; 5th ed., 1939; 6th ed., 1948; 7th ed., 1957; 8th ed., 1974. Bergey's Manual of Determinative Bacteriology; and Vol 2, 1986. Bergey's Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore, London.

BERNHEIMER, A.W. (1965).
Staphylococcal alpha-toxin. Annals of the New York Academy of Sciences. 128: 112-123.

BERNHEIMER, A.W. (1974).
Interactions between membranes and cytolytic bacterial toxins. Biochimica et Biophysica Acta. 344: 27-50.

BERNHEIMER, A.W; AVIGAD, L.S. and KIM, K.S. (1974).
Staphylococcal sphingomyelinase (beta haemolysin). Annals of the New York Academy of Sciences. 236: 292-306.

BERNHEIMER, A.W. and SCHWARTZ, L.L. (1963).
Isolation and composition of staphylococcal alpha-toxin. Journal of General Microbiology. 30: 455-468.

BERNHEIMER, A.W. and SCHWARTZ, L.L. (1964).
Lysosomal disruption of bacterial toxins. Journal of Bacteriology. 87: 1100-1104.

BERNHEIMER, A.W. and SCHWARTZ, L.L. (1965).
Effect of staphylococcal and other bacterial toxins on platelets in vitro. Journal of Pathology and Bacteriology. 89: 209-223.

BEUCHAT, L.R. (1974).
Preparation and evaluation of a microbiological growth medium formulated from catfish waste peptone. Journal of Milk and Food Technology 37: 277-281.

BHAGAVAN, N.V. (1974).
Biochemistry: a comprehensive review, pp 592-686. Lippincott Company, Phladelphia, Toronto.

BHAKDI, S. and MUHLY, M. (1985).
Decomplementation antigen, a possible determinant of staphylococcal pathogenicity. Infection and Immunity 47: 41-46.

BIGGER, J.W; BOLAND, C.R. and O'MEARA, R.A.Q. (1927).
A new method of preparing staphylococcal haemolysin. Journal of Pathology and Bacteriology. 30: 271-277.

BIRKBECK, T.H. and WHITELAW, D.D. (1980).
Immunogenicity and molecular characterisation of staphylococcal delta-haemolysin. Journal of Medical Microbiology. 13: 213-221.

BOROWSKI, J. and TYBUSZ, R. (1963).
The effect of human sera on coagulase-positive and coagulase-negative staphylococci. Pathologia et Microbiologia. 26: 89-99.

BOYCE, J.M. and CAUSEY, W.A. (1982).
Increasing occurrence of methicillin-resistant Staphylococcus aureus in the United States. Infection Control. 3: 377-383.

BREED, R.S. (1956).
Staphylococcus pyogenes Rosenbach. International Bulletin of Bacteriological Nomenclature and Taxonomy. 6: 35-42.

BROCK, J.H. and REITER, B. (1976).
Chemical and biological properties of extracellular slime produced by Staphylococcus aureus grown in high-carbohydrate, high-salt medium. Infection and Immunity 13: 653-660.

BROCK, J.H; TURVEY, A. and REITER, B. (1973).
Virulence of two mastitis strains of Staphylococcus aureus in bovine skin: enhancement by growth in high carbohydrate high salt medium or in raw milk. Infection and Immunity. 7: 865-872.

BROWDER, H.P; TAVORMINA, P.A. and ZYGMUNT, W.A. (1968).
Optical configuration of staphylococcal cell wall serine. Journal of Bacteriology, 96: 1452-1453.

BRYANT, R.E. (1969).
The effect of divalent cation depletion on phagocytosis of staphylococci. Yale Journal of Biology and Medicine. 41: 303-310.

BURNET, F.M. (1930).
Production of staphylococcal toxin. Journal of Pathology and Bacteriology. 33: 1-16.

CARTER, C.W; COXON, R.V; PARSONS, D.S. and THOMPSON, R.H.S. (1959).
Biochemistry in relation to medicine. 3rd edition, pp 421-422, London, Longmans, Green and Co.

CAWDERY, M; FOSTER, W.D; HAWGOOD, B.C. and TAYLOR, C. (1969).
The role of coagulase in the defence of Staphylococcus aureus against phagocytosis. British Journal of Experimental Pathology. 50: 408-412.

CHAO, L-P. and BIRKBECK, T.H. (1978).
Assay of staphylococcal delta-haemolysin with fish erythrocytes. Journal of Medical Microbiology. 11: 303-313.

CHESBRO, W.R; HEYDRICK, F.P; MARTINEAU, R. and PERKINS, G.N. (1965).
Purification of staphylococcal beta-haemolysin and its action of staphylococcal and streptococcal cell walls. Journal of Bacteriology. 89: 378-389.

CHRISTIE, R; ATKINS, N.E. and MUNCH-PETERSEN, E. (1944).
A note on a lytic phenomenon shown by group B streptococci. Australian Journal of Experimental Biology and Medical Science. 22: 197-200.

CHRISTIE, R; NORTH, E.A. and PARKIN, B.J. (1946).
Criteria of pathogenicity in staphylococci. Australian Journal of Experimental Biology and Medical Science. 24: 73-78.

CIBOROWSKI, P; GARLINSKI, P; ROSZKOWSKI, W. and JELJASZEWICZ, J. (1988).
Phagocytosis of staphylococci preincubated in subinhibitory concentration of selected antibiotics In: In vitro studies on Gram-positive bacteria, PS-2 44. 6th Mediterranean Congress of Chemotherapy. Abstract, Italy.

CIBOROWSKI, P. and JELJASZEWICZ, J. (1985).
Staphylocccal enzymes and virulence. In: Bacterial Enzymes and Virulence, I.A. Holder (ed), pp 145-183. CRC Press.

CLARK, L.A. and EASMON, C.S.F. (1986).
Opsonic requirements of Staphylococcus epidermidis. Journal of Medical Microbiology 22: 1-7.

CLUFF, L.E; REYNOLDS, R.C; PAGE, D.L. and BRECKENRIDGE, J.L. (1968).
Staphylococcal bacteremia and altered host resistance. Annals of Internal Medicine. 69: 859-873.

COHN, F. (1872).
Untersuchungen uber Bacterien. Beitrage zur Biologie der Pflanzen 2, 1: 127-224.

COHN, Z.A. and MORSE, S.I. (1959).
Interactions between rabbit polymorphonuclear leucocytes and staphylococci. Journal of Experimental Medicine. 110: 419-443.

COLEMAN, G. (1983).
The effect of glucose on the differential rates of extracellular protein and alpha-toxin formation by Staphylococcus aureus (Wood 46). Archives of Microbiology. 134: 208-211.

COLEMAN, G. (1981).
Pleiotropic compensation in the regulation of extracellular protein formation by a low alpha-toxin producing variant of Staphylococcus aureus (Wood 46). Journal of General Microbiology 122: 11-15.

COLEMAN, G. and ABBAS-ALI, B. (1977).
Comparison of the patterns of increase in alpha-toxin and total extracellular protein by Staphylococcus aureus (Wood 46) grown in media supporting widely differing growth characteristics. Infection and Immunity. 17: 278-281.

COOPER, L.Z: MADOFF, M.A. and WEINSTEIN, L. (1964).
Haemolysis of rabbit erythrocyes by purified staphylococcal alpha-toxin. Journal of Bacteriology. 87: 127-135.

COVE, J.H; HOLLAND, K.T. and CUNLIFFE, W.J. (1980).
The vitamin requirements of staphylococci isolated from human skin. Journal of Applied Bacteriology, 49: 29-37.

COWAN, S.T; SHAW, C. and WILLIAMS, R.E.O. (1954).
Type strain for Staphylococcus aureus Rosenbach. Journal of General Microbiology. 10: 174-176.

COWAN, S.T. and STEEL, K.J. (1974).
Cowan and Steel's Manual for the identification of medical bacteria. 2nd edition, revised by S.T. Cowan, Cambridge University Press.

CRUICKSHANK, R. (1970).
Cultivation of microorganisms: use of culture media. In: Medical Microbiology, 11th edition, pp 790-811. Edinburgh and London, UK: Livingstone.

CYBULSKA, J. and JELJASZEWICZ, J. (1966a).
Bacteriostatic activity of serum against staphylococci. Journal of Bacteriology. 91: 953-962.

CYBULSKA, J. and JELJASZEWICZ, J. (1966b).
Bacteriostatic activity of normal serum against staphylococci. Postepy Mikrobiologii. 5: 261-265. (Symposium on staphylococci and staphylococcal infections, Warsaw, 1965).

DALEN, A.B. (1973a).
Production of staphylococcal alpha-toxin in a defined medium and identification of a stimulating factor from yeast extract. Journal of General Microbiology, 74: 53-60.

DALEN, A.B. (1973b).
The induction of staphylococcal alpha-toxin by histidine. Journal of General Microbiology. 74: 61-69.

DALEN, A.B. (1973c).
The influence of pH and histidine dipeptides on the production of staphylococcal alpha-toxin. Journal of General Microbiology. 79: 265-274.

DAVIS, G.H.G. and HOYLING, B. (1973).
Use of a rapid acetoin test in the identification of staphylococci and micrococci. International Journal of Systematic Bacteriology. 23: 281-282.

DENYS, J. and LECLEF, J. (1895).
Sur le mechanisme de l'immunite chez le lapin vaccine contre le streptocoque pyogene. Cellule 11: 177-221.

DEVRIESE, L.A. (1977).
Isolation and identification of Staphylococcus hyicus. American Journal of Veterinary Research. 38: 787-792.

DEVRIESE, L.A. and HAJEK, V. (1980).
Identification of pathogenic staphylococci isolated from animals and foods. Derived from animals. Journal of Applied Bacteriology. 49: 1-11.

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: 295-301.

DOERY, H.M; MAGNUSSON, B.J; CHEYNE, I.M. and GULASEKHARAM, J. (1963).
A phospholipase in staphyloccocal toxin which hydrolyzes sphingomyelin. Nature (London). 198: 1091-1092.

