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Aspects of Pathogenicity of *Streptococcus pyogenes* Group A

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Presented for the Degree of Doctor of Philosophy in the Faculty of  
Science , University of Glasgow .

School of Biological Sciences  
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Dedication

I should like to dedicate this thesis to my late respected father ,  
Allah dino M. Memon for his vigorous determination to scatter  
knowledge and education .

### Acknowledgements

I should like to take this opportunity to thank Professor John. H. Freer for giving me placement to do research in this department and for his guidance, supervision, stimulating discussions throughout this work and for reading and commenting of this manuscript.

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Finally, my thanks also go to Yasmeen Kazi for her good humour and valuable discussions during my research.

Bacteria.....and We!

You the mini enemies.....

You the mini enemies, unavoidable !

Adverse effects donated, but undesirable !

Life and health without you, miserable !

You the mini.....

Equipped with conventional and ultra-mutated arms,

Can walk and dance with graceful movements ,

Rest, react, and rule, still survive with distinct postures!

you the mini.....

Behave as friends, foes and non-aligned,

Opportunists, thou, but limited and needed,

Criminals, thieves, bandits, yet undefeated!

you the mini.....

Potent and powerful, harassed and hunters,

Survive and strive; even engulfed, increase and inducers,

Localized or generalized, shambles or worse !

You the mini.....

Badar Memon

### Summary

This thesis has sought to characterize some of the factors that influence the production, purification and toxicity of streptolysin S (SLS) from *Streptococcus pyogenes* group A. Two strains, C203S and 55903M, were examined for the production of SLS. Of these, C203S produced the highest yield. Production of the lysin in brain heart infusion broth (BHI, Difco) supplemented with 1% (w/v) maltose and 2% (w/v) sodium bicarbonate (BHI-BM), was maximal between the early and late exponential phases of growth. SLS production was examined in both strains grown in BHI-BM (Oxoid) and BHI-BM (Difco). Cultures in the latter medium gave the higher yields of SLS.

For intensive production of SLS, a procedure involving fourteen "inductions" was carried out on the same pellet of bacteria, which was repeatedly resuspended in induction buffer and stimulated each time with RNA-core. SLS was synthesized *de novo* during each induction cycle, and inhibitors of protein synthesis such as chloramphenicol blocked its formation. The combined material from the 14 inductions (crude SLS) was further purified by hydroxylapatite column chromatography. The purified product had a specific activity of  $3.5 \times 10^5$  haemolytic units (mg protein)<sup>-1</sup>. The homogeneity of the final product could not be estimated because it was carrier-bound; 5-8 bands originating from the carrier RNA-core were also observed when the final product was analyzed by SDS and native polyacrylamide gel electrophoresis. The molecular weight proved difficult to determine as the SLS migrated at the dye front. Although the zymogram technique was used successfully to locate SLS by its haemolytic activity on native PAGE, it was not possible to estimate mol. wt.

In stability and storage studies, SLS was completely stable in 0.1% (w/v) bovine serum albumin supplemented with 20% (v/v) glycerol at -20°C for more than 6 months.

The effect of iron on the growth of *S. pyogenes* group A and *S. milleri* and its influence on SLS production was investigated. Successful growth of 27 strains of *S. pyogenes* Gr A and 3 strains of *S. milleri* was obtained in media containing a 20-fold molar excess of chelators such as EDDA, transferrin,  $\alpha$ - $\alpha'$ -dipyridyl and desferal, over Fe. This suggested that iron was not an absolute requirement for growth of these bacteria. None of the streptococcal strains investigated produced siderophores as assayed by conventional methods. Positive control tests with *Staphylococcus aureus* and *Escherichia coli* MW gave siderophores. Finally, the yield of SLS was not affected by the concentration of iron in the growth medium.

In toxicity studies, SLS inhibited the chemiluminescent response of rabbit peritoneal neutrophils induced by FMLP (N-Formyl-L-Methionyl-L-leucyl-L-phenylalanine). This inhibition was dose-dependent. SLS also inhibited the opsonization of zymosan, an effect which might have arisen by two possible mechanisms :-

- (i) by inhibition of a step common to both the classical and the alternate pathway of complement.
- (ii) by serum components acting as carriers of SLS, which was released on exposure to neutrophils.

The possibility that SLS and the streptococcal M-protein may act synergistically or antagonistically was investigated by the chemiluminescence assay. No synergism or antagonism was evident. Further work is required to determine the structure of SLS so that its

interactions with carriers, cell membranes and serum proteins can be better understood.

	<u>Page</u>
Declaration	ii
Acknowledgements	iv
Summary	vii
Index of Tables	xviii
Index of Figures	xix
Abbreviations	xxii
<u>PART I: Introduction</u>	1
1.    The Genus <i>Streptococcus</i>	1
1.1.    Historical Background	2
1.2.    Classification	3
2. <i>Streptococcus pyogenes</i> group A	7
2.1.    Microscopy	7
2.2.    Group Specific Polysaccharides	8
2.3.    Type-Specific M-antigen	10
2.4.    Putative Virulence Factors of	13
<i>Streptococcus pyogenes</i> group A	
2.4.1.  Erythrogenic Toxins	13
2.4.2.  Streptokinase	15
2.4.3.  Nuclease	17
2.4.4.  Hyaluronidase	17
2.4.5.  Serum Opacity Factor	18
2.4.6.  NADase	19
2.4.7.  Mitogen	19
2.4.8.  Streptolysin O (SLO)	19
2.5.    Pathogenesis	21

	<u>Page</u>
3. Streptolysin S	22
3.1. Definition	22
3.2. Discovery and Historical Background	23
3.3. Evidence for Multiple forms of SLS	24
3.4. Biological Effects of SLS	29
4. Neutrophils: Activities and Functions	33
4.1. Classification	33
4.2. Origin : development and deployment of PMNs	34
4.3. Chemotaxis and activation of PMNs	35
4.4. Adherence	36
4.5. Phagocytosis	38
4.6. Microbiocidal Activity	42
5. Iron in Man and Microbes	43
5.1. Availability of Iron	47
5.2. Acquisition of Iron	53
5.3. The role of Iron in the Growth and Production of SLS in <i>Streptococcus pyogenes</i> group A	58
<u>OBJECT OF RESEARCH</u>	60
<u>PART II: Materials and Methods</u>	61
6. Toxin Production	61
6.1. Strains	61
6.2. Media	61
6.3. Cultural Conditions	62
6.4. Measurement of Bacterial Growth	62

	<u>Page</u>
6.5. Induction of Streptolysin S	62
6.6. Purification of Streptolysin S	63
6.7. Assays	66
6.7.1. Haemolytic Assay	66
6.7.2. Protein Estimation	67
6.8. Polyacrylamide Gel Electrophoresis of Protein	67
6.8.1. Molecular-Weight Estimation	67
6.8.2. Native Polyacrylamide Gel Electrophoresis of Protein	68
6.8.3. Immunoblotting	68
6.9. Blood Agar Overlay (Zymogram)	69
6.10. Blood Agar Diffusion Assay	69
6.11. Stability and Storage Study of SLS	70
6.12. Effect of Chloramphenicol on the Synthesis of SLS	70
7. Neutrophil Preparation	71
7.1. Chemiluminescence (CL) Assay	71
7.1.1. Opsonization Procedures	72
7.1.2. Effect of SLS on opsonization of Zymosan	73
7.1.3. Effect of 56°C for 30 min and Mg <sup>++</sup> EGTA on the ability of Rabbit Serum to Opsonize Zymosan	73
7.1.4. Agglutination of Rabbit Serum and Zymosan	74
7.1.5. Ability of SLS to Bind Zymosan	75
7.1.6. Effect of SLS on Bound Serum Opsonin	75
8. Effect of Iron on the Growth of <i>Streptococcus pyogenes</i> group A	76
8.1. Bacteria	77

	<u>Page</u>
8.2. Measurement of Bacterial Growth	77
8.3. Biochemical Assays	77
8.3.1. Estimation of Fe <sup>+</sup> present in culture medium	77
8.3.2. Assay for Detection of Phenolate and Hydroxamate-type siderophores	79
8.3.3. The ferric perchlorate test for Hydroxamate -type siderophores	79
8.3.4. Csaky test for Hydroxamate-type Siderophores	80
8.3.5. Universal Chemical Assay for Detection and Determination of Siderophores (Blue Plate Assay)	80
8.3.6. Thin Layer Chromatography	81
8.3.7. Detection of Phenolate-type Siderophores by Thin Layer Chromatography	82
8.3.8. Detection of hydroxamate-type Siderophores by Thin Layer Chromatography	83
8.3.9. Detection of Siderophores on Thin Layer Chromatography plates	83
8.3.10. Inhibition of Growth by Ethylenediamine-di-O- hydroxyphenyl acetic acid (EDDA)	83
8.4. Chemical Defined Medium (CDM)	84
8.4.1. Ion-Exchange Chromatography on Chelex 100 Resin Preparation and Regeneration of Resin	85
8.5. Effect of Potassium cyanide (KCN) and Sodium azide (NaN <sub>3</sub> ) on the growth of <i>Streptococcus</i> <i>pyogenes</i> Gr A	85
8.6. Effect of Potassium cyanide (KCN) or Sodium azide	86

	<u>Page</u>
(NaN <sub>3</sub> ) Plus Iron chelator EDDA on the growth of <i>Streptococcus pyogenes</i> Gr A	
8.7. Crude membrane Preparation and SDS-PAGE	87
<u>PART III: Results</u>	88
9. Production of Streptolysin S	88
9.1. Induction of Streptolysin S	88
9.2. Streptolysin S Production in Relation to Growth rate	88
9.3. Effect of growth medium on SLS levels	89
9.4. Purification of Streptolysin S	93
9.5. Criteria of Purity	93
9.5.1. SDS-Polyacrylamide Gel Electrophoresis	93
9.5.2. Native-Polyacrylamide Gel Electrophoresis	97
9.5.3. Zymogram	97
9.5.4. Agar Diffusion Assay	99
9.6. Western Blotting	99
9.7. Stability and Storage Study	102
9.8. Effect of Chloramphenicol on the synthesis of SLS	102
9.9. Iron and haemolysin (SLS) production in <i>Streptococcus pyogenes</i>	104
10. The influence of purified SLS on the chemiluminescence response of rabbit peritoneal neutrophils to FMLP	108
10.1. Effect of SLS at Sublethal doses	110
10.2. Effect of SLS on Opsonophagocytosis (washed zymosan)	114

	<u>Page</u>
10.3. Effect of SLS on opsonization of zymosan by rabbit serum (unwashed zymosan)	114
10.4. Effect of Heating and Mg <sup>++</sup> EGTA on Rabbit serum	117
10.5. Effect of SLS on Bound Opsonin	119
10.6. Effect of SLS on responsiveness of PMNs	119
10.7. Effect of SLS on lucigenin amplified CL response	122
10.8. Sensitivity of cells towards SLS	122
10.9 Possible synergism between M-protein and SLS	124
11. Effect of Transferrin on the Growth of <i>Streptococcus pyogenes</i> group A	128
11.1 Effect of EDDA on the Growth of <i>Streptococcus pyogenes</i> group A	128
11.2. Effect of $\alpha$ - $\alpha$ -dipyridyl on the Growth of <i>Streptococcus pyogenes</i> group A	131
11.3. Effect of Desferal on the Growth of <i>Streptococcus pyogenes</i> group A	131
11.4. Inhibition of Growth by EDDA in the Agar Plate Assay	135
11.5. Search for Siderophores under Different Growth Conditions	135
11.6. Assay for Phenolate and Hydroxamate-type Siderophores in cultures of Streptococcal isolates grown in three different iron-limiting media	138
11.7. Assay for Phenolate-type Siderophores by Thin	140

	<u>Page</u>
Layer Chromatography	
11.8. Detection of Hydroxamate-type Siderophores by Thin Layer Chromatography	140
11.9. Assay for Siderophores by a Universal Assay (Blue Agar Plate Assay)	141
12. Effect of Potassium cyanide (KCN) and Sodium azide (NaN <sub>3</sub> ) on the Growth of <i>Streptococcus pyogenes</i> Gr A	141
12.1. Effect of Potassium cyanide (KCN) or Sodium azide NaN <sub>3</sub> ) Plus Iron chelator EDDA on the growth of <i>Streptococcus pyogenes</i> Gr A	146
12.2. Membrane Protein profile under Iron-restricted conditions	146
<u>PART IV: Discussion</u>	154
13. Production (induction and release) , purification and characterization of SLS	152
13.1. The growth of <i>Streptococcus pyogenes</i> in the presence of chelators	159
13.2. Absence of siderophore production	161
13.3. Effect of KCN and NaN <sub>3</sub> on the growth of <i>Streptococcus pyogenes</i> group A	162
13.4. Effect of KCN or NaN <sub>3</sub> plus iron chelator EDDA on the growth of <i>Streptococcus pyogenes</i> group A	164
13.5. Iron and SLS production in <i>Streptococcus</i> <i>pyogenes</i> group A	164
13.6. The influence of purified SLS on the chemiluminescence response of rabbit peritoneal	165

	<u>Page</u>
neutrophils to FMLP	
13.7. Effect of SLS on opsonophagocytosis	166
13.8. Possible synergism between M-protein and SLS	168
13.9. Brief evaluation of the role of SLS in the pathogenicity of group A streptococci	169
14. <u>PART V: References</u>	174
15. <u>PART VI: Appendices</u>	205
15.1. Media and Diluents	205
15.2. Buffers and gels for SDS-PAGE	207
15.3. Buffer and Diluents	210
16. Permission to use copyright material	213

Index of Tables

<u>Table</u>	<u>Title</u>	<u>Page</u>
1.	Differential reactions of species of Streptococcus	6
2.	Comparison of biological properties of streptolysins O and S	31
3.	Chemotactic factors for neutrophils	37
4.	Role of iron in microorganisms	46
5.	Bacteria whose virulence in experimental infections is enhanced by iron compounds	48
6.	Locations of host iron-binding glycoproteins	52
7.	Effect of 56°C for 30 min and Mg <sup>++</sup> -EGTA on rabbit serum	74
8.	Agglutination of rabbit serum and zymosan	75
9.	Effect of SLS on bound serum opsonin	76
10.	Bacterial strains: designation and origin	78
11.	Purification and recovery of SLS	94
12.	Loss of SLS activity under different conditions	105
13.	Effect of chloramphenicol on SLS production	106
14.	Effect of SLS on bound serum opsonin	120
15.	Growth of streptococci in presence of iron-chelators	129
16.	Assay for siderophore production in the presence of iron-chelators in BHIB, THB, and CD broth	137
17.	Detection of siderophores by different assays	139
18.	Growth inhibitory concentration of sodium azide and potassium cyanide for streptococci and other organisms	145

<u>Flow sheet</u>	<u>Index of Flow sheets</u>	<u>Page</u>
1. Induction of streptolysin S		64
2. Purification of streptolysin S		65
3. Preparation and regeneration of chelex 100 resin column		86

<u>Figure</u>	<u>Index of Figures</u>	
1. $\beta$ -haemolysis of <i>Streptococcus pyogenes</i> gr A		4
2. Schematic diagram of Group A streptococcus		9
3. M-protein projections on the cell surface		11
4. Extracellular macromolecular substances identified in culture fluids of group A streptococci		14
5. Spread and multiplication of Streptococcus		16
6. A highly simplified scheme of PMN activation after FMLP stimulation		40
7. Details of the classical and alternate pathways of complement activation		41
8. Schematic drawing of steps in phagocytosis		41
9. Schematic representation of two ways by which pathogenic bacteria obtain iron from iron-binding proteins		56
10. Schematic model of low and high affinity iron assimilation pathways		56
11. SLS production in relation to growth rate (Strain C203S)		90
12. SLS production in relation to growth rate (Strain 55903M)		91
13. Comparative studies of SLS production in Difco and Oxoid medium		92
14. Elution profile of SLS from hydroxylapatite column		95
15. Analysis of crude preparation of SLS by SDS-PAGE		96

<u>Figure</u>	<u>Index of Figures</u>	<u>Page</u>
16.	Analysis of purified preparation of SLS by SDS-PAGE	96
17.	Analysis of crude preparation of SLS by native-PAGE	98
18.	Analysis of purified fractions of SLS by native-PAGE	98
19.	Zymogram (Blood agar overlay)	100
20.	Blood agar diffusion assay for SLS	101
21.	Transfer of SLS on nitrocellulose paper by Western blotting	103
22.	Effect of SLS on rabbit peritoneal neutrophils-FMLP-induced CL response	111
23.	Effect of SLS at sublethal doses: FMLP-induced CL response	113
24.	Effect of SLS on opsonization of zymosan (washed zymosan)	115
25.	Effect of SLS on opsonization of zymosan (unwashed zymosan)	116
26.	Effect of heating and MgEGTA on normal rabbit serum	118
27.	Effect of SLS on responsiveness of PMNs	121
28.	Effect of SLS on lucigenin-amplified CL assay	123
29.	Effect of M-protein on opsonization of zymosan	126
30.	Growth curve of strain 60343X in presence of transferrin	130
31.	Growth curve of strain 54359V in presence of transferrin	130
32.	Growth curve of strain 55093M in presence of EDDA	132
33.	Growth curve of strain 52942 in presence of EDDA	132
34.	Growth curve of strain 02750 in presence of $\alpha, \alpha$ -dipyridyl	133
35.	Growth curve of <i>E. coli</i> MW in presence of $\alpha, \alpha$ -dipyridyl	133
36.	Growth curve of strain 55903M in presence of Desferal	134
37.	Plate assay for inhibition of growth by EDDA	136
38.	Assay for phenolate-type siderophores by TLC	142
39.	Assay for hydroxamate-type siderophores by TLC	143

<u>Figure</u>	<u>Index of figures</u>	<u>Page</u>
40.	Universal assay for siderophores	145
41.	Growth curve of strain 55903M in presence of KCN	147
42.	Growth curve of <i>E. coli</i> MW in presence of KCN	148
43.	Growth curve of strain 55903M in presence of EDDA plus KCN	149
44.	Analysis of Crude membrane preparation on SDS-PAGE	151

Abbreviations

A	Absorbance
Bis	N' N' -methylenebisacrylamide
BSA	Bovine serum Albumin
BHIB	Brain Heart Infusion Broth
BHI-BM	Brain Heart Infusion Broth supplemented with 2% sodium bicarbonate and 1% maltose
CDM	Chemical Defined Medium
CL	Chemiluminescence
Cm	Chloramphenicol
D	Daltons
EDDA	Ethylenediamine-di-o-hydroxyphenyl acetic acid
<i>et al</i>	<i>et alios</i> (and others)
Fig	Figure
FMLP	N' Formyl-Methionyl-L-Leucine-Phenylalanine
g	gravity
h	hour
HBS	Hepes Buffered saline
HEPES	N-2-hydroxyethyl piperazine N'-2-ethane sulphonic acid
HU	Haemolytic Unit
IB	Induction Buffer
KCN	Potassium Cyanide
L	Litre
mV	milliVolts
NB	Nutrient Broth
nm	nanometre
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate Buffer Saline
PMNs	Polymorphonuclear Neutrophils
Rf	Relative Mobility
RNA	Ribonucleic acid
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
SLO	Streptolysin O
SLS	Streptolysin S
TEMED	NNNN'-tetra-methyl-1,2-diamino-ethane
Tf	Transferrin
THB	Todd Hewit Broth
$\mu$ g	Microgram
$\mu$ l	Microlitre
V	volts

## INTRODUCTION

### 1. The genus Streptococcus

Members of the genus *Streptococcus* have predominantly spherical to ovoid-shaped cells which associate in pairs or chains of varying length. Each cell is approximately 1.0  $\mu\text{m}$  in diameter. These bacteria divide in only one plane and have a tendency to remain together and form a chain. The length of the chain depends to some extent on whether the organisms are grown on a solid or a liquid medium and how roughly they are manipulated or handled before microscopy. In general, the longest chains are seen in a wet mount of a liquid culture.

The streptococci are almost always frankly gram-positive. Motile strains are rare. Capsule formation is common in some species. The genus consists of asporogenous, chemoorganotrophic, catalase-negative and oxidase-negative bacteria. For the most part, streptococci are aerotolerant organisms, but a few obligate anaerobes do exist. They are demanding in their growth requirements because they have lost the ability to synthesize many of the nutrients they need. For example, some streptococci require more than 15 amino acids, all of the known B vitamins, some purines and pyrimidines plus asparagine or glutamine for growth. For routine culturing of streptococci, a complex medium containing peptones, meat infusion, salts and glucose is used.

The members of this genus are widely distributed in nature, largely as parasites and pathogens of man and animals and in various dairy products. They make a large contribution to the normal bacterial flora of the human respiratory, alimentary and female genital tracts.