DOERY, H.M; MAGNUSSON, B.J; GULASEKHARAM, J. and PEARSON, J.E. (1965).
The properties of phospholipase enzymes in staphylococcal toxins. Journal of General Microbiology. 40: 283-296.

DONAHUE, J.A. (1969).
Antistaphylococcal haemolysins and delta-haemolysin inhibitor in adult human serum. Canadian Journal of Microbiology. 15: 957-959.

DOSSETT, J.H; KRONVALL, G; WILLIAMS, R.C. (Jnr). and QUIE, P.G. (1969).
Antiphagocytic effects of staphylococcal protein A. Journal of Immunology. 103: 1405-1410.

DOWNEY, R.J. and KEJIMA, M. (1967).
Influence of serum on intracellular digestion of Staphylococcus aureus by polymorphonuclear neutrophils from the guinea pig. Journal of Reticuloendothelial Society. 4: 168-176.

DRANGSHOLT, C. (1948).
Preparation of peptone and amino-acid containing extracts from press water. British Patent 641,120.

DREIZEN, S; MOSNY, J.J; GILLEY, E.J. and SPIES, T.D. (1954).
The amino acid requirements of oral acidogenic microorganisms associated with human dental caries. Journal of Dental Research, 33: 339-345.

DUNCAN, J.L. and CHO, G.J. (1971).
Production of staphylococcal alpha-toxin. I: Relationship between cell growth and toxin formation. Infection and Immunity. 4: 456-461.

DUNCAN, J.L. and CHO, G.J. (1972).
Production of staphylococcal alpha-toxin. II: Glucose repression of toxin formation. Infection and Immunity. 6: 689-694.

EDGAR, J.B. and STOCKER, B.A.D. (1961).
Metabolic and genetic investigations of nutritionally exacting strains of Staphylococcus pyogenes. Nature, 191: 1121-1122.

EDITORIAL BOARD (1958).
Conservation of the generic name Staphylococcus Rosenbach, designation of Staphylococcus aureus Rosenbach, as the nomenclatural type of the genus Staphylococcus Rosenbach, and designation of a neotype culture of Staphylococcus aureus Rosenbach. Opinion 17. International Bulletin of Bacteriological Nomenclature and Taxonomy. 8: 138-153.

EHRENKRANZ, N.J; ELLIOTT, D.F. and ZARCO, R. (1971).
Serum bacteriostasis of Staphylococcus aureus. Infection and Immunity. 3: 664-670.

EKSTEDT, R.D. (1956).
Further studies on the antibacterial activity of human serum on Micrococcus pyogenes and its inhibition by coagulase. Journal of Bacteriology. 72: 157-161.

EKSTEDT, R.D. and BERNHARD, J.M. (1973).
Preparation and characterisation of a slime layer material produced by Staphylococcus aureus. Proceedings of the Society for Experimental Biology and Medicine. 142: 86-91.

EKSTEDT, R.D. and NUNGESTER, W.J. (1955).
Coagulase in reversing antibacterial activity of normal human serum on Micrococcus pyogenes. Proceedings of the Society for Experimental Biology and Medicine. 89: 90-94.

EKSTEDT, R.D. and YOSHIDA, K. (1969).
Immunity to staphylococcal infection in mice: effect of living versus killed vaccine. Role of circulating antibody, and induction of protection-inducing antigen(s) in vitro. Journal of Bacteriology. 100: 745-750.

ELEK, S.D. (1959).
Staphylococcus pyogenes and its relation to disease. E. and S. Livingstone, Edinburgh and London.

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.

ELEK, S.D. and LEVY, E. (1950).
Distribution of haemolysins in pathogenic and non-pathogenic staphylococci. Journal of Pathology and Bacteriology. 62: 541-545.

ELLWOOD, D.C. and TEMPEST, D.W. (1972).
Effects of environment on bacterial wall content and composition. Advances in Microbiology and Physiology. 7: 83-117.

ENG, R.H.K; WANG, C; PERSON, A; KIEHN, T.E. and ARMSTRONG, D. (1982).
Species identification of coagulase-negative staphylococcal isolated from blood cultures. Journal of Clinical Microbiology. 15: 439-442.

EVANS, J.B. (1976).
Anaerobic growth of Staphylococcus species from human skin: Effects of uracil and pyruvate. International Journal of Systematic Bacteriology. 26: 17-21.

EVANS, J.B; BRADFORD, W.L. and NIVEN, C.F. (Jnr) (1955).
Comments concerning the taxonomy of the genera Micrococcus and Staphylococcus. International Bulletin of Bacteriological Nomenclature and Taxonomy. 5: 61-66.

FACKRELL, H.B. and WISEMAN, G.M. (1974).
Immunogenicity of the delta haemolysin of Staphylococcus aureus. Journal of Medical Microbiology. 7: 411-414.

FACKRELL, H.B. and WISEMAN, G.M. (1976).
Properties of the gamma haemolysin of Staphylococcus aureus Smith 5R. Journal of General Microbiology. 92: 11-24.

FILDES, P. and RICHARDSON, G.M. (1937).
The nutrition of Staphylococcus aureus: sulphur requirements. British Journal of Experimental Pathology. 18: 292-304.

FILDES, P; RICHARDSON, G.M; KNIGHT B.C.J.G. and GLADSTONE, G.P. (1936).
A nutrient mixture suitable for the growth of Staphylococcus aureus. British Journal of Experimental Pathology. 17: 481-484.

FINLAND, M. (1973).
Excursions into epidemiology: selected studies during the past four decades at Boston City Hospital. Journal of Infectious Diseases. 128: 76-124.

FINLAND, M; PETERSON, O.L. and STRAUSS, E. (1942).
Staphylococcal pneumonia occurring during an epidemic of influenza. Archives of Internal Medicine. 70: 183-205.

FLETCHER, S. (1962).
The growth of staphylococci in human serum. Journal of Pathology and Bacteriology. 84: 327-335.

FORSGREN, A. and QUIE, P.G. (1974).
Effects of Staphylococcal protein A on heat labile opsonins. Journal of Immunology. 112: 1177-1180.

FOSTER, E.A. (1967).
Tissue injury by toxins in experimental staphylococcal infections. Effect of polyvalent antitoxin on developing renal infections. American Journal of Pathology. 51: 913-928.

FRASER, G. (1964).
Studies on staphylococci of canine origin with particular reference to the nature and distribution of their haemolysins. Research in Veterinary Science. 5: 365-375.

FREEMAN, B.A. (1979).
Burrows Textbook of Microbiology. 21st Edition. W.B. Saunders Company, Philadelphia, London, Toronto.

FREER, J.H. and ARBUTHNOTT, J.P. (1983).
Toxins of Staphylococcus aureus. Pharmacology and Therapeutics. 19: 55-106.

FREER, J.H; ARBUTHNOTT, J.P. and BILLCLIFFE, B. (1973).
Effects of staphylococcal alpha-toxin on the structure of erythrocyte membranes: A biochemical and freeze-etching study. Journal of General Microbiology. 75: 321-332.

FRITSCHE, D. (1970).
Untersuchungen zur Funktion des beta-hamolysins von Staphylococcus aureus. Zentralblat fuer Bakteriologie Parasitenkun De Infektionskrankeiten und Hygiene Abteilung 1: originale Reihe B. 213: 348-355.

FRITSCHE, D. and ZITZ, M. (1973).
The effect of uptake of free fatty acids on the growth and the beta-haemolysin production of Staphylococcus aureus. Medical Microbiology and Immunology. 158: 185-191.

FROBISHER, M; HINSDILL, R.D; CRABTREE, K.T. and GOODHEART, C.R. (1974).
Fundamentals of microbiology. 9th Edition. W.B. Saunders Company, Philadelphia, London, Toronto.

GEMMELL, C.G. (1983).
Extracellular toxins and enzymes of coagulase-negative staphylococci. In: Staphylococci and Staphylococcal Infections, C.S.F. Easmon and C. Adlam (Eds), Vol. 2, Academic Press, London. pp 809-827.

GEMMELL, C.G; PETERSON, P.K; SCHMELING, D.J. and QUIE, P.G. (1982a).
Effect of staphylococcal alpha-toxin on phagocytosis of staphylococci by human polymorphonuclear leukocytes. Infection and Immunity. 38: 975-980.

GEMMELL, C.G; PETERSON, P.K; TOWNSEND, K; QUIE, P.G. and KIM, Y. (1982b).
Biological effects of the interaction of staphylococcal alpha-toxins with human serum. Infection and Immunity. 38: 981-985.

GEMMELL, C.G. and SCHUMACHER-PERDREAU, F. (1986).
Extracellular toxins and enzymes elaborated by coagulase-negative staphylococci. In: Coagulase-negative staphylococci. P.A. Mardh and K.H. Schleifer (Eds), pp 109-121. Almquist and Wiksell International Stockholm, Sweden.

GEMMELL, C.G. and THELESTAM, M. (1981).
Toxinogenicity of clinical isolates of coagulase-negative staphylococci towards various animal cells. Acta Pathologica et Microbiologica Scandinavica (B), 89: 417-421.

GEMMELL, C.G; THELESTAM, M. and WADSTROM, T. (1976).
Toxigenicity of coagulase-negative staphylococci. In: Staphylococci and staphylococcal diseases, J. Jeljaszewicz, (Ed), Zentralblatt fur Bakteriologie, Mikrobiologie und Hygiene Supplemente 5, Gustav Fischer Verlag, Stuttgart, New York. pp 133-136.

GENGOU, O. (1935).
De l'action des secretions staphylococciques sur les hematoblastes: leurs role dans la production des thrombi post-operatoires. Annales de l'Institut Pasteur, t. 54: 428-441.

GIEHL, T.J; QORONFLEH, M.W. and WILKINSON, B.J. (1987).
Transport, nutritional and metabolic studies of taurine in staphylococci. Journal of General Microbiology. 133: 849-856.

GIGLI, I. and NELSON, R.A. (1968).
Complement dependent immune phagocytosis. I: Requirements for C1, C4, C2, C3. Experimental Cell Research 51: 45-67.