The organisms may be intracellular or extracellular. Their metabolism is fermentative and they metabolise sugars with the production mainly of lactic acid, and do not form gas. The pattern of carbohydrate metabolism more or less resembles that of the homofermentative lactobacilli.

### 1.1. Historical background

The term *Streptococcus* (combination of two Greek words, *Strepto*, winding, twisted plus *Kokkos*, berry) was first applied by the famous surgeon Billroth in 1874 to the chain-forming coccus that he saw in infected wounds. The similar coccus responsible for causing erysipelas was described by Fehleisen in 1883. The term '*Streptococcus*' and *Streptococcus* were subsequently used by various authors to designate a particular kind of cell congregation and were not used in the generic sense. Rosenbach (1884) first applied the name *Streptococcus pyogenes* to cocci that grew in chains and had been isolated from suppurative lesions in man.

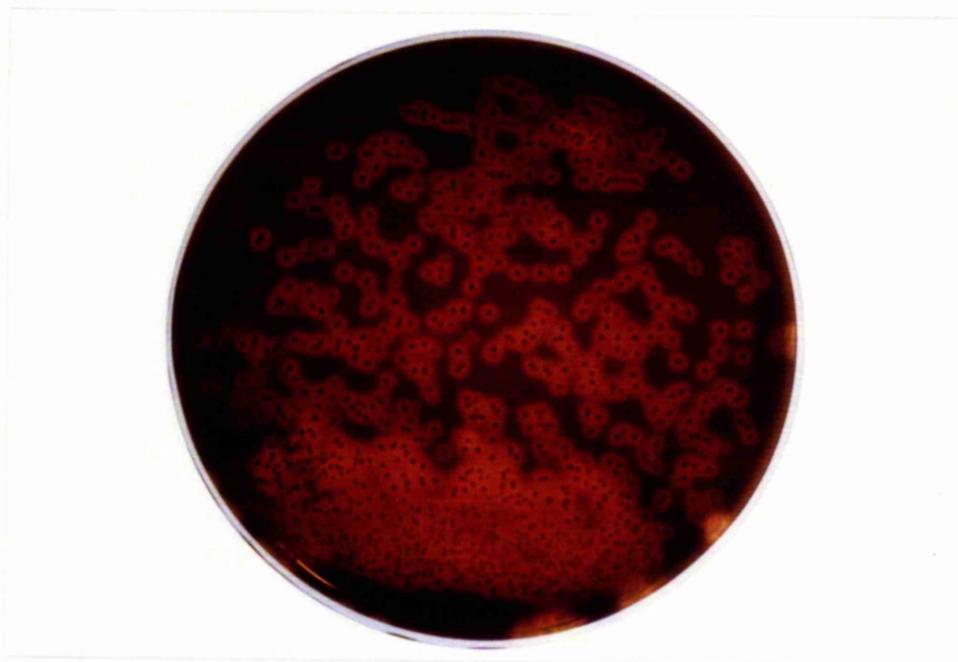
The account by Pasteur, Chamberland, and Roux (1881) of a septicæmic infection of rabbits inoculated with human saliva is probably the earliest reference to the pneumococcus, though no clearly identifiable description of the organism was published before the independent studies of Frankel and Weichselbaum in 1886. Nocard and Mollereau (1887) reported the production of mastitis in the cow and goat by inoculation into the udder of a streptococcus from the milk of a cow with this disease. Schutz (1887) described streptococci isolated from the lesions of equine pneumonia and strangles.

### 1.2. Classification

The members of this genus exist in nature either as parasites, saprophytes or part of body's normal flora. Being parasites, they cause a variety of diseases in man or his domestic animals. Hence, such strains can more or less be classified and characterized accurately whereas strains which occur as a part of the normal body flora or others which survive saprophytically are still largely unclassified. Thus, precise, acceptable and satisfactory classification is still awaited. Conventionally, in the classification of this genus, three major schemes or general characters have been investigated in the past. These are :

- i) The appearance of streptococci on blood agar: Not all streptococci are able to grow on blood agar and those that grow do not necessarily cause haemolysis. On the basis of this valuable and differentiating character, one can screen the pathogenic species. To supplement this statement, most streptococci that cause the common septic infections produce true or  $\beta$ - haemolysis on blood agar plates under appropriate conditions (Fig. 1). Other members of the genus produce greenish or  $\alpha$ -haemolysis on blood-containing media, and some produce no change at all.
- ii) To subdivide the streptococci by their ability to cause specific biochemical changes, such as fermentation of particular sugars, is of little value. However, individual tests may be useful to screen and characterize particular strains or species. For example, the ability to grow at extremes of pH, or in the presence of certain inhibitory chemicals, may be used to

Fig. 1



$\beta$ -haemolysis of *S. pyogenes* group A

differentiate species within this genus.

iii) In practice, detection and classification by group-specific antigens is of great value for pathogenic species, but is not so useful in grouping non-pathogenic species.

Although there have been many attempts to devise satisfactory schemes for the classification of streptococci, including Sherman's (1937), it is Jones's classification (1978) which is most widely accepted (Bergeys Manual of Systematic Bacteriology, 1986). Jones divided the genus into 7 groups designated pyogenic, pneumococci, oral, faecal, lactic, anaerobic and other streptococci. Although such groupings are admittedly artificial and, in most cases, have no strict taxonomic validity, they provide a convenient framework within which to describe the various organisms. A similar arrangement to that of Jones (1978) has been adopted here, except that *S. pneumoniae* has been included in the pyogenic group (Table 1).

A recent trend in streptococcal taxonomy has been towards the more extensive application of numerical, chemotaxonomic and genetic techniques, with less emphasis on serological criteria for classification. Although serology remains important for identification and typing of some major pathogens, it cannot be used as the main basis for establishing fundamental taxonomic relationships between streptococci. Schleifer and Kilpper-Balz (1987) made a more comprehensive revision of the genus based on the structure of the cell-wall peptidoglycan together with the G+C content of the DNA and the results of DNA pairing. In their classification, over-riding importance is given to the results of the genetic studies by current

Table 1 Differential reactions of species of *Streptococcus*

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Differential reactions of species of *Streptococcus*\*

Characteristics	Pyogenic streptococci			Oral streptococci			Enterococci			Lactic strepto-cocci			Anaerobic streptococci			Other streptococci																
	1. <i>S. pyogenes</i>	2. <i>S. agalactiae</i>	3. <i>S. equi</i>	4. <i>S. iniae</i>	5. <i>S. pneumoniae</i>	6. <i>S. salivarius</i>	7. <i>S. sanguis</i>	8. " <i>S. mitior</i> "	9. " <i>S. milleri</i> "	10. <i>S. mutans</i>	11. <i>S. rattus</i>	12. <i>S. cricetus</i>	13. <i>S. sobrinus</i>	14. <i>S. ferus</i>	15. <i>S. faecalis</i>	16. <i>S. faecium</i>	17. <i>S. avium</i>	18. <i>S. gallinarum</i>	19. <i>S. lactis</i>	20. <i>S. raffinosus</i>	21. <i>S. mordillorum</i>	22. <i>S. hansenii</i>	23. <i>S. pleomorphus</i>	24. <i>S. parvulus</i>	25. <i>S. acidominimus</i>	26. <i>S. uberis</i>	27. <i>S. bovis</i>	28. <i>S. equinus</i>	29. <i>S. thermophilus</i>			
Growth at 10°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
Growth at 45°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
Growth at 6.5% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Growth at pH 9.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Growth with 40% bile	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
α-Hemolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
β-Hemolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Arginine hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hippurate hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculin hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Obligate anaerobe	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

\* Symbols: see Table 12.2; also NT, not tested.

† Outer ring of α-hemolysis.

‡ Usually weak reaction.

§ Strains called *S. anginosus* may be positive.

¶ Often slow.

‡ Some strains are microaerophilic or require added CO<sub>2</sub>.

methods, which give only a partial view of the base sequences of the genome, and which are to some extent influenced by the techniques employed (Colman, 1990). For example, differences in the temperature at which DNA reassociation is performed led to the suggestion by Farrow and Collins (1984) that *S. salivarius* and *S. thermophilus* should be reclassified as a single species, but Schleifer and Kilpper-Balz (1987) considered they should continue to form 2 species.

A number of taxonomic and nomenclatural uncertainties remain to be resolved within the genus *streptococcus*. Further work is required to clarify the species designation of some of the streptococci presently described as Lancefield groups C, E, G, L, M, P, U and V. Problems still exist among the oral streptococci, notably those named *S. mitis* and "*S. mitior*" and also with the "*S. milleri*" group (Bergey's Manual of Systematic Bacteriology 1986).

## 2. *Streptococcus pyogenes* group A

### 2.1. Microscopy

The cells are about 0.8  $\mu\text{m}$  in diameter and grow in chains, with the number of cells per chain depending on the environment. In fluid cultures (or purulent exudates), long chains are formed, whereas pus and preparations made from solid cultures tend to contain short chains or pairs (diplococci). Electron microscopic examination of thin sections of streptococci show a profile of gram-positive cells with homogeneous cell wall which may have fimbriae attached, a cytoplasmic membrane, ribosomes, chromatin and occasional cytoplasmic granules. A capsule may also be present. The role of the capsule as a virulence factor and its formation will be discussed later under virulence

factors of GrA streptococci.

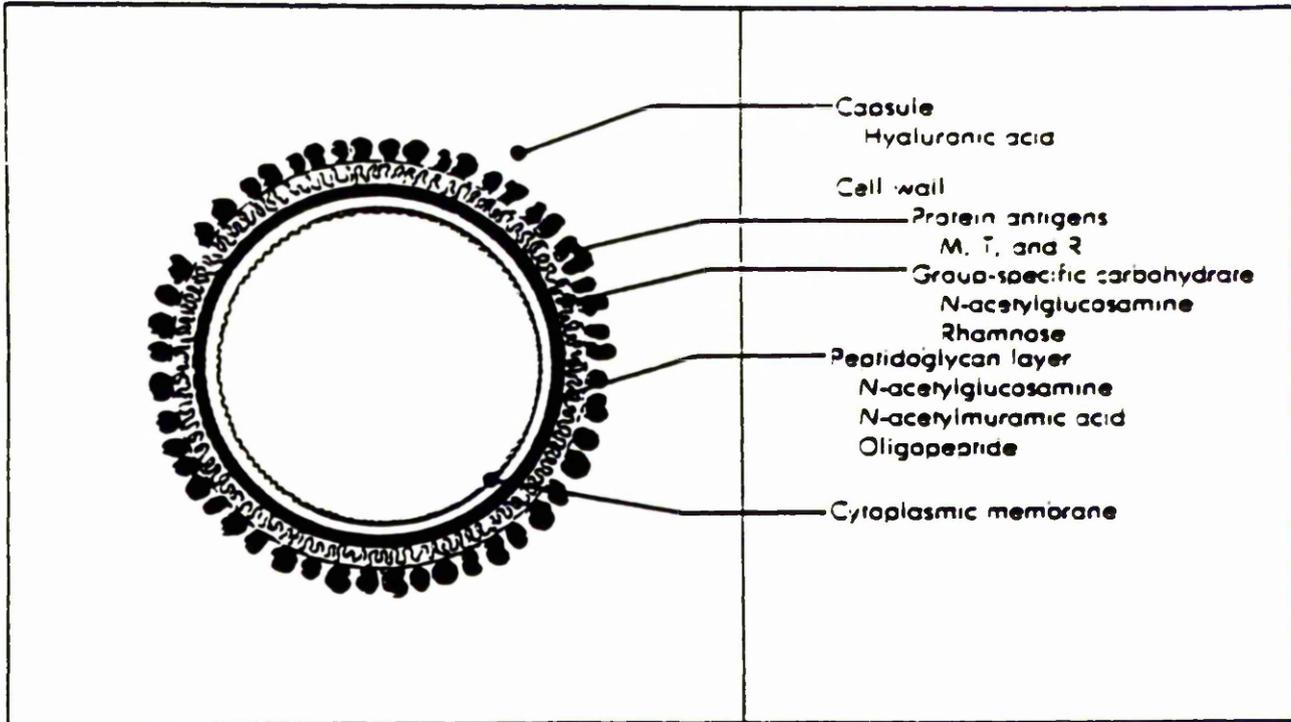
The basic structural framework of the cell wall is the peptidoglycan layer, similar to that found in other gram-positive bacteria. Associated with the cell surface are the group- and type-specific antigens (Fig. 2)

## 2.2. Group-specific polysaccharide

Serologically specific and distinct polysaccharides have served as the accurate means of classifying beta-haemolytic streptococci from various sources into numerous groups (Lancefield, 1933). These soluble carbohydrate antigens are located in the cell wall and are extracted from the organism by boiling at pH 2, the method used for routine typing of streptococci (Lancefield, 1942).

Group A carbohydrate contains 60% rhamnose and 30% glucosamine, the terminal D-N-acetylglucosamine residues being the antigenic determinants of the molecule (Krause, 1963). Immunity and protection to streptococcal infection are not related to the carbohydrate antigen; however, it was shown that the peptidoglycan-C-polysaccharide complex of the cell wall caused chronic relapsing lesions in rabbits (Schwab and Cromartie, 1960). When separated, neither the peptidoglycan nor the C-polysaccharide alone produced the chronic relapsing lesion (Krause and McCarty, 1961). Cell wall antigen was localised in the areas of chronic inflammation and the lesion correlated with the persistence of the antigen (Ohanian and Schwab, 1967). The peptidoglycan is considered to be the "toxic" moiety while the C-polysaccharide masks the peptidoglycan, thereby

Fig. 2

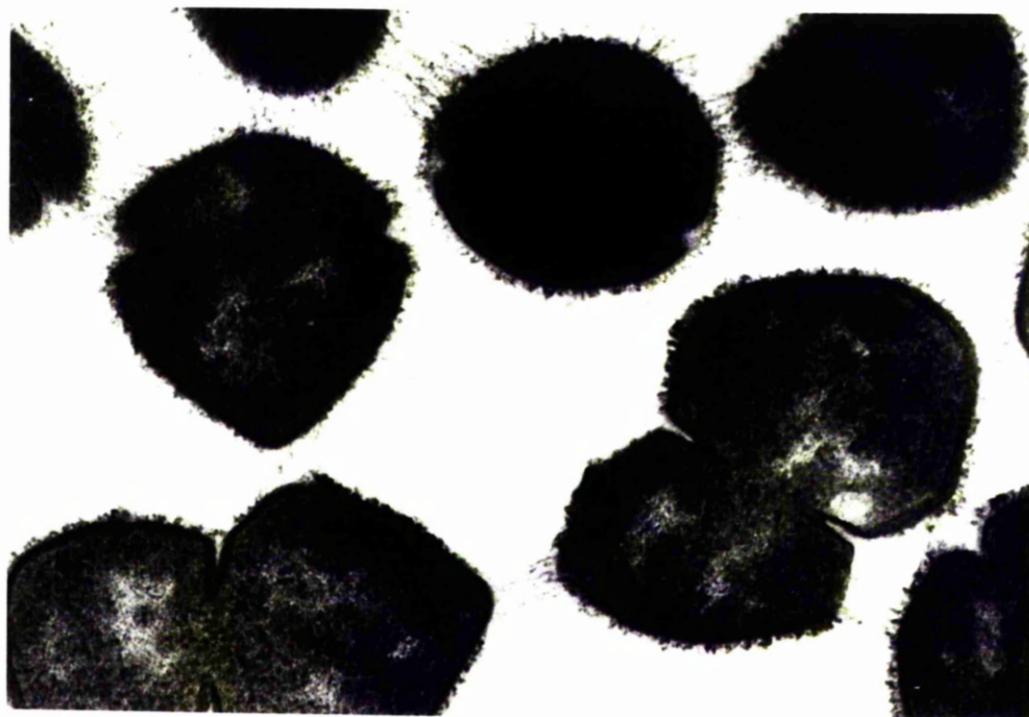
Schematic diagram of group A *Streptococcus*.

facilitating the persistence of the irritating agent (Schwab and Ohanian, 1966).

### 2.3. Type specific M-antigen

The M-protein (Lancefield, 1928) belongs<sup>to</sup> a family of heat-stable, trypsin-sensitive protein antigens that form part of the cell surface of group A streptococci. When viewed by transmission electron microscopy they appear as projections which may result from interactions between several adjacent M-protein fibrils forming tuftlike structures (Fig. 3) (Fischetti, 1989). Whether these surface structures are composed exclusively of M-protein or result from complex formation between M-proteins and other surface components is still not determined. However, ferritin-labelled anti-M antibodies show that these protrusions contain M-protein (Fischetti, 1989). M-protein-negative mutants lacked these structures. The M-antigens possess type specificity in that, with very rare exceptions, each strain of streptococcus has a single M-antigen type that is serologically distinct from all other M-antigens. They are to be distinguished from the T antigens and the R antigens, which are also surface proteins but appear not to be of any significance in relation to pathogenicity. The distribution of M, T and R antigens among streptococci follows certain predictable patterns, so that all of them are of use in typing group A streptococci, but the T and R antigens are not restricted to this streptococcal group. Only M-antigens divide the group into a series of distinct clones of strains (Maxted, 1978). Over 70 different M-antigens have been recognized, but this is thought to be far from the total that exist. Even with a comprehensive set of M-typing sera, a considerable proportion

Fig. 3



Electron micrograph of ultrathin sections of group A streptococci exhibiting M-protein fibrils on the cell surface .

of strains remain untypable.

For many years the method used to prepare crude M-protein for further purification was based on that developed by Lancefield (1928) for the preparation of extracts for streptococcal typing (Fox, 1974, and Lancefield, 1962). According to this procedure, organisms are suspended in dilute HCl at pH 2.0 and placed in a boiling-water bath for 10 min. After neutralization, the resulting extract is centrifuged and the supernate is used as starting material for the further characterization of the M-antigen.

While the procedure effectively removes M-protein from the cell, it is unlikely that the final purified product represents the native M-protein molecule (Fox and Wittner, 1965). Other procedures for extracting M-protein from whole streptococci or their isolated cell walls include sonic oscillation (Ofek *et al.*, 1969), extraction with alkali (Fox and Wittner, 1969), treatment with group C bacteriophage-associated lysin (Fischetti *et al.*, 1974), and digestion with pepsin at a sub-optimal pH of 5.8 (Manjula *et al.*, 1986) to yield fragments termed Pep M-molecules. Depending on the serotype, the size of the extracted PepM molecule varies from 20,000 to 40,000 in molecular weight (Manjula *et al.*, 1986).

The ability of group A streptococci to persist in infected tissues is primarily due to the cell surface M-protein, a molecule which confers on the streptococcus the ability to resist phagocytosis by polymorphonuclear leukocytes in the absence of type-specific antibodies to the M-molecule (Lancefield, 1959).

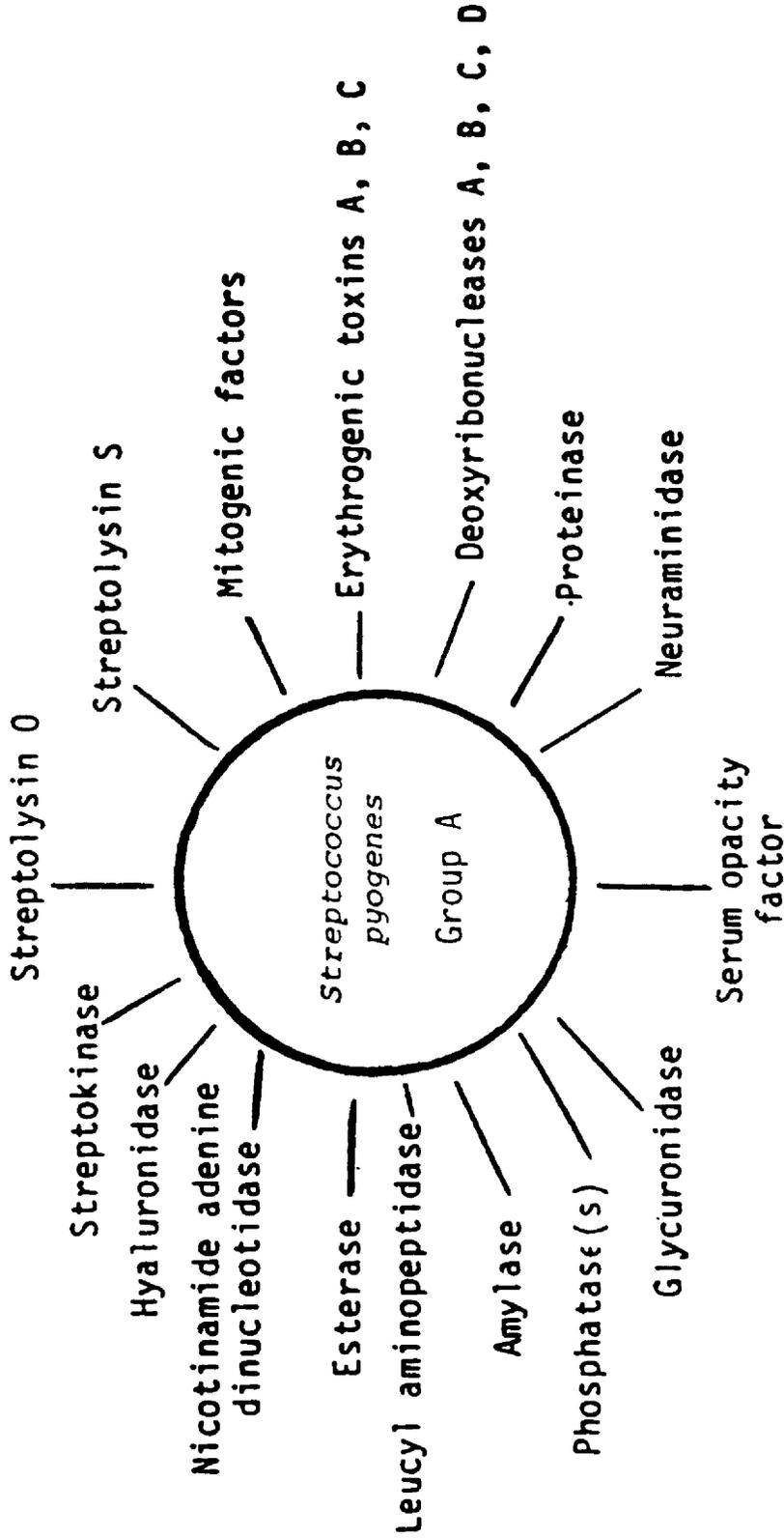
## 2.4. Putative virulence factors of *Streptococcus pyogenes* gr A