GILL, D.M. (1982).
Bacterial toxins: A table of lethal amounts. Microbiological Reviews. 46: 86-94.

GILLESPIE, W.A. and ALDER, V.G. (1952).
Production of opacity in egg-yolk media by coagulase-positive staphylococci. Journal of Pathology and Bacteriology. 64: 187-200.

GLADSTONE, G.P. (1937).
The nutrition of Staphylococcus aureus nitrogen requirements. British Journal of Experimental Pathology. 18: 322-333.

GLADSTONE, G.P. (1938).
The production of staphyloccocal alpha-haemolysin in a chemically defined medium. British Journal of Experimental Pathology. 19: 208-226.

GLADSTONE, G.P. (1973).
The effect of iron and haematin on the killing of staphylococci by rabbit polymorphonuclear leucocytes: with notes on the inhibition by iron of serum b-lysins and staphylocidal cationic proteins from polymorphonuclear leucocytes. In: Staphylococci and Staphylococcal Infections, J. Jeljaszewicz (Ed), Vol. 1, Contributions to Microbiology and Immunology. S. Karger. Basel, London. pp 222-243.

GLADSTONE, G.P. and VAN HEYNINGEN, W.E. (1957).
Staphylococcal leukocidins. British Journal of Experimental Pathology. 38: 123-137.

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 leukocytes. British Journal of Experimental Pathology. 55: 427-447.

GLADSTONE, G.P. and YOSHIDA, A. (1967).
The cytopathic action of purified staphylococcal delta haemolysin. British Journal of Experimental Pathology. 48: 11-19.

GLENNY, A.T. and STEVENS, M.F. (1935).
Staphylcoccus toxins and antitoxins. Journal of Pathology and Bacteriology. 40: 201-210.

GRETLER, A.C. and EVANS, J.B. (1951).
Vitamin requirements of staphylococci. Bacterial Proceedings. 1951: 147-148.

GRETLER, A.C; MUCCIOLO, P; EVANS, J.B. and NIVEN, C.F. (Jnr) (1955)
Vitamin nutrition of the staphylococci with special reference to their biotin requirements. Journal of Bacteriology. 70: 44-49.

GROV, A. (1976).
The effect of specific antibodies on the inhibition of leukocyte migration caused by staphylococcal peptidoglycan. Acta Patholologica et Microbiologica Scandinavica. Section B. 84: 315-317.

GROV, A; HELGELAND, S. and ENDRESEN, C. (1976).
Inhibition of leucocyte migration by peptidoglycan fragments. In: Staphylococci and Staphylococcal Diseases and Staphylococcal Infections. J. Jeljaszewicz (Ed). Zentralblatt fur Bakteriologie: Abteilung 1. Supplemente 5. Gustavfischer Verlag. Stuttgart, New York. pp 389-392.

GRUN, V.L. (1968).
Zur bestimmung von standartvarianten der staphylokokken humaner und boviner herkunft. Milchwissenschaft 23: 604-608.

GUIRARD, B. (1974).
The amino acid requirements of microorganisms for growth. In: Handbook of Microbiology: Vol. IV: Microbial Metabolism, Genetics and Immunology, A.I. Laskin and H.A. Lechevalier, (Eds). Cleveland, Ohio, The Chemical Rubber Co. pp 9.

GULASEKHARAM, J; CHEYNE, I; DOERY, H.M. and MAGNUSSON, B.J. (1963).
Serum opacity reaction produced by staphylococci. Nature, 198: 1114.

GUYONNET, F. and PLOMMET, M. (1970).
Haemolysine gamma de Staphylococcus aureus: Purification et proprietes. Annales de l'Institut Pasteur. 118: 19-33.

HACH, W. and POLULACH, N. (1936).
Nutritive media from fish products. Archives of Biological Science, USSR. 41: 163-165.

HAJEK, V. and MARSALEK, E. (1971).
The differentiation of pathogenic Staphylococcus and a suggestion for their taxonomic classification. Zentralblatt fur Bakteriologie, parasitenkunde, Infecktionskrankheiten und Hygiene (Abteilung, I), A217: 176-182.

HALE, J.H. and SMITH, W. (1945).
The influence of coagulase on the phagocytosis of staphylococci. British Journal of Experimental Pathology. 26: 209-217.

HALEY, R.W; HIGHTOWER, A.W; KHABBAZ, R.F; THORNSBERRY, C; MARTONE, W.J; ALLEN, J.R. and HUGHES, J.M. (1982).
The emergence of methicillin-resistant Staphylococcus aureus infections in United States hospitals. Annals of Internal Medicine. 97: 297-308.

HALLANDER, H.O. and BENGTSSON, S. (1967).
Studies on the cell toxicity and species specificity of purified staphylococcal toxins. Acta Patholologica et Microbiologica Scandinavica. 70: 107-119.

HAMMES, W; SCHLEIFER, K.H. and KANDLER, O. (1973).
Mode of action of glycine on the biosynthesis of peptidoglycan. Journal of Bacteriology. 116: 1029-1053.

HARRIGAN, W.F. and McCANCE, M.E. (1969).
Laboratory methods in microbiology. Academic Press, London and New York.

HARSHMAN, S. and SUGG, N. (1985).
Effect of calcium ions on staphylococcal alpha-toxin-induced haemolysis of rabbit erythrocytes. Infection and Immunity. 47: 37-40.

HEATLEY, N.G. (1971).
A new method for the preparation and some properties of staphylococcal delta haemolysin. Journal of General Microbiology. 69: 269-278.

HED, J. and STENNDAHL, O. (1982).
Differences in the ingestion mechanisms of IgG and C_3b particles in phagocytosis by neutrophils. Immunology. 45: 727-736.

HEYMER, B. (1975).
Biological properties of the peptidoglycan. Zeitschrift fuer Immunitaetsforschung Experimentelle und Klinische Immunologie. 149: 245-257.

HILL, L.R. (1981).
Taxonomy of the staphylococci. In: The Staphylococci. Proceedings of the Alexander Ogston Centennial Conference. A. Macdonald and G. Smith (eds). Aberdeen University Press, UK. pp 33-62.

HIRSCH, J.G. (1960).
Comparative bactericidal activities of blood serum and plasma serum. Journal of Experimental Medicine. 112: 15-22.

HOF, D.G; REPINE, J.E; PETERSON, P.K. and HOIDAL. J.R. (1980).
Phagocytosis by human alveolar macrophages and neutrophils: Qualitative differences in the opsonic requirements for uptake of Staphylococcus aureus and Streptococcus pneumoniae in vitro. American Review of Respiratory Disease. 121: 65-71.

HOIDAL, J.R: SCHMELING, D. and PETERSON, P.K. (1981).
Phagocytosis, bacterial killing and metabolism by purified human lung phagocytes. The Journal of Infectious Disease. 144: 61-71.

HOLT, R.J. (1971).
The colonization of ventriculoatrial shunts by coagulase-negative Staphylococci. In: Bacterial Infections: Changes in their Causative Agents, Trends and Possible Basis. M. Finland, W. Marget and K. Bartmann (eds). Springer Verlag, Berlin. pp 81-87.

HUBALEK, Z. (1969).
Numerical taxonomy of genera Micrococcus Cohn and Sarcina Goodsir, Journal of General Microbiology. 57: 349-363.

HUMPHREYS, D.W; WHEAT, L.J. and WHITE, A. (1974).
Staphylococcal heat-stable opsonins. Journal of Laboratory and Clinical Medicine. 84: 122-128.

INTERNATIONAL Committee on Nomenclature of Bacteria. Subcommittee on Taxonomy of Staphylococci and Micrococci (1968). Minutes. International Journal of Systematic Bacteriology. 21: 161-163.

JACKSON, A.W. and LITTLE, R.M. (1957).
Leucocidal effects of staphylococcal dela-lysin. Canadian Journal of Microbiology. 3: 101-102.

JASSIM, S.A.A. (1984).
Growth and toxin production of Staphylococcus aureus in dairy products. MPhil Thesis, University of Nottingham, School of Agriculture.

JASSIM, S; SALT, W.G. and STRETTON, R.J. (1988).
The preparation and use of media based on a simple fish waste extract. Letters in Applied Microbiology. 6: 139-143.

JELJASZEWICZ, J. (1960).
Studies on staphylococcal coagulase. VI. Occurrence of toxic and biochemical activities among coagulase-negative and coagulase-positive staphylococci. Medycyna Doswiadczalnai Mikrobiologia. 12: 33-41.

JELJASZEWICZ, J. (1972).
Toxins (haemolysins). In: The Staphylococci. J.O. Cohen, (Ed), Wiley Interscience, New York, 249-280.

JELJASZEWICZ, J; NIEWIAROWSKI, S; POPLAWSKI, A. and BLAWAT, L. (1966).
Platelet aggregation by staphylococcal toxins. Thrombosis et Diathesis Haemorrhagica. 15: 69-83.

JELJASZEWICZ, J; SZMIGIELSKI, S. and HRYNIEWICZ, W. (1978).
Biological effects of staphylococcal and streptococcal toxins. In: Bacterial toxins and cell membranes. J. Jeljaszewicz and T. Wadstrom, (eds). Academic Press, London and New York. pp 185-227.

JONES, D; DEIBEL, R.H. and NIVEN, C.F. (Jnr) (1963).
Identity of Staphylococcus epidermidis. Journal of Bacteriology. 85: 62-67.

JOZEFCZYK, Z. and RACZKA, A. (1985).
In vitro effect of staphylococcal enterotoxins A-F and specific antibodies on phagocytosis of latex by human PMN granulocytes. In: The Staphylococci. J. Jeljaszewicz (Ed). Zentralblatt fur Bakteriologie, Mikrobiologie und Hygiene Supplemente; 14. Gustav Fischer Verlag, Stuttgart, New York, pp 443-445.

KANTOR, H.S; TEMPLE, B. and SHAW, W.V. (1972).
Staphylococcal delta haemolysin: purification and characterisation. Archives of Biochemistry and Biophysics. 151: 142-156.