*S. pyogenes* GrA produces a number of exoproteins that may play a part in streptococcal pathogenesis and are thought to be biologically important. These products represent the adaptation of such organisms to a particular survival strategy. The products, as shown in Fig.4, may be virulence factors and the evidence linking them with particular aspects of streptococcal infection is still not well understood. Most strains of *S. pyogenes* can produce most of these products under appropriate conditions.

2.4.1. Erythrogenic Toxins (Scarlet fever toxin, Streptococcal pyrogenic exotoxins (SPES, Dick toxin ): These are responsible for eliciting the rash in scarlet fever and have been implicated in a toxic shock-like syndrome (TSLs, Cone, *et al.*, 1987; Wannamaker and Schlievert, 1988 ; Lee and Schlievert, 1989). Four erythrogenic toxins (streptococcal pyrogenic exotoxins) are known and most strains of *S. pyogenes* produce one or more (Colman, 1990). Hooker and Follensby (1934) described the toxins A and B, Watson (1960) type C and McMillan *et al.*, (1987) type D. They are proteins with mol.wt. of 8000, 17000, 13000 and 13000 respectively. The biological properties of the toxins include pyrogenicity, enhancement of lethal shock and myocardial damage due to endotoxin or streptolysin O, and induction of non-specific T lymphocyte mitogenicity (Lee and Schlievert, 1989). The last effect probably contributes to the development of scarlet fever rash in a susceptible host (Schlievert and Watson, 1979).

*S. pyogenes* strains carrying one or other of the M-antigens

Fig. 4



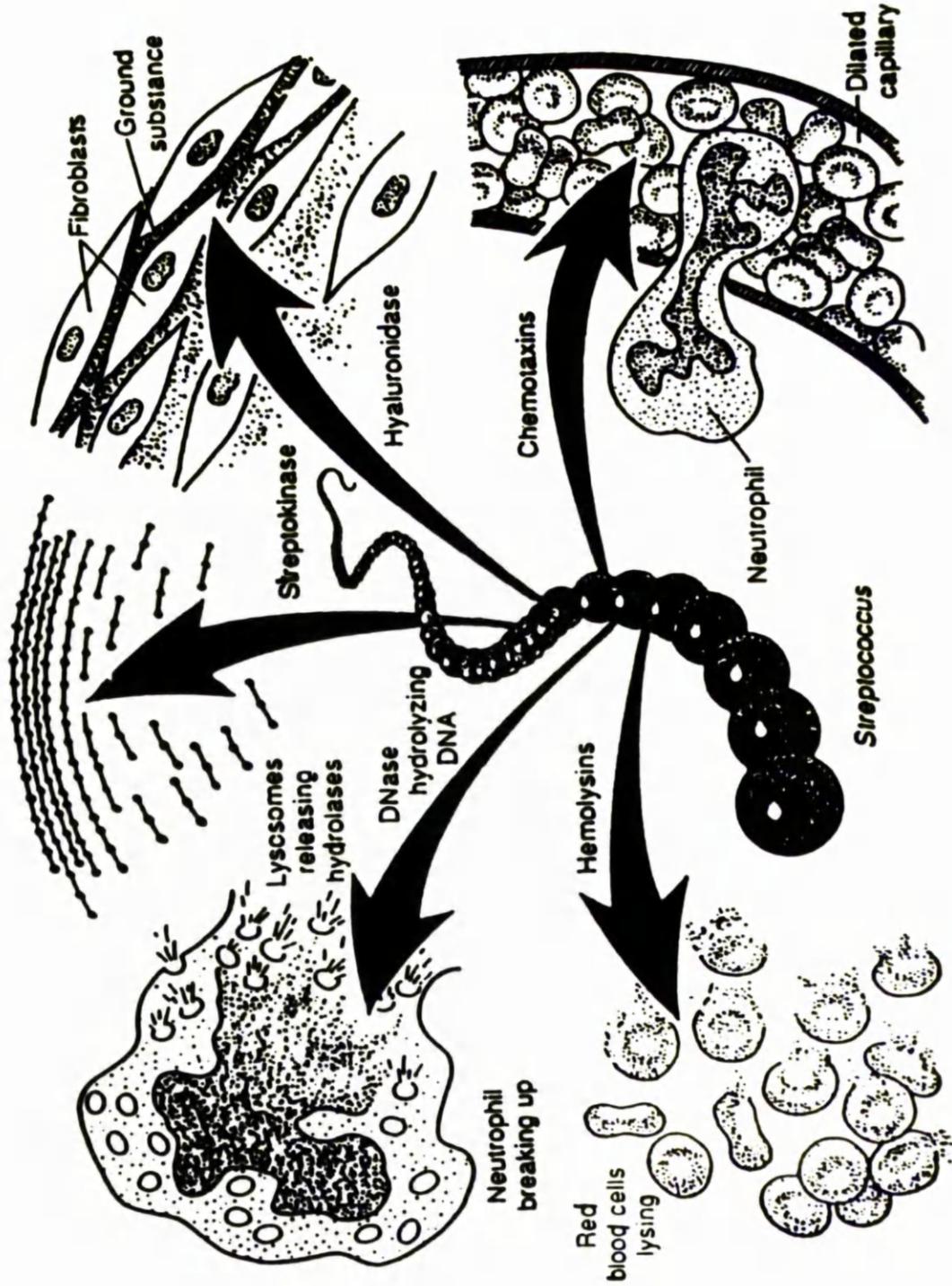
Extracellular macromolecular substances identified in culture fluids of group A streptococci.

1, 3 or 4 are the most numerous isolates from scarlet fever in the UK (Gaworzewska and Colman 1988) ; members of M-type 22 were prominent among scarlet fever strains from Australia and New Zealand, and had earlier caused an epidemic in East Germany (Parker, 1967). The possibility of involvement of bacteriophage in the production of exotoxin was suggested by Cantacuzene and Bonciu (1926). Zabriskie (1964) converted non-lysogenic, non-toxigenic strains of *S. pyogenes* to the production of type-A toxin with a temperate bacteriophage ; the mechanism of this conversion is unknown (Feretti and Yu, (1987). The gene for the type-A toxin has been cloned ; it shows extensive homology to the staphylococcal enterotoxins B (Johnson *et al.*, 1986) and C (Hynes and Weeks 1987).

2.4.2. Streptokinase (Fibrinolysin): Filtrates of streptococci of the groups A, C, and G cause dissolution of fibrin clots by means of fibrinolysin or streptokinase activity (Tillet and Garner, 1933). This acts on a factor present in normal human plasma (plasminogen) which is converted into a protease (plasmin) which in turn lyses the fibrin (Kaplan, 1944; Christensen, 1945) (Fig.5). The binding of streptokinase to plasminogen seems to be specific and plasminogen from domestic animals other than cat is not activated. Dillon and Wannamaker (1965) showed that streptokinase remained active after heating at 100°C for 50 minutes and also found that two immunologically distinct streptokinases were produced by group-A strains ; these could be distinguished from that produced by group C strains in spite of general similarity of amino acid composition (Gerlach and Kohler 1979).

Fig. 5

# Spread and Multiplication



Antibody to Streptokinase appears in man after streptococcal infections, but its measurement is often complicated by the presence of other factors in serum that render the fibrin clot resistant to lysis by inducing a non-specific anti-protease that appears during the acute phase of certain fevers (Kaplan, 1946).

An extracellular protein of mol. wt 46 000 was isolated from group-A streptococci associated with glomerulonephritis but was generally not produced by strains isolated from other clinical states (Villarreal *et al.*, 1979). This protein was identified as streptokinase and was antigenically distinct from that produced by a group-C strain (Johnston and Zebriskis, 1986).

2.4.3 Nuclease : All strains of *S. pyogenes* form at least one deoxyribonuclease (DNAase) and ribonuclease (RNAase) (Tillet *et al.*, 1948). Groups B, C, G and L streptococci also produce DNAase (Deibel, 1963) but usually in smaller amounts. There are at least four antigenic variants designated A, B, C and D (Wannamaker *et al.*, 1967) but DNAase B is the predominant nuclease in *S. pyogenes*. Antibodies to the B enzyme appear in the blood after most infections with group A streptococci (Wannmaker, 1959). The B and D enzymes, but not the A and C have ribonuclease activity.

2.4.4 Hyaluronidase (Spreading Factor): The ability to produce hyaluronidase is present in nearly all group-A streptococci (Benchetrit *et al.*, 1984); antibodies to the enzyme are formed after human infections (Friou and Wanner 1947). It splits hyaluronic acid, an important component of the ground substance of connective tissue (Fig.5). Many streptococci, including *S. pyogenes* (Meyer *et al.*, 1940)

form hyaluronic acid during the early stage of growth " *in vitro* " and subsequently form hyaluronidase which destroys it (Pike, 1948).