KAPRAL, F.A. (1967).
Neutralization of Staphylococcus aureus delta haemolysin by phospholipids. Bacteriological Proceedings. 1967: 64.

KAPRAL, F.A. and SHAYEGANI, M.G. (1959).
Intracellular survival of staphylococci. Journal of Experimental Medicine. 110: 123-138.

KARAKAWA, W.W. and YOUNG, D.A. (1979).
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; YOUNG, D.A. and KANE, J.A. (1978).
Structural analysis of the cellular constituents of a fresh clinical isolate of Staphylococcus aureus and their role in the interaction between the organisms and polymorphonuclear leukocytes in the presence of serum factors. Infection and Immunity. 21: 496-505.

KARCHMER, A.W; ARCHER, G.L. and DISMUKES, W.E. (1983).
Staphylococcus epidermidis causing prosthetic valve endocarditis: microbiologic and clinical observations as guides to therapy. Annals of Internal Medicine. 98: 447-455.

KARNOVSKY, M.L. and BOLIS, L. (Eds) (1982).
Phagocytosis - past and future. London and New York, Academic Press.

KERN, R.A; KINGKADE, M.J; KERN, S.F. and BEHRENS, O.K. (1951).
Characterization of the action of lysozyme on Staphylococcus aureus and on Micrococcus lysodeikticus. Journal of Bacteriology. 61: 171-178.

KILTON, L.J; FOSSIECK, B.E; COHEN, M.H. and PARKER, R.H. (1979).
Bacteremia due to gram-positive cocci in patients with neoplastic disease. American Journal of Medicine. 66: 596-602.

KING, E.O; FROBISHER, M. and PARSONS, E.I. (1949).
In vitro test for virulence of Corynebacterium diphtheriae. American Journal of Public Health. 39: 1314-1320.

KIRBY, W.M.M. (1965).
Therapeutic aspects of staphylococcal disease. Annals of New York Academy of Sciences. 128: 443-450.

KLEBANOFF, S.J. and CLARK, R.A. (1978).
The neutrophil: Function and clinical disorders. North Holland Publishing, Amsterdam, New York, Oxford.

KLOOS, W.E. (1980).
Natural population of the genus Staphylococcus. Annual Review of Microbiology. 34: 559-592.

KLOOS, W.E. and JORGENSEN, J.H. (1985).
Staphylococci. In: Manual of Clinical Microbiology. E.H. Lennette, A. Balows, W.J. Hausler Jr, H.J. Shadomy (Eds). Fourth edition. American Society for Microbiology. pp 143-153.

KLOOS, W.E. and PATTEE, P.A. (1965).
A biochemical characterization of histidine-dependent mutants of Staphylococcus aureus. Journal of General Microbiology. 39: 185-194.

KLOOS, W.E. and SCHLEIFER, K.H. (1975a).
Simplified scheme for routine identifications of human Staphylococcus species. Journal of Clinical Microbiology. 1: 82-88.

KLOOS, W.E. and SCHLEIFER, K.H. (1975b).
Isolation and chacterization of Staphylococci from human skin. II. Descriptions of four new species: Staphylococcus warneri, Staphylococcus capitis, Staphylococcus hominis and Staphylococcus simulans. International Journal of Systematic Bacteriology. 25: 62-79.

KLOOS, W.E. and SCHLEIFER, K.H. (1986).
Staphylococcus. In: Bergey's Manual of Systematic Bacteriology. Williams and Wilkins, Baltimore. pp 1011-1035.

KLOOS, W.E. and SCHULTES, L.M. (1969).
Transformation in Micrococcus lysodeikticus. Journal of General Microbiology. 55: 307-317.

KNIGHT, B.C.J.G. (1937).
The nutrition of Staphylococcus aureus: Nicotinic acid and vitamin B_1. Biochemical Journal. 31: 731-737.

KNOX, K.W. and WICKEN, A.J. (1973).
Immunological properties of teichoic acids. Bacteriological Reviews. 37: 215-257.

KOENIG, M.G. (1972).
The Phagocytosis of staphylococci. In: The Staphylococci. J.O. Cohen (Ed). Wiley-Interscience, London. pp 365-383.

KOENIG, M.G; MELLY, M.A. and ROGERS, D.E. (1962).
Factors relating to the virulence of staphylococcus. II. Observations on four mouse-pathogenic strains. Journal of Experimental Medicine. 116: 589-599.

KOLAWOLE, D.O. (1983).
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. 91: 211-222.

KORMAN, R.Z. (1966).
Elevated cell wall serine in pleiotropic staphylococcal mutants. Journal of Bacteriology. 92: 762-768.

KREGER, A.S. (1970).
Cited by Jeljaszewicz, J; Szmigielski, S. and Hryniewicz, W. (1978). Biological effects of staphylococcal and streptococcal toxins. In: Bacterial Toxins and Cell Membranes. J. Jeljaszewicz and T. Wadstrom, (eds). Academic Press, London. pp 185-227.

KREGER, A.S; KIM, K.S; ZABORETZKY, F. and BERNHEIMER, A.W. (1971).
Purification and properties of staphylococcal delta haemolysin. Infection and Immunity. 3: 449-465.

KRONVALL, G. and GEWURZ, H. (1970).
Activation and inhibition of IgG mediated complement fixation by staphylococcal protein A. Clinical and Experimental Immunology. 7: 211-220.

KUREK, M; PRYJMA, K; BARTKOWSKI, S. and HECZKO, P.B. (1977).
Anti-staphylococcal gamma haemolysin antibodies in rabbits with staphylococcal osteomyelitis. Medical Microbiology and Immunology. 163: 61-65.

LADISCH, S. and PIZZO, P.A. (1978).
Staphylococcus aureus sepsis in children with cancer. Pediatrics. 61: 231-234.

LAUE, P. and MacDONALD, R.E. (1968).
Growth stimulation of Staphylococcus aureus by L. Cystine or L. Djenkolic acid. Journal of Bacteriology. 95: 2420-2421.

LAXDAL, T; MESSNER, R.P; WILLIAMS, R.C. and QUIE, P.G. (1968).
Opsonic, agglutinating and complement fixing antibodies in patients with subacute bacterial endocarditis. Journal of Laboratory and Clinical Medicine. 71: 638-653.

LEBOEUF-TRUDEAU, T; De REPENTIGNY, J; FRENETTE, R.M. and SONEA, S. (1969).
Tryptophan metabolism and toxin formation in Staphylococcus aureus Wood 46 strain. Canadian Journal of Microbiology, 15: 1-7.

LEIJH, P.C.J; Van den BARSELAAR, M.T; Van ZWET, T.L; DAHA, M.R. and Van FURTH, R. (1979).
Requirement of extracellular complement and Immunoglobulin for Intracellular killing of micro-organisms by human monocytes. Journal of Clinical Investigation. 63: 772-784.

LEIJH, P.C.J; Van FURTH, R. and Van ZWET, T.L. (1986).
In vitro determination of phagocytosis and intracellular killing by polymorphonuclear and mononuclear phagocytes. In: Handbook of Experimental Immunology; Cellular Immunology. D.M. Weir, L.A. Herzenberg, C. Blackwell and L.A. Herzenberg, (Eds). Blackwell Scientific Publications, Oxford, London. Vol. 2, pp 46.1-46.21.

LEINONEN, M. (1985).
Serological methods for the study of bacterial surface antigens. In: Enterobacterial surface antigens: methods for molecular characterisation. T.K. Korhonen, E.A. Dawes and P.H. Makela, eds. Elsevier Science Publishers (Biomedical Division). pp 179-206.

LI, I.W. and MUDD, S. (1965).
The heat-labile serum factor associated with intracellular killing of Staphylococcus aureus. Journal of Immunology. 94: 852-857.

LI, I.W; MUDD, S. and KAPRAL, F.A. (1963).
Dissociation of phagocytosis and intracellular killing of Staphylococcus aureus by human blood leukocytes. Journal of Immunology. 90: 804-809.

LO, C.Y. and FACKRELL, H.B. (1979).
Immunologic evidence that staphylococcal alpha-toxin is oriented on membranes. Canadian Journal of Microbiology. 25: 686-692.

LOW, D.K-R. and FREER, J.H. (1977a).
Biological effect of highly purified beta-lysin (sphingomyelinase C) from Staphylococcus aureus. Federation of European Microbiological Societies, Microbiology Letters. 2: 133-138.

LOW, D.K-R. and FREER, J.H. (1977b).
The purification of beta-lysin (sphingomyelinase C) from Staphylococcus aureus Federation of European Microbiological Societies, Microbiology Letters. 2: 139-143.

LOW, D.K-R; FREER, J.H; ARBUTHNOTT, J.P; MOLLBY, R. and WADSTROM, T. (1974).
Consequences of sphingomyelin degradation in erythrocyte ghost membranes by staphylococcal beta-toxin (sphingomyelinase C). Toxicon. 12: 279-285.

LUCAS, P.R. and SEELEY, H.W. (1955).
A catalase negative Micrococcus pyogenes var aureus. Journal of Bacteriology. 69: 231.

LUKASZKIEWICZ, Z. and CHOJNOWSKI, W. (1985).
Characteristics of methicillin-resistant clinical isolates of Staphylococcus aureus. In: The Staphylococci. J. Jeljaszewicz, (ed). Zentralblatt fur Bakteriologie Mikrobiologie und Hygiene. I. Abteilung supplemente 14. Gustav Fischer Verlag, Stuttgart, New York. pp 599-600.

LYONS, C. (1937).
Antibacterial immunity Staphylococcus pyogenes. British Journal of Experimental Pathology. 18: 411-422.

MAH, R.A; FUNG, D.Y.C. and MORSE, S.A. (1967).
Nutritional requirements of Staphylococcus aureus S-6. Applied Microbiology. 15: 866-870.

MAHESWARAN, S.K; FROMMES, S.P. and LINDORFER, R.K. (1969).
Ultrastructural changes in neutrophils treated with staphylococcal alpha-toxin. Canadian Journal of Microbiology. 15: 128-129.