Hyaluronidase production is stimulated by the addition of hyaluronic acid to a growing culture (McClellan, 1941). Certain M-types of *S. pyogenes* (e.g. type 4 and 22) in which capsulation is rarely seen, form large amounts of hyaluronidase (Parker, 1983). The formation of hyaluronidase occurs not only in Group A and C, but also in group B, in the large-colony form of group G, in *S. suis* and *S. anginosus*, and the pneumococci.

Hyaluronidase is antigenic, but the enzyme produced by group A strains is immunologically distinct from that of streptococci of group C and G. There is no evidence that it favours invasion of tissues by streptococci (Colman, 1990).

2.4.5. Serum opacity factor (SOF): Many group A streptococci give rise to opacity factor in serum broth (Parker, 1983) or around colonies on serum agar. This is probably attributable to the enzymic release of lipids from <sup>u</sup>serum lipoprotein (Top and Wannamaker, 1968, Johnson and Kaplan, 1988). The opacity factor is closely related to M-proteins. Among the group-A streptococci, all members of some M-types but no members of others form it (Top and Wannamaker, 1968). It is formed also by streptococci of group L. The activity is neutralized by antisera, particularly those prepared in guinea-pigs (Fraser, 1982). The specificity of the reaction almost exactly parallels that of the corresponding M-antigens; thus opacity-neutralization test are a useful adjunct to M-typing procedures (Maxted *et al.*, 1973). Antibodies to OF persist in human sera for 4-12 years (Johnson and Kaplan, 1988).

Recently, Parkash and Dutta (1991) reported the finding of antibodies to more than one OF type, suggesting repeated streptococcal infection but with different OF types. The opacity factor is extractable with hot acid from M-positive strains but not from M-negative variants of them; it is also present extracellularly in broth cultures. Purification of M-antigen from opacity-forming strains yielded material progressively richer in opacity factor (Hallas and Widdowson, 1983).

2.4.6. NADase (Nicotinamide adenine dinucleotidase): This extracellular enzyme (Carlson *et al.*, 1957) is formed by all members of some M-types of group A streptococci but not by members of other types (Lazarides and Bernheimer 1957, Lutticken *et al.*, 1976). It is also formed by some streptococci of groups C and G (Green, 1979). It is doubtful whether NADase plays any part in pathogenesis, but antibodies to it may be formed in man as a result of infection (Kellner *et al.*, 1958).

2.4.7. Mitogen: Group A streptococci under appropriate conditions produce an extracellular factor that can bring about transformation of lymphocytes into large, blastlike cells capable of division (Taranta, *et al.*, 1968; Ginsburg, 1972). The streptococcal factor (mitogen) acts non-specifically like certain lectins (such as phytohaemagglutinin). In 1969, Taranta, *et al.*, reported that streptococcal mitogen is exclusively produced in group A streptococci and is found in culture supernates, in cell walls, in cell membranes (Keiser, *et al.*, 1971).

2.4.8. Streptolysin O (SLO): Streptolysin O (oxygen labile) is one of a series of closely related lysins formed by a variety of

bacteria that include the human pyogenic streptococci of groups A, C, or G (Streptolysin O), *S. pneumoniae* (pneumolysin), *Clostridium tetani* (tetanolysin), *Clostridium perfringens* ( $\theta$ -toxin), *Bacillus cereus* (cereolysin) and *Listeria monocytogenes* (Listerolysin). All are activated by sulphhydryl compounds, are inhibited by cholesterol and related sterols (Howard and Wallace, 1953) and have some antigenic similarities as indicated by precipitin and inhibition tests employing horse antisera to SLO (Cowell and Bernheimer, 1977). The gene for streptolysin O has been cloned into *Escherichia coli* but no homology could be detected in Southern blot hybridization experiments between the cloned DNA sequences and DNA isolated from other bacterial species producing a comparable haemolysin (Kehoe and Timmis, 1984). The nucleotide sequence of the cloned gene has been determined (Kehoe and Miller 1987). It is predicted to have a mol.wt of 70,000-75,000 and may be composed of a chain 538 amino acids.

There is good evidence that the site of attachment of the lysin to the cell membrane is the cholesterol molecule (Alouf, 1980). Red cells of all readily available animals, except the mouse, are lysed (Howard and Wallace, 1953) as are polymorphonuclear leucocytes and platelets. The reduced lysin is bound rapidly to erythrocytes at 0°C. The oxidized form does not become attached to cells. Lysis occurs when the temperature is raised but the way in which it occurs is a matter of debate (Alouf, 1980).

The development of circulating antibodies to streptolysin O after an attack of streptococcal pharyngitis indicates production of the toxin in the host. The consequences of its release are not known. Intravenous injection of potent toxin into mice, rabbits and guinea-

pigs is followed by death within seconds. Death is from acute toxin action on the heart (Halbert *et al.*, 1961). These authors suggested that the progressive tissue damage that occurs in rheumatic fever could be due to the dissociation, in the circulation or the myocardium, of immune complexes containing streptolysin O.

### 2.5. Pathogenesis

Streptococci are responsible for a large number of important diseases of man (Parker, 1978) and animals (Wilson and Salt, 1978). Streptococci are important pathogens both because of the many severe infections they produce and because of complications that may occur after recovery from the acute infection.

Man is one of the most susceptible of all animals to streptococcal infections which are among the most common of human bacterial infections. Among the diseases of major importance caused by these organisms are the following :

- Acute sore throat (tonsillitis and pharyngitis) and peritonsillar abscess (quinsy)
- Scarlet fever
- Ear infections (otitis media and mastoiditis)
- Puerperal sepsis
- Meningitis
- Skin infections such as cellulitis, erysipelas (inflammation of the skin and underlying tissues usually of the face and scalp ) and occasionally impetigo (blistering of the skin )
- Necrotizing fasciitis , pyomyositis, spontaneous gangrenous my<sup>o</sup>sitis
- Septicaemia and endocarditis

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Post streptococcal or secondary infections

- Acute glomerulonephritis
- Rheumatic fever

3. Streptolysin S :

3.1. Definition: Streptolysin S (SLS) is the non-antigenic (non-immunogenic), oxygen-insensitive cytolysin which is produced by Group A streptococci. It is largely responsible for a zone of beta-haemolysis surrounding colonies on blood agar media (Alouf and Loridan 1986). Streptolysin S is so-called because it is produced in serum and is oxygen-stable in contrast to streptolysin O, the oxygen labile, sterol-dependent cytolysin also produced by streptococci (Wannamaker, 1983). SLS is an unstable polypeptide which associates with various kinds of carrier molecules that act as stabilizers. It is thought to be active only in the carrier state. It has never been produced in a carrier-free state, and always requires the addition of a carrier to the culture or resting cell suspension (Wannamaker , 1983). However, Alouf and Loridan (1986) for the first time reported purification of RNA-core-induced SLS and isolation and haemolytic characteristics of the carrier-free toxin. The carriers (also called inducers) include many apparently unrelated substances such as serum albumin , RNA, ribonuclease-resistant RNA core, Trypan blue,  $\alpha$ -lipoprotein, and some non-ionic detergents such as Tween 40, 60 , 80 and Triton X-205 (Wannamaker, 1983). These apparently unrelated molecules to which the haemolytic moiety complexes serve to remove the active peptide from the bacterial surface and bind it in an active conformation (Duncan and Mason, 1976). The haemolytic peptide can be transferred from one

carrier to another , and destruction of either the haemolytic moiety or the carrier molecule inactivates the haemolytic activity of the toxin (Duncan and Mason, 1976, Alouf and Loridan, 1988). The active peptide has been estimated to consist of 32 amino-acids (Alouf and Loridan, 1988) with a high content of glycine and proline (Wannamaker 1983). However, Bernheimer (1967) reported that the most abundant amino acids were glutamic acid (or glutamine) and serine. The polypeptide lacks six amino acids viz histidine, arginine, cysteine, valine, methionine and isoleucine (Wannamaker, 1983).

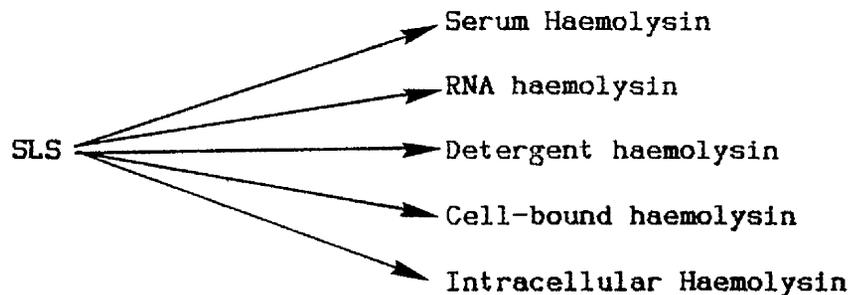
### 3.2. Discovery and Historical Background of SLS

Marmorek (1895) was the first to discover that filtrates of certain streptococcal culture possess haemolytic activity towards red blood cells of various animal species. Todd (1938) demonstrated that streptococci isolated from human sources produced two distinct haemolysins. He named the oxygen-labile lysin streptolysin O (SLO), to indicate its sensitivity to oxygen, and the oxygen-stable lysin, SLS to indicate its Stability to oxygen and its high stability in serum. Herbert and Todd (1944) and Ginsburg and Harris (1963) found that the oxygen-stable haemolysin could be extracted from washed streptococci with a serum protein fraction. Okamoto (1939) discovered that yeast nucleic acid induced the formation of a potent SLS-type haemolysin in growing streptococcal culture as well as resting streptococci. Subsequently, Bernheimer and Rodbart (1948) studied the factors affecting the formation of SLS in cultures containing RNA. They found that it was possible to form SLS through the interaction of RNA with resting streptococci by employing the system used by Herbert and Todd

(1944). This haemolysin showed a number of similarities to the serum haemolysin investigated by Humphrey (1949), and Bernheimer (1954). None of the haemolysins were immunogenic. Haemolytic material could be obtained from washed group A streptococci by treatment with ultrasound for long periods (Schwab (1956). This haemolytic material, named intracellular haemolysin (IH) was similar in certain respects to the haemolysin produced in the presence of yeast RNA. Both Ginsburg and Grossowicz (1958) and Taketo and Taketo (1967) subsequently showed that washed streptococci haemolyzed red blood cells, but that no extracellular haemolysin could be demonstrated. Thus, this haemolytic factor was named cell-bound haemolysin (CBH) by Ginsburg and Grossowicz (1958). Ginsburg and Grossowicz (1958) also reported that the cell-bound factor could be released from the streptococcal cells by certain detergents, serum albumin and by lipoproteins (Ginsburg and Harris, 1963) and named it streptolysin D (D stand for detergent). It had many properties similar to the haemolysins produced by streptococci in the presence of serum or yeast RNA. Ginsburg (1970) established that the lytic moiety of the RNA-streptolysin or that of the albumin-streptolysin complexes could be transferred to Tween or Triton. They further showed that the haemolytic activity of albumin-streptolysin was transferrable to RNA and vice versa. Their findings led to the unifying concept that RNA, Tween, albumin and other components in lysis complexes functioned as carriers for a haemolytic moiety, which under appropriate conditions, could be transferred from one carrier to another.

### 3.3. Evidence for multiple forms of SLS : It is now well-

established that SLS can be formed by streptococci in the presence of different inducers , and the properties of the different forms of



oxygen-stable haemolysin are discussed here briefly.

3.3.1. Serum haemolysin : Weld (1934) showed that a potent haemolysin indistinguishable from the serum haemolysin described by Todd (1938) in growing cultures could be obtained by shaking washed streptococci with horse or human serum. He also demonstrated that a single batch of streptococci could be extracted at least 5 times, each extraction yielding approximately the same amount of haemolysin. The extracted haemolysin was thought to be present on streptococcal cells and solubilized by the serum. Synder (1960) studied the effect of sera from various species on the production of SLS by strain C203S. While sera of horses and dogs induced the formation of 300 HU/ml of SLS, sera of humans and monkeys formed 74 and 68 HU/ml, respectively. Sera of rabbit and guinea pigs under similiar condition each formed only 15 HU/ml of SLS.

3.3.2. RNA haemolysin : Okamoto (1939) , Okamoto et al., (1941) and Bernheimer and Rodbart (1948) demonstrated that the formation of haemolytic toxin by streptococci was greatly enhanced by addition of yeast ribonucleic acid to the culture medium. The amount of haemolysin was a function of the concentration of RNA, the optimal amount of RNA

being approximately 1% (w/v) Hosoya *et al.*, (1949) Bernheimer (1949) further demonstrated that the enhancement of haemolysin formation by yeast RNA could also be obtained by shaking resting streptococci with solutions containing a mixture of nucleic acids. Bernheimer (1954) and Humphrey (1949) both reported that haemolysin produced in this fashion was inhibited by lecithin, trypan blue, congo-red, papain and chymotrypsin, yet was unaffected by trypsin, pepsin, cholesterol, or sera of animals previously injected with preparations of SLS.

Many different sources of RNA have been used to induce lysin production. These include streptococci, mammalian liver (Okamoto, 1962), wheat germ (Bernheimer and Rodbart, 1948), muscle tissues and Ehrlich ascites tumor cells (Okamoto, 1962), *Azotobacter vinelandii* Tanaka *et al.*, (1958) tobacco leaves (Bernheimer, 1954). Several groups (Bernheimer, 1949; Hosoya *et al.*, (1949) and Tanaka *et al.*, 1958) showed that following digestion of yeast RNA with pancreatic ribonuclease and precipitation with ethanol, the precipitate markedly increased haemolysin formation by streptococci. Bernheimer and Rodbart (1948) further showed that the active fraction (AF) thus obtained was associated with the ribonuclease-resistant core and had approximately 100 times the activity of the starting material. The AF appeared to be a polynucleotide. Tanaka *et al.*, (1958) hydrolysed yeast RNA with weak alkali at 0°C and separated from the resulting mixture a fraction which precipitated with 25% (v/v) acetone. This fraction, a polynucleotide, was several-fold more active than whole RNA in SLS induction. Further treatment with RNase increased its potency. Alkaline hydrolysis resulted in cleavage of all internucleotide linkages of RNA with the formation of 2' and 3'

mononucleotides rich in adenine and guanine.

RNA-core haemolysin has a reported molecular weight of 12,000 (Bernheimer, 1967), 18,500 daltons (Calandra and Oginski, 1975) or below 4000 (Koyama and Egami, 1964). Activity of RNA-SLS complex, according to Ginsburg and Harris (1964), is abolished by an *Aspergillus* ribonuclease. However, Koyama and Egami (1964) and Hryniewicz *et al.*, (1980) demonstrated resistance of SLS to numerous ribonucleases. These findings may be explained by possible masking of the carrier molecule, when attached to the haemolytic moiety, thus making SLS not susceptible to the enzymes. An active haemolytic moiety without a carrier has been isolated for the first time with reported molecular weight below 4000 on the basis of SDS-PAGE and 20 000 by gel filtration in guanidine.HCL (Alouf and Loridan, 1986).

The production of a haemolysin from *Treponema hyodysenteriae* and *Treponema innocens* which is oxygen-stable was increased by an improved culture method and by repeated incubation of these spirochaetes in a buffer containing RNA-core (Saheb *et al.*, 1981; Kent *et al.*, 1988; Kent *et al.*, 1991). Martin *et al.*, (1985) reported that several strains of *Haemophilus pleuropneumoniae* were also capable of producing RNA-dependent haemolysin (s) and reported that RNA or RNA-core (from *Torula* yeast RNA) were effective "carriers" for *H. pleuropneumoniae* haemolysin just as they were for SLS. However, serum albumin, Tween 80, and, for some unknown reason, RNA-core from bakers' yeast RNA, all of which are functional carriers for SLS (Calandra *et al.*, 1976) and *T. hyodysenteriae* haemolysin (Lemcke and Burrows, 1982) were ineffective as "carriers" for *H. pleuropneumoniae* haemolysin (Martin *et al.*, 1985). Nevertheless, like

SLS and the haemolysins produced by *T. hyodysenteriae* and *T. innocens*, the *H. pleuropneumoniae* haemolysin was sensitive to inhibition by trypan blue and sensitive to destruction by pronase. Interestingly, *H. pleuropneumoniae* haemolysin was sensitive to proteolytic digestion by both trypsin and chymotrypsin, whereas SLS is sensitive only to the latter (Ginsburg, 1970) and *T. hyodysenteriae* to neither (Saheb et al., 1981); these results may reflect differences in the exposure of specific amino acid residues on the surfaces of these haemolysins (Martin et al., 1985).

3.3.3. Detergent haemolysin : Ginsburg and Harris (1963) showed that a potent haemolytic factor was obtained by incubating washed group A streptococci with Tween 40, 60, or 80, (polyoxyethylene sorbitan monopalmitate, stearate, or oleate, respectively), Triton X-205 (octylphenol polyethylene oxide), or with trypan blue (Ginsburg and Harris, 1965). Calandra and Cole (1981) using the detergents Tween 20, 40, 60, and 80, BriJ 56, and Lubrol WX reported that activation of precursor in the membrane was better with a detergent, whereas that in the cytoplasm was better with RNA-core. Therefore, precursor from two different cellular locations can be differentiated by the effects of RNA-core and detergents on precursor titre. Streptococcal strains (e.g., C-203U) incapable of producing RNA or serum haemolysin also failed to produce detergent haemolysin.

3.3.4. Cell-bound haemolysin (CBH) : As demonstrated previously (Smith, 1937), various strains of group A streptococci possess a cell-bound haemolysin. The cell-bound haemolysin can be demonstrated by incubation of red blood cells of various animals species with washed

streptococci in the presence of glucose, Mg<sup>+</sup> and sulphhydryl compounds (Ginsburg and Harris, 1965). Under such conditions, haemolysis occurs within a short time although no extracellular haemolysin can be demonstrated in supernatant fluids of the incubation mixture or in extracts of streptococci disrupted by sonic oscillation. Ofek *et al.*, (1970) also reported that there was a direct correlation between the capacity of the streptococcal isolates to haemolyze red blood cells and their cytopathic effects on leucocytes.

### 3.3.5. Intracellular haemolysin (IH) : Schwab (1956)

demonstrated that group A streptococci subjected to sonic oscillation released a haemolytic factor which was designated intracellular haemolysin (IH). The amount of IH released steadily increased with the time of sonic treatment. Calandra and Cole (1976) <sup>and</sup> Jeljaszewicz *et al.*, (1978) revealed the existence of intracellular haemolysins, of which two seem to function as SLS precursors. One of them, named 'labile active cellular haemolysin, is released from cells by phage-associated lysin (muralysin) and is stabilized by RNA-core, whereas the second, termed 'latent haemolysin, is activated by sonic treatment in the presence of RNA-core.

### 3.4. Biological effects of SLS

SLS is a very potent membrane-damaging agent and lyses a wide variety of living cells and organelles (Alouf and Loridan, 1986) (Table 3). All eukaryotic cells that have been tested, including erythrocytes, lymphocytes (Jeljaszewicz *et al.*, 1978), polymorphonuclear leucocytes (Bernheimer, 1972), platelets (Bernheimer and Schwartz, 1965), various tissue culture cells and

tumour cells (Okamoto, 1976) are damaged by the toxin (Table 2). Intracellular organelles such as mitochondria and lysosomes are also disrupted by SLS. Its lytic spectrum is somewhat broader than SLO, it is lytic or cytotoxic not only for eukaryotic cells but also for wall-less forms of some bacteria notably protoplasts and L-forms from various species (Bernheimer, 1972).

#### In vitro effects

3.4.1. Erythrocytes: The haemolytic activity of SLS is well-established. It causes swelling of erythrocytes, followed by haemoglobin escape (Ginsburg, 1970) (Fig. 5). Erythrocytes of various animals differ in their susceptibility to SLS (Ginsburg, 1970) which may be attributed to differences in phospholipid content of the cell membrane. The kinetics of SLS-induced haemolysis has been studied by several investigators (Bernheimer, 1944; Duncan and Mason 1976, and Hryniewicz and Pryjma (1977). SLS is inhibited by several phospholipids, including phosphatidylcholine, phosphatidylethanolamine and phosphatidic acid (Elias *et al.*, 1966). These phospholipids may serve as SLS receptors in the cell membrane. Elias *et al.*, (1966) found that treatment of erythrocytes and their ghosts with phospholipase C, followed by exposure to SLS, resulted in diminished binding of SLS suggesting a role for membrane phospholipids in SLS action. According to Bernheimer (1972), SLS probably brings about a relatively subtle alteration in the organization of the lipid and /or protein molecules comprising the membrane, an alteration which abolishes selective permeability, thereby permitting free passage of ions. The retained haemoglobin exerts osmotic pressure which draws water into the cell

## Comparison of biological properties of streptolysins O and S

Table 2

Effect	Streptolysin O	Streptolysin S
Erythrocyte haemolysis	+	+
by osmotic swelling	-	+
"functional hole" formation	+	-
Leucocyte damage	+	+
Inhibition of phagocytosis	+	+
Inhibition of chemotaxis	+	?
Lymphocyte cytotoxicity	+	+
Blastoid transformation of lymphocytes	-	-
Lysis of platelets	+	+
Lysis of intact bacteria	-	-
Lysis of wall deficient bacteria	-	+
Lysis of mycoplasma	+ <sup>a</sup>	+
Somatic cells <i>in vitro</i> cytotoxicity	+	+
Inhibition of virus adsorption	?	+
Damage of mitochondria	+	+
Lysosome disruption or labilization	+	+
<i>In vivo</i> effects:		
Cardiotoxicity	+	-
Haemolysis	+	?
Dermonecrototoxicity	+	?
Lethality	+	-
Antitumour activity	-	+
Arthritis induction	-	+

<sup>a</sup> Strains containing cholesterol.

causing increased cell volume and rupture of the membrane. Thus lysis by SLS involves an osmotic mechanism and is similar in this respect to several other haemolytic systems. That SLS-induced lysis is a colloid-osmotic process is well documented by the studies of Duncan and Mason (1976) on rabbit erythrocytes and Hryniewicz and Pryjma (1977) on human erythrocytes as shown by the release of  $^{86}\text{Rb}$  prior to the escape of haemoglobin from the cells.