MALTMAN, J.R; ORR, J.H. and HINTON, N.A. (1960).
The effect of desiccation on Staphylococcus pyogenes with special reference to implications concerning virulence. American Journal of Hygiene. 72: 335-342.

MAMO, W; ROZGONYI, F; BROWN, A; HJERTEN, S. and WADSTROM. T. (1987).
Cell surface hydrophobicity and charge of Staphylococcus aureus and coagulase-negative staphylococci from bovine mastitis. Journal of Applied Bacteriology. 62: 241-249.

MARKS, J. (1951).
The standardization of staphylococcal alpha-antitoxin with special reference to anomalous haemolysins including gamma-lysin. Journal of Hygiene. (Cambridge) 49: 52-66.

MARKS, J. (1952).
Recognition of pathogenic staphylococci with notes on non-specific staphylococcal haemolysin. Journal of Pathology and Bacteriology. 64: 175-186.

MARKS, J. and VAUGHAN, A.C.T. (1950).
Staphylococcal delta-haemolysin. Journal of Pathology and Bacteriology. 62: 597-615.

MATHIEU, L.G; De REPENTIGNY, J; TURGEON, S. and SONEA, S. (1968).
Thymineless death of Staphylococcus aureus and formation of its alpha-toxin. Canadian Journal of Microbiology. 14: 983-987.

McCARTNEY, C.A. and ARBUTHNOTT, J.P. (1978).
Mode of action of membrane damaging toxins produced by staphylococci. In: Bacterial Toxins and Cell Membranes. J. Jeljaszewicz and T. Wadstrom, (eds). Academic Press, London, pp 89-127.

McNIVEN, A.C. and ARBUTHNOTT, J.P. (1972).
Cell associated alpha-toxin from Staphylococcus aureus. Journal of Medical Microbiology. 5: 123-127.

MELLY, M.A; DUKE, L.J; LIAU, D.F. 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. (1893).
Lectures on the comparative pathology of inflammation. Trench Trubner and Co Ltd, London, Kegan Paul.

MILES, A.A. and MISRA, S.S. (1938).
The estimation of the bactericidal power of the 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.

MOLLBY, R. (1976).
Effect of staphylococcal beta-haemolysin (sphingomyelinase C) on cell membranes. In: Staphylococci and Staphylococcal Diseases J. Jeljaszewicz, (ed). Gustav Fischer Verlag, Stuttgart, New York. pp 665-677.

MOLLBY, R. (1983).
Isolation and properties of membrane damaging toxins. In: Staphylococci and Staphylococcal Infections. C.S.F. Easmon and C. Adlam (eds). Academic Press, London. Vol. 2, pp 619-669.

MONTGOMERIE, J.Z; KALMANSON, G.M. and GUZE, L.B. (1968).
Renal failure and infection. Medicine. 47: 1-32.

MORSE, S.I. (1960).
Isolation of a phagocytosis inhibiting substance from culture filtrates of an encapsulated Staphylococcus aureus. Nature. 186: 102-103.

MORSE, S.I. (1973).
Pathogenesis of Staphylococcal infections. In: Symposium on New Approaches for Inducing Natural Immunity to Pyogenic Organisms J.B. Robbins, R.E. Horton and R.M. Krause, (eds). DHEW Publishing No. 74, 553, Washington, DC. pp 131-136.

MUDD, S; LUCKE, B; McCUTCHEON, M. and STRUMIA, M. (1930).
On the mechanism of opsonin and bacteriotropin action. VI. Agglutination and tropin action by precipitin sera. Characterisation of the sensitised surface. Journal of Experimental Medicine. 52: 313-329.

MUDD, S; McCUTCHEON, M. and LUCKE, B. (1934).
Phagocytosis, Physiological Reviews. 14: 210-275.

MULLER-EBERHARD, H.J. (1965).
The role of antibody, complement and other humoral factors in host resistance to infections. In: Bacterial Mycotic Infections of Man R.J. Dubos and J.G. Hirsch (Eds). Pitman Medical Co., London. 4th ed., pp 181-214.

MUNCH-PETERSEN, E. and CHRISTIE, R. (1947).
On the effect of the interaction of Staphylococcal beta-toxin and group-B streptococcal substance on red blood corpuscles and its use as a test for the identification of Streptococcus agalactiae. Journal of Pathology and Bacteriology. 59: 367-371.

MUSHER, D.M. and McKENZIE, S.O. (1977).
Infections due to Staphylococcus aureus. Medicine, 56: 383-409.

NAHMIAS, A; SAKURAI, N; BLUMBERG, R; DOEGE, A. and SULZER, C. (1961).
The Staphylococcus "80/81 Complex": Epidemiological and laboratory observations. Journal of Infectious Diseases. 109: 211-222.

NESBITT, W.E; DOYLE, R.J. and TAYLOR, K.G. (1982).
Hydrophobic interactions and the adherence of Streptococcus sanguis to hydroxylapatite. Infection and Immunity 38: 637-644.

NOLAN, C.M. and BEATY, H.N. (1976).
Staphylococcus aureus bacteremia: current clinical patterns. American Journal of Medicine. 60: 495-500.

O'BRIEN, A.D. and KAPRAL, F.A. (1977).
Effect of Staphylococcus aureus delta-toxin on Chinese hamster ovary cell morphology and Y-1 adrenal cell morphology and steroidogenesis. Infection and Immunity. 16: 812-816.

OEDING, P. and GROV, A. (1972).
Cellular antigens. In: The Staphylococci, J.O. Cohen (Ed). Wiley-Interscience, London, pp 333-356.

OEDING, P; MARADON, J.L; HAJEK, V. and MARSALEK, E. (1971).
A comparison of phage patterns and antigenic structure with biochemical properties of Staphylococcus aureus strains isolated from cattle. Acta Pathologica et Microbiologica Scandinavica. 79B: 357-364.

OGSTON, A. (1880).
Uber Abscesse. Archiv fur Klinische Chirurgie. 25: 588-600.

OGSTON, A. (1882).
Micrococcus poisoning. Journal of Anatomy (London). 17: 24-58.

OWENS, J.J. (1974).
The egg-yolk reaction produced by several species of bacteria. Journal of Applied Bacteriology. 37: 137-148.

OWENS, J.J. and JOHN, P.C.L. (1975).
The egg-yolk and lipolytic reactions of coagulase positive Staphylococci. Journal of Applied Bacteriology. 39: 23-30.

PARISI, J.T. (1985).
Coagulase-negative staphylococci and the epidemiological typing of Staphylococcus epidermidis. Microbiological Reviews. 49: 126-139.

PARKER, M.T. (1983).
Staphylococcus and Micrococcus; the anaerobic Gram-positive cocci. In: Principles of bacteriology, virology and immunity. Systematic Bacteriology. M.T. Parker (Ed). Edward Arnold, London. Seventh editon, Vol. 2: pp 218-245.

PASSET, J. (1885).
Uber Mikroorganismen er eiterigen Zellgewebsentzundung des Menschen. Fortschritte der Medical. 3: 33-43.

PASTEUR, L. (1880).
De l'extension de la theorie des germes a l'etiologie de quelques maladies communes. Comptes Rendus des Seances de la Societe de Biologie. Paris, 90: 1035-1044.

PATTEE, P.A. and NEVELN, D.S. (1975)
Transformation analysis of the linkage groups in Staphylococcus aureus. Journal of Bacteriology. 124: 201-211.

PATTERSON, L.T. and HIGGINBOTHAM, R.D. (1965).
Mouse C-reactive protein and endotoxin-induced resistance. Journal of Bacteriology. 90: 1520-1524.

PAUL, B.B; STRAUSS, R.R; JACOBS, A.A. and SBARRA, A.J. (1970).
Function of H_2O_2, myeloperoxidase, and hexose monophosphate shunt enzymes in phagocytizing cells from different species. Infection and Immunity. 1: 338-344.

PEARCE, J.H; KALAWOLE, D.O. and SCRAGG, M.A. (1976).
Mechanisms of resistance of staphylooccci grown in vivo to polymorph bactericidins. In: Staphylococci and Staphylococcal Disease. J. Jaljaszewicz, (Ed). Gustav Fischer Verlag, Stuttgart. pp 849-854.

PETERS, G; LOCCI, R. and PULVERER, G. (1982).
Adherence and growth of coagulase-negative staphylococci on surfaces of intravenous catheters. Journal of Infectious Diseases. 146: 479-482.

PETERSON, P.K; KIM, Y; WILKINSON, B.J; SCHMELING, D; MICHAEL, A.F. and QUIE, P.G. (1978a).
Dichotomy between opsonization and serum complement activation by encapsulated staphylococci. Infection and Immunity. 20: 770-775.

PETERSON, P.K; VERHOEF, J; SABATH, L.D. and QUIE, P.G. (1976).
Extracellular and bacterial factors influencing staphylococcal phagocytosis and killing by human polymorphonuclear leukocytes. Infection and Immunity. 14: 496-501.

PETERSON, P.K; VERHOEF, J; SABATH, L.D. and QUIE, P.G. (1977a).
Effect of protein A on staphylococcal opsonization. Infection and Immunity. 15: 760-764.

PETERSON, P.K; VERHOEF, J; SCHMELING, D. and QUIE, P.G. (1977b).
Kinetics of phagocytosis and bacterial killing by human polymorphonuclear leukocytes and monocytes. Journal of Infectious Diseases. 136: 502-508.

PETERSON, P.K; WILKINSON, B.J; KIM, Y; SCHMELING, D; DOUGLAS, S.D; QUIE, P.G. and VERHOEF, J. (1978b).
The key role of peptidoglycan in the opsonization of Staphylococcus aureus. Journal of Clinical Investigation. 61: 597-609.

PETERSON, P.K; WILKINSON, B.J; KIM, Y; SCHMELING, D. and QUIE, P.G. (1978c).
Influence of encapsulation on staphylcoccal opsonization and phagocytosis by human polymorphonuclear leukocytes. Infection and Immunity. 19: 943-949.

PHILLIPS, W.E. Jr; KING, R.E. and KLOOS, W.E. (1980).
Isolation of Staphylococcus hyicus subsp. hyicus from a pig with septic polyarthritis. American Journal of Veterinary Research. 41: 274-276.

PHILLIPS, W.E. Jr. and KLOOS, W.E. (1981).
Identification of coagulase-positive Staphylococcus intermedicus and Staphylococcus hyicus subsp. hyicus isolates from veterinary clinical specimens. Journal of Clinical Microbiology. 14: 671-673.

PROCTOR, A.R. and KLOOS, W.E. (1970).
The tryptophan gene cluster of Staphylococcus aureus. Journal of General Microbiology. 64: 319-327.

QUIE, P.G. (1973).
Infections due to neutrophil malfunction. Medicine 52: 411-417.

QUIE, P.G; MESSNER, R.P. and WILLIAMS, R.C. Jr. (1968).
Phagocytosis in subacute bacterial endocarditis: Localisation of the primary opsonic site to Fc fragment. Journal of Experimental Medicine. 128: 553-570.

RAHAL, J.J. (1972)
Comparative effects of purified staphylococcal alpha and delta toxins on mitochondrial metabolism. Journal of Infectious Diseases. 126: 96-103.

RAUS, J. and LOVE, D.N. (1983).
Characterization of coagulase-positive Staphylococcus intermedius and Staphylococcus aureus isolated from veterinary clinical specimens. Journal of Clinical Microbiology. 18: 789-792.

REIFSTECK, F; WEE, S. and WILKINSON, B.J. (1987).
Hydrophobicity-hydrophilicity of staphylococci. Journal of Medical Microbiology. 24: 65-73.

RESCIGNO, A. and MACCACARO, G.A. (1961).
The information content of biological classifications. In: Information Theory. C. Cherry (Ed). Butterworth, London. pp 437.

RICHARDSON, G.M. (1936).
The nutrition of Staphylococcus aureus necessity for uracil in anaerobic growth. Biochemical Journal. 30: 2184-2190.

ROGERS, D.E. and MELLY, M.A. (1960).
Further observations on the behaviour of staphylococci within human leukocytes. Journal of Experimental Medicine. 111: 533-558.

ROGERS, D.E. and TOMPSETT, R. (1952).
The survival of staphylococci within human leucocytes. Journal of Experimental Medicine. 95: 209-235.

ROSENBACH, F.J. (1884).
Mikro-Organismen bei den Wund Infections - krankheiten des Menschen. J.F. Bergmann, Wiesbaden, Germany, pp 132.

ROSYPAL, S; ROSYPALOVA, A. and HOREJS, J. (1966).
The classification of micrococci and staphylococci based on their DNA base composition and adansonian analysis. Journal of General Microbiology. 44: 281-292.

ROTTA, J. (1975).
Endotoxin-like properties of the peptidoglycan. Zeitschrift fuer Immunitaetsforschung Experimentelle und Klinische Immunologie. 149: 230-244.

RUBIN, J; RODGERS, W.A; TAYLOR, H.M; EVERETT, E.D; PROWANT, B.F; FRUTO, L.V. and NOLPH, K.D. (1980).
Peritonitis during continuous ambulatory peritoneal dialysis. Annals of Internal Medicine. 92: 7-13.

RUSSELL, R.J; WILKINSON, P.C; McINROY, R.J; McKAY, S; McCARTNEY, A.C. and ARBUTHNOTT, J.P. (1976).
Effects of staphylococcal products on locomotion and chemotaxis of human blood neutrophils and monocytes. Journal of Medical Microbiology. 9: 433-449.

SALL, T. (1962).
Interrelationship of extracellular enzymes and pseudocapsulation in a strain of Staphylococcus aureus. Journal of Bacteriology. 83: 1238-1243.

SALL, T; MUDD, S. and TAUBLER, J. (1961).
Concerning the surface of cells of Staphylococcus pyogenes. I. A pseudocapsulation phenomenon under certain experimental conditions. Journal of Experimental Medicine. 113: 693-700.

SANDERSON, A.R; STROMINGER, J.L. and NATHENSON, S.G. (1962).
Chemical structure of teichoic acid from Staphylococcus aureus, strain Copenhagen. Journal of Biological Chemistry, 237: 3603-3613.

SCHADE, A.L. (1963).
Significance of serum iron for the growth, biological characteristics and metabolism of Staphylococcus aureus. Biochemische Zeitschrift. 338: 140-148.

SCHLEIFER, K.H. (1969).
Substrate dependent modifications of the amino acid sequence of the murein of staphylococci. Journal of General Microbiology. 57: XIV.

SCHLEIFER, K.H. (1983).
The cell envelope. In: Staphylococci and Staphylococcal Infections C.S.F. Easmon and C. Adlam, (Eds). Academic Press, London. Vol. 2, pp 385-428.

SCHLEIFER, K.H. (1986).
Taxonomy of coagulase-negative staphylococci. In: Coagulase-negative staphylococci. P-A. Mardh and K.H. Schleifer, (Eds). Almqvist and Wiksell International, Stockholm, Sweden. pp 11-26.

SCHLEIFER, K.H. and KANDLER, O. (1972).
Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriological Reviews. 36: 407-477.

SCHLEIFER, K.H; HAMMES, W.P. and KANDLER, O. (1976).
Effect of endogenous and exogenous factors on the primary structures of bacterial peptidoglycan. Advances in Microbial Physiology. 13: 245-292.

SCHLEIFER, K.H; HUSS, L. and KANDLER, O. (1969).
The effect of nutrition on the amino acid sequence of the serine containing murein of Staphylococcus epidermidis strain 24. Archiv fur Mikrobiologie. 68: 387-404.

SCHLEIFER, K.H. and KLOOS, W.E. (1975).
Isolation and characterization of staphylococci from human skin. I. Amended descriptions of Staphylococcus epidermidis and Staphylococcus saprophyticus and descriptions of three new species: Staphylococcus cohnii, Staphylococcus haemolyticus and Staphylococcus xylosus. International Journal of Systematic Bacteriology. 32: 50-61.

SCHLEIFER, K.H. and KOCUR, M. (1973).
Classification of staphylococci based on chemical and biochemical properties. Archiv fur Mikrobiologie. 93: 65-85.

SCHLEIFER, K.H; MEYER, S.A. and RUPPRECHT, M. (1979).
Relatedness among coagulase-negative staphylococci: Deoxyribonucleic acid reassociation and comparative immunological studies. Archiv fur Mikrobiologie. 122: 93-101.

SCHLEIFER, K.H. and STACKEBRANDT, E. (1983).
Molecular systematics of prokaryotes. Annual Review of Microbiology. 37: 143-187.

SCHLIEVERT, P.M; SHANDS, K.N; DAN, B.B; SCHMID, G.P. and NISHIMURA, R.D. (1981).
Identification and characterization of an exotoxin from Staphylococcus aureus associated with toxic shock syndrome. Journal of Infectious Diseases. 143: 509-516.

SCHMELING, D.J; GEMMELL, C.G; CRADDOCK, P.R; QUIE, P.G. and PETERSON, P.K. (1981).
Effect of staphylococcal alpha-toxin on neutrophil migration and adhesiveness. Inflammation 5: 313-322.

SCHMELING, D.J; PETERSON, P.K; HAMMERSCHMIDT, D.E; KIM, Y; VERHOEF, J; WILKINSON, B.J. and QUIE, P.G. (1979).
Chemotaxigenesis by cell surface components of Staphylococcus aureus. Infection and Immunity. 26: 57-63.

SCRIBNER, D.J. and FAHRNEY, D. (1976).
Neutrophil receptors for IGg and complement: Their roles in the attachment and ingestion phases of phagocytosis. Journal of Immunology. 116: 892-897.

SEGAL, A.W; HARPER, A.M; GARCIA, R.C. and MERZBACH, D. (1982)
The action of cells from patients with chronic granulomatous disease on Staphylococcus aureus. Journal of Medical Microbiology. 15: 441-449.

SEWELL, C.M; CLARRIDGE, J.E; YOUNG, E.J. and GUTHRIE, R.K. (1982).
Clinical significance of coagulase-negative staphylococci. Journal of Clinical Microbiology. 16: 236-239.

SHARMA, B.S. and HAQUE, R. (1973).
A chemically defined medium for the production of staphylococcal beta haemolysin. Canadian Journal of Microbiology. 19: 1319-1323.

SHAYEGANI, M.G. (1970).
Failure of immune sera to enhance significantly phagocytosis of Staphylococcus aureus: Non-specific adsorption of phagocytosis-promoting factors. Infection and Immunity. 2: 742-749.

SHAYEGANI, M; HISATSUNE, K. and MUDD, S. (1970).
Cell wall component which affects the ability of serum to promote phagocytosis and killing of Staphylococcus aureus. Infection and Immunity. 2: 750-756.

SHAYEGANI, M.G; KAPRAL, F.A. and MUDD, S. (1964).
Phagocytosis and intracellular killing of Staphylococcus aureus by human and rabbit blood leukocytes. Journal of Immunology. 93: 88-93.

SHAYEGANI, M.G. and MUDD, S. (1966).
Role of serum in the intracellular killing of staphylococci in rabbit monocytes. Journal of Bacteriology. 91: 1393-1398.

SIEGEL, I. and COHEN, S. (1964).
Action of staphylococcal toxin on human platelets. Journal of Infectious Diseases. 114: 488-502.

SJOQUIST, J. and STALENHEIM, G. (1969).
Protein A from Staphylococcus aureus. IX. Complement-fixing activity of protein A-IgG complexes. Journal of Immunology. 103: 467-473.

SMITH, C.D. and PATTEE, P.A. (1967).
Biochemical and genetic analysis of isoleucine and valine bio-synthesis in Staphylococcus aureus. Journal of Bacteriology. 93: 1832-1838.

SMITH, E.W; GOSHI, K; NORMAN, P.S. and CLUFF, L.E. (1963).
Studies on the pathogenesis of staphylococcal infection. VII. The human cutaneous reaction to injection of alpha-haemolysin. Bulletin of the Johns Hopkins Hospital, 113: 247-260.

SMITH, H. (1964).
Microbial behaviour in natural and artificial environments. Symposium of the Society for General Microbiology. 14: 1.

SMITH, H. (1977).
Microbial surfaces in relation to pathogenicity. Bacteriological Reviews. 41: 475-500.

SMITH, H. and FITZGEORGE, R.B. (1964).
The chemical basis of the virulence of Brucella abortus. V. The basis of intracellular survival and growth in bovine phagocytes. British Journal of Experimental Pathology. 45: 174-186.

SMITH, M.L. and PRICE, S.A. (1938).
Staphylococcus gamma-haemolysin. Journal of Pathology and Bacteriology. 47: 379-393.

SMITH, R.M; PARISI, J.T; VIDAL, L. and BALDWIN, J.N. (1977).
Nature of the genetic determinant controlling encapsulation in Staphylococcus aureus Smith. Infection and Immunity. 17: 231-234.

SOLBERG, C.O; CHRISTIE, K.E; LARSEN, B. and TONDER, O. (1976).
Influence of antibodies and therolabile serum factors on the bactericidal activity of human neutrophil granulocytes. Acta Pathologica et Microbiologica Scandinavica. Sect. C . 84: 112-118.

SOMPOLINSKI, D; GLUSKIN, J. and ZIV, G. (1969).
Pantothenate requiring dwarf colony variants of Staphylococcus aureus as the etiological agent in bovine mastitis. Journal of Hygiene. (Cambridge). 67: 511-516.

SPINK, W.W. and PAINE, J.R. (1940).
The bactericidal power of blood from patients and normal controls for staphylococci. Journal of Immunology. 38: 383-398.

SRINIVASAKUMAR, N. and RAO, R.S. (1986).
Staphylococcus aureus protein A-antibody-mediated haemagglutination for the analysis of cross-reactivity between phenol-water extracts of Bacteroides fragilis. Journal of Medical Microbiology. 21: 343-347.

STALENHEIM, G. (1971).
Protein A from Staphylococcus aureus. XI. Fixation of human complement and of complement from guinea pig and rabbit. Acta Pathologica et Microbiologica Scandinavica. Sect B. 79: 665-672.

STALENHEIM, G; GOTZE, O; COOPER, N.R; SJOQUIST, J. and MULLER-EBERHARD, H.J. (1973).
Consumption of human complement components by complexes of IgG with protein A of Staphylococcus aureus. Immunochemistry. 10: 501-507.

STALENHEIM, G. and SJOQUIST, J. (1970).
Protein A from Staphylococcus aureus. X. Complement-fixing activity of complexes between protein A and myeloma IgG or papain fragments of IgG. Journal of Immunology. 105, 944-948.

STANIER, R.Y; ADELBERG, E.A. and INGRAHAM, J.L. (1977).
General Microbiology. The Macmillan Press Ltd., London and Basingstoke, pp 789.

STEWART, G.T. (1965).
The lipase and pigments of staphylococci. Annals of the New York Academy of Sciences. 128: 132-151.

STINEBRING, W.R. (1962).
Characteristics of intracellularly grown Brucella abortus. Journal of Infectious Diseases. 111: 17-24.

STORMONTH, D.A. and COLEMAN, G. (1972).
A rapid and convenient method for determining the dry weight of bacteria which show a tendency to form large aggregates. Journal of General Microbiology. 71: 407-408.

STOSSEL, T.P. (1974a).
Phagocytosis. New England Journal of Medicine. 290: 717-723.

STOSSEL, T.P. (1974b).
Phagocytosis. New England Journal of Medicine. 290: 833-839.

STOSSEL, T.P: FIELD, R.J; GITLIN, J.D; ALPER, C.A. and ROSEN, F.S. (1975).
The opsonic fragment of the third component of human complement (C3). Journal of Experimental Medicine. 141: 1329-1347.

SURGALLA, M.J. (1947).
A study of the production of Staphylococcal enterotoxin in chemically defined mediums. Journal of Infectious Diseases. 81: 97-111.

SZMIGIELSKI, S; JELJASZEWICZ, J; KOBUS, M; LUCZAK, M; LUDWICKA, A; MOLLBY, R. and WADSTROM, T. (1976).
Cytotoxic effects of staphylococcal alpha-, beta- and gamma-haemolysins. In. Staphylococci and Staphyloccocal Diseases. J. Jeljaszewicz, (Ed). Gustav Fischer Verlag, Stuttgart and New York. pp 691-706.

TARR, H.L.A and DEAS, C.P. (1948).
Utilisation of fish wastes. Bacteriological peptones from fish. Progress Reports of the Fisheries Research Board of Canada, Pacific Station. 75: 43-45.

TARR, H.L.A. and DEAS, C.P. (1949).
Bacteriological peptones from fish flesh. Journal of the Fisheries Research Board of Canada. 7: 552-560.

TAUBLER, J.H; KAPRAL, F.A. and MUDD, S. (1963).
Role of alpha-toxin in lesion formation by Staphylococcus aureus on sutures subcutaneously implanted in mice. Journal of Bacteriology. 86: 51-57.

TAYLOR, A.G. and BERNHEIMER, A.W. (1974).
Further characterization of staphylococcal gamma-haemolysin. Infection and Immunity. 10: 54-59.

TAYLOR, D. and HOLLAND, K.T. (1988).
Effect of dilution rate and Mg^{2+} limitation on toxic shock syndrome toxin-1 production by Staphylococcus aureus grown in defined continuous culture. Journal of General Microbiology. 134: 719-723.

TEJLER, T. (1937).
Some experiments on the bactericidal effect of normal serum of Staphylococcus pyogenes aureus. Acta Pathologica et Microbiologica Scandinavica. 14: 1-14.

THATCHER, F.S. and SIMON, W. (1957).
Some physiological and toxigenic properties of members of the genus Micrococcus in relation to taxonomy. International Bulletin of Bacteriological Nomenclature and Taxonomy. 7: 21-36.

THELESTAM, M; MOLLBY, R. and WADSTROM, T. (1973).
Effects of staphylococcal alpha-, beta-, delta-, and gamma-haemolysin on human diploid fibroblasts and Hela cells: Evaluation of a new quantitative assay for measuring cell damage. Infection and Immunity. 8: 938-946.

TOMPSETT, R. (1954).
The survival of staphylococci within phagocytic cells. Bulletin of the New York Academy of Medicine. 30: 480.

TORII, M; KABAT, E.A. and BEZER, A.E. (1964).
Separation of teichoic acid of Staphylococcus aureus into two immunologically distinct specific polysaccharides with alpha- and beta-N-acetylglucosaminyl linkages respectively. Journal of Experimental Medicine. 120: 13-29.

TOTAKE, K. (1979).
Effect of various carbon sources on the interaction of growth and alpha-toxin production in Staphylococcus aureus (Wood 46). Journal of Saitama Medical School. 6: 215-221.

TOTAKE, K. and ICHIKAWA, Y. (1983).
Correlation between the production of alpha-toxin and growth rate in Staphylococcus aureus. Microbiology and Immunology. 27: 389-394.

TRIPPEN, B; HAMMES, W.P; SCHLEIFER, K.H. and KANDLER, O. (1976).
Mode of action of D-amino acids on the biosynthesis of peptidoglycan. Archiv fur Mikrobiologie. 109: 247-261.

TSCHAPE, H. (1973).
Genetic studies on nutrient markers and their taxonomic importance. In: Staphylococci and Staphylococcal Infections. J. Jeljaszewicz (Ed). Vol. 1. Contributions to Microbiology and Immunology. S. Karger, Basel, London. pp 57-62.

TURNER, W.H. (1978a).
Purification and characterisation of an immunologically distinct delta-haemolysin from a canine strain of Staphylococcus aureus. Infection and Immunity. 20: 485-494.

TURNER, W.H. (1978b).
The effect of medium volume and yeast extract diffusate on delta-haemolysin production by five strains of Staphylococcus aureus. Journal of Applied Bacteriology. 45: 291-296.

TURNER, W.H. and PICKARD, D.J. (1979).
Immunological relationship between delta-haemolysins of Staphylococcus aureus and coagulase-negative strains of Staphylococci. Infection and Immunity. 23: 910-911.

TURNER, W.H. and PICKARD, D.J. (1980).
A new haemolysin from Staphylococcus aureus which lyses horse erythrocytes. Journal of General Microbiology. 116: 237-241.

VAN ESELTINE, W.P. (1955).
On the inadvisability of separating the genera Micrococcus and Staphylococcus. International Bulletin of Bacteriological Nomenclature and Taxonomy. 5: 53-60.

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; VAN DIJK, W.C; PETERS, R; VAN DER TOL, M.E. and VERHOEF, J. (1979).
The role of. Staphylococcus aureus cell wall peptidogylcan, teichoic acid, and protein A in the processes of complement activation and opsonization. Immunology, 37: 615-621.

VERBRUGH, H.A; VAN DIJK, W.C; PETERS, R; VAN ERNE, M.E; DAHA, M.R; PETERSON, P.K. and VERHOEF, J. (1980).
Opsonic recognition of staphylococci mediated by cell wall peptidoglycan: Antibody-independent activation of human complement and opsonic activity of peptidoglycan antibodies. Journal of Immunology. 124: 1167-1173.

VERHOEF, J; PETERSON, P.K; KIM, Y; SABATH, L.D. and QUIE, P.G. (1977a).
Opsonic requirements for staphylococcal phagocytosis: Heterogenecity among strains. Immunology. 33: 191-197.

VERHOEF, J; PETERSON, P.K. and QUIE, P.G. (1977b).
Human polymorphonuclear leukocyte receptors for staphylococcal opsonins. Immunology. 33: 231-239.

VON RECKLINGHAUSEN, N.D. (1871).
Cited by Baird-Parker, A.C. (1972). In: The Staphylococci. J.O. Cohen (Ed). Wiley-Interscience, London. pp 1-20.

WADSTROM, T. (1983).
Biological effects of cell damaging toxins. In: Staphylococci and Staphylococcal Infections. C.S.F. Easmon and C. Adlam (Eds). Academic Press Inc. London. Vol. 2, pp 671-704.

WADSTROM, T; KJELLGREN, M. and LJUNGH, A. (1976).
Extracellular proteins from different Staphylococcus species: A preliminary study. In: Staphylococci and Staphylococcal Infections. J. Jeljaszewicz, (Ed), Gustav Fischer Verlag, Stuttgart, and New York. pp 623-634.

WADSTROM. T. and MOLLBY, R. (1972).
Some biological properties of purified staphylococcal haemolysins. Closing remarks, Toxicon, 10: 511-519.

WADSTROM, T; THELESTAM, M. and MOLLBY, R. (1974).
Biological properties of extracellular proteins from Staphylococcus. Annals of the New York Academy of Sciences. 236: 343-361.

WALBUM, L.E. (1921).
Action de la staphylolysine sur les globules de chevre. Comples Rendus des Seances de la Sociiete de Biologie. 85: 1205-1206.

WALBUM, L.E. (1922).
Studien uber die Bildung der Backterellen Toxine. 1. Mitteilung. Staphylolysin. Biochemische Zeitschrift. 129: 367-443.

WALDVOGEL, F.A; MEDOFF, G. and SWARTZ, M.N. (1970).
Osteomyelitis: A review of clinical features, therapeutic considerations and unusual aspects. New England Journal of Medicine. 282: 198-206.

WALTON, E. and GLADSTONE, G.P. (1975).
A study of the action of the cationic proteins from rabbit polymorphonuclear leucocytes on the staphylococcal cell membrane. British Journal of Experimental Pathology. 56: 459-465.

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.

WATANAKUNAKORN, C. and BAIRD, I.M. (1977).
Staphylococcus aureus bacteremia and endocarditis associated with a removable infected intravenous device. American Journal of Medicine. 63: 253-256.

WEINSTEIN, D.B; MARSH, J.B; GLICK, M.C. and WARREN, L. (1969).
Membranes of animal cells. IV. Lipids of the L cell and its surface membrane. Journal of Biological Chemistry. 244: 4103-4111.

WEKSLER, B.B. and HILL, M.J. (1969).
Inhibition of leucocyte migration by a staphylococcal factor. Journal of Bacteriology. 98: 1030-1035.

WELLMAN, W.E. and SENFT, R.A. (1964).
Bacterial meningitis. III. Infections caused by Staphylococcus aureus. Mayo Clinic Proceedings. 39: 263-269.

WHALEY, K. and SINGH, H. (1973).
In vitro studies on the phagocytosis of Staphylococcus aureus by peritoneal macrophages of New Zealand. Mice. Immunology 24: 25-35.

WHEAT, L.J; HUMPHREYS, D.W. and WHITE, A. (1974).
Opsonization of staphylococci by normal human sera: The role of antibody and heat-labile factors. Journal of Laboratory and Clinical Medicine. 83: 73-78.

WILEY, B.B. (1961).
A new virulence test for Staphylococcus aureus and its application to encapsulated strains. Canadian Journal of Microbiology. 7: 933-943.

WILEY, B.B. and WONNACOTT, J.C. (1962).
Isolation and partial characterization of a capsular material from Staphylococcus aureus. Journal of Bacteriology. 83: 1169-1176.

WILKINSON, B.J. (1983).
Staphylococcal capsules and slime. In: Staphylococci and Staphylococcal Infections. C.S.F. Easmon and C. Adlam (Eds). Academic Press, London. Vol. 2, pp 481-523.

WILKINSON, B.J; KIM, Y; PETERSON, P.K; QUIE, P.G. and MICHAEL, A.F. (1978).
Activation of complement by cell surface components of Staphylococcus aureus. Infection and Immunity. 20: 388-392.

WILKINSON, B.J; PETERSON, P.K. and QUIE, P.G. (1979).
Cryptic peptidoglycan and antiphagocyte effect of the Staphylococcus aureus capsule: Model for the antiphagocytic effect of bacterial cell surface polymers. Infection and Immunity. 23: 502-508.

WILKINSON, P.C. (1977).
Action of sphingomyelinase C and other lipid specific agents as inhibitors of Fc binding and locomotion in human leucocytes. Immunology. 33: 407-412.

WILLIAMS, M.R; HIBBITT, K.G; FIELD, T.R. and BUNCH, K.J. (1984).
Further studies on the variation among cows, bulls and calves in the ability of their blood polymophonuclear leucocytes to kill Staphylococcus aureus. British Veterinary Journal. 140: 307-313.

WILLIAMS, P; DENYER, S.P. and FINCH, R.G. (1988).
Protein antigens of Staphylococcus epidermidis grown under iron-restricted conditions in human peritoneal dialysate. Federation of European Microbiological Societies, Microbiology Letters. 50: 29-33.

WILLIAMS, R.E.O. and HARPER, G.J. (1947).
Staphylococcal haemolysins on sheep-blood agar with evidence for a fourth haemolysin. Journal of Pathology and Bacteriology. 59: 69-78.

WILSON, A.T; WILEY, G.G. and BRUNO, P.(1957).
Fate of non-virulent group A streptococci phagocytized by human and mouse neutrophils. Journal of Experimental Medicine. 106: 777-786.

WILSON, G.S. and MILES, A. (1975).
Clostridium: The spore-bearing anaerobes. In: Topley and Wilson's Principles of Bacteriology, Virology and Immunity. Edward Arnold, London. Sixth ed., Vol. 1, pp 1109-1157.

WILSON, P.D; SALVATI, E.A; AGLIETTI, P. and KUTNER, L.J. (1973).
The problem of infection in endoprosthetic surgery of the hip joint. Clinical Orthopaedics and Related Research. 96: 213-221.

WISEMAN, G.M. (1965).
Factors affecting the sensitization of sheep erythrocytes to staphylococcal beta-lysin. Canadian Journal of Microgiology. 11: 463-471.

WISEMAN, G.M. (1970).
The beta- and delta-toxins of Staphylococcus aureus. In: Microbial toxins. III. Bacterial Protein Toxins. T.C. Montie, S. Kadis and S.J. Ajl, (Eds). Academic Press, New York. pp 237-265.

WISEMAN, G.M. (1975).
The haemolysins of Staphylococcus aureus. Bacteriological Reviews. 39: 317-344.

WISEMAN, G.M. and CAIRD, J.D. (1970).
Mode of action of the alpha-toxin of Staphylococcus aureus. Canadian Journal of Microbiology. 16: 47-50.

WISEMAN, G.M. and CAIRD, J.D. (1972).
Further observations on the mode of action of the alpha-toxin of Staphylococcus aureus Wood 46. Canadian Journal of Microbiology. 18: 987-992.

WISEMAN, G.M; CAIRD, J.D. and FACKRELL, H.B. (1975).
Trypsin-mediated activation of the alpha-haemolysin of Staphylococcus aureus Wood 46. Journal of Medical Microbiology. 8: 29-38.

WOOD, W.B. Jr. (1960).
Phagocytosis, with particular reference to encapsulated bacteria. Bacteriological Reviews. 24: 41-49.

WRIGHT, A.E. and DOUGLAS, S.R. (1904).
An experimental investigation of the role of the body fluids in connection with phagocytosis. Proceedings of the Royal Society of London. 72: 357-370.

WRIGHT, J. (1936).
Staphylococcal leucocidin (Neisser-Wechsberg type) and antileucocidin. The Lancet, 230: 1002-1004.

YAMAGUCHI, Y. and KUROKAWA, M. (1972).
Nutritional requirements of Staphylococcus aureus. Gunma Reports on Medical Sciences. 3: 367-371.

YOSHIDA, K. and EKSTEDT, R.D. (1968).
Relation of mucoid growth of Staphylococcus aureus to clumping factor reaction: morphology, in serum-soft agar and virulence. Journal of Bacteriology. 96: 902-908.

YOSHIDA, K; ICHIMAN, Y. and OHTOMO, T. (1976).
Mouse virulent strain of Staphylococcus epidermidis. Relation of antiphagocytic activity of the protection-inducing antigen. Japanese Journal of Microbiology. 20: 209-217.

YOSHIDA, K; NAKAMURA, A; OHTOMO, T. and IWAMI, S. (1974).
Detection of capsular antigen production in unencapsulated strains of Staphylococcus aureus. Infection and Immunity. 9: 620-623.

YOSHIDA, K. and TAKEUCHI, Y. (1970).
Comparison of compact and diffuse variants of strains of Staphylococcus aureus. Infection and Immunity. 2: 523-527.

YOTIS, W.W. and EKSTEDT, R.D. (1959).
Studies on staphylococci: I. Effect of serum and coagulase on the metabolism of coagulase positive and coagulase negative strains. Journal of Bacteriology. 78: 567-574.

YOTIS, W.W. and LENTINO, J.R. (1973).
Further studies on a serum nonantibody alpha-globulin with antistaphylococcal activity. Journal of Applied Bacteriology 36: 147-155.

YOTIS, W.W. and STANKE, R. (1966).
Bacteriostatic action of progesterone on staphylococci and other microorganisms. Journal of Bacteriology. 92: 1285-1289.

ZURIER, R.B; WEISSMANN, G; HOFFSTEIN, S; KAMMERMAN, S. and TAI, H.H. (1974).
Mechanisms of lysosomal enzyme release from human leukocytes. II. Effects of cAMP and cGMP, autonomic agonists and agents which affect microtuble function. Journal of Clinical Investigation. 53: 297-309.

ZYGMUNT, W.A; BROWDER, H.P. and TAVORMINA, P.A. (1967).
Lytic action of lysostaphin on susceptible and resistant strains of Staphylococcus aureus. Canadian Journal of Microbiology. 13: 845-853.