3.4.2. Lymphoid cells Hryniewicz and Pryjma (1977, 1978, 1980) and Hryniewicz *et al.*, (1986) showed that lymphoid cells involved in immune reactions are susceptible to various degrees to SLS. Lymphocytes appeared to be the most sensitive cell type to the cytotoxic action of SLS, as measured by  $^{51}\text{Cr}$  release and the trypan blue exclusion test. This study showed that T lymphocytes (human, mice) were more sensitive than B lymphocytes.

3.4.3. Polymorphonuclear leucocytes : The reported studies on SLS effects on leukocytes have been reviewed by Bernheimer and Schwartz (1960) and Hirsch *et al.*, (1963), Jeljaszewicz *et al.*, (1978), showed that SLS was cytotoxic and caused degranulation of intracellular organelles and changes in the cytoplasm. Ofek *et al.*, (1970) reported that cell-bound haemolysin killed mouse peritoneal macrophages within 30 min whereas RNA-SLS or serum-SLS required 60-180 min for complete killing. Addition of 10% mouse or rabbit serum to RNA-SLS or cell-bound haemolysin delayed its cytotoxic action.

3.4.4. Other cells : In addition to red and white blood cells, many other cell types undergo lysis after exposure to SLS. These include platelets (Bernheimer and Schwartz, 1965), Ehrlich ascites

tumour cells (Ginsburg and Grossowicz, 1960), heart cells of the rabbit and rat, renal cells, McCoy, HeLa and KB cells (Ginsburg, 1972).

3.4.5. Subcellular organelles : SLS disrupts membrane-bound organelles such as lysosomes, nuclei, and mitochondria (Weissmann *et al.*, 1963, 1964 ; Keiser *et al.*, 1964).

3.4.6. Antitumour activity : The antitumour activity of SLS-forming streptococci has been reviewed by Okamoto (1976, and Okamoto *et al.*, 1978). Such activity was observed on a variety of animal tumours including carcinoma, sarcoma, hepatoma, fibrosarcoma and lymphatic leukemia cells and appears to be due to two different effects, namely a direct cytotoxic effect on tumour cells and a host-mediated antitumour effect which probably involves the immune response and reticuloendothelial system stimulating factor.

#### 4. Neutrophils : Activities and functions

The role of PMNs in health and diseases is well-established. Bacterial pathogenesis is heavily dependent on the capacities of microbial cells to avoid activating or to resist antimicrobial mediators of neutrophil polymorphonuclear granulocytes, the first line of phagocytic defence against infection (Spitznagel, 1983). To understand the encounters between streptococci and neutrophils, it is indispensable to understand the activities and functions of neutrophils.

##### 4.1. Classification

White blood cells (WBC) may be divided into two major

populations on the basis of the form of their nuclei : single nuclei (mononuclear or round cells) or segmented nuclei (polymorphonuclear). Mononuclear cells are further divided into large (macrophage or monocytes) and small (lymphocytes) cell types. Lymphocytes may be further subdivided into two major populations, T cells and B cells, on the basis of functions and cell surface phenotype. The large mononuclear cells (macrophages) are phagocytic cells and in peripheral blood are termed monocytes , whereas in tissues they are called histocytes. Polymorphonuclear white blood cells are subdivided into three major populations on the basis of staining properties of their cytoplasmic granules in standard haematologic blood smears or tissue preparations : neutrophil-pink, eosinophil-red, basophil-blue. Polymorphonuclear cells take part in both immune specific and non-inflammatory reactions.

#### 4.2. Origin , development and deployment of PMNs

PMNs originate from pluripotent stem cells in the bone marrow (Quessenberry and Levitt, 1979). Granulocytes begin as myeloblasts, which differentiate by division into myelocytes. Subsequently, during a 7-day postmitotic period, cytoplasmic and nuclear changes occur that result in a fully mature and functional PMN (Sawyer *et al.*, 1989). Two major populations of granules which PMN acquires are the azurophilic and the specific granules. The azurophilic granules (also known as primary granules) contain myeloperoxidases, lysozyme, cationic proteins, and neutral proteases, including elastase. Primary granules are released into the phagosome following phagocytosis and provide the PMN with localized microbicidal activity. The secondary (also called specific) granules are formed during the 7-day maturation period and

contain lactoferrin, lysozyme, vitamin B<sub>12</sub>-binding protein, cytochrome b, collagenase and certain receptor molecules. The specific granules which discharge much of their contents to the outside of the cell, are thought to provide a regulatory function in the inflammatory response (Weissmann *et al.*, 1980). In 1982, Dewald *et al.*, and in 1987, Petrequin *et al.*, identified tertiary granules in PMNs. These granules contain gelatinase, cytochrome b, and the MAC-1 glycoprotein adherence receptors. The role of these novel granules in PMNs is not fully understood (Petrequin *et al.*, 1987). Circulating PMNs represent only 5% of the total body pool. About one half of the intravascular population of PMNs are not circulating but are adherent to the endothelium of small vessels, a phenomenon called margination. After a half-life in the blood stream of 6-8 hours, PMNs enter the tissues, where they remain functional for 1-2 days (Sawyer *et al.*, 1989). An increase in both total number and percentage of circulating PMNs can be induced by acute infection, endotoxin, and steroids. This increase occurs via increased bone marrow production, accelerated bone marrow release, and demargination (Bishop *et al.*, 1968).

#### 4.3. Chemotaxis and activation of PMNs

Neutrophils may be attracted to sites of inflammation by a number of chemotactic factors (Table 3). The significance of these chemotactic factors *in vivo* is less clear. These chemotactic factors include low molecular weight peptides (e.g. FMLP), intermediate molecular weight compounds (C5a fragment of complement) to high molecular weight compounds such as immunoglobulins and lymphokines.

The existence of receptors for some of these factors, such

as the complement component C<sub>3b</sub> and IgG, are well-established (Roos *et al.*, 1981) as are receptors for complement chemotactic factor C<sub>5a</sub> (Smith, *et al.*, 1979) and formylated chemotactic peptides (Zigmond, 1978). Acute inflammatory reactions need not be initiated by immune mechanisms and are frequently associated with bacterial infections (such as staphylococcal and streptococcal infections) or traumatic tissue injury. In these situations neutrophils are attracted into sites of inflammation by chemotactic factors released by the infecting organisms (f-Met peptide) or by products of damaged tissue such as fibronectin, fibrin, collagen degradation products, or factors produced by other inflammatory cells (Stewart, 1987). In immune complex reactions, neutrophils are attracted by formation of activated complement components following Ab-Ag reaction in tissues. Upon attraction to sites of inflammation, neutrophils attempt to engulf and digest complexes e.g. consisting of bacteria coated with Ab and complement and other products of damaged tissues (Clark, 1990). The steps between stimulation of receptors and activation of PMNs have been best delineated for the chemoattractant FMLP (Fig. 6) (Sawyer, *et al.*, 1989).

#### 4.4. Adherence

Adherence to endothelial surfaces by PMNs is an important early step in the response of the cell to inflammatory stimuli (Atherton and Born, 1972). A family of cell-surface glycoproteins have been identified, which enhance adherence of leukocytes to a variety of targets, especially after the leukocytes have been stimulated (Anderson *et al.*, 1985). The MAC-1 receptor, also termed MO-1 and OKM1,

Table 3      Chemotactic Factors for Neutrophils

---

Complement
C <sub>5a</sub>
C <sub>5a</sub> des-Arg
Fibrinopeptide B
F-Met tripeptides
Collagen peptides
Transfer factor (Lymphokine)
Neutrophil chemotactic factors of :
Fibroblasts
Macrophages (Interleukin 1)
Lymphocytes
Platelet-activating factor
Leukotriene B <sub>4</sub>

---

is found on PMNs as well as on monocytes, NK cells, and some lymphocytes (Sawyer, *et al.*, 1989). This receptor functions as the receptor for C3bi (CR3) (Dana *et al.*, 1984). The lymphocyte function-associated antigen-1 (LFA-1) and receptors are found on PMNs as well as on monocytes and lymphocytes. Like the MAC-1 receptor, these receptors consist of  $\alpha$  and  $\beta$  subunits, a structural form common to all three receptors (Sanchez-Madrid *et al.*, 1983).

Patients with genetic deficiencies of these glycoproteins

have been identified whose clinical course has been marked by delayed umbilical cord separation, impaired pus formation, gingivitis, periodontitis, and recurrent bacterial infections of the skin and soft tissues (Abramson *et al.*, 1981). A variety of adherence-related white cell functions have been shown *in vitro* to be abnormal in these patients, including motility, post-stimulation adherence, spreading, aggregation, chemotaxis, and antibody-dependent cellular cytotoxicity (ADCC). Phagocytosis of particles opsonized by C3bi, post-phagocytic oxidative metabolism, and degranulation are also markedly abnormal. The severity of the clinical syndrome correlates closely with the degree of receptor deficiency ; those patients with more severe clinical courses have been shown to have virtually no expression of MAC-1, LFA-1 (Anderson *et al.*, 1985).

#### 4.5. Phagocytosis

PMN ingestion of microbes isolates them from host tissues and permits more efficient killing. Calcium and magnesium ions are required for optimal phagocytosis (Wilks and Bangham 1964) ; anaerobic glycolysis is the energy source. In general, bacteria must be coated or opsonized for attachment and ingestion to occur. The major opsonins are IgG (the only class of immunoglobulin (Fig. 7) that promotes phagocytosis in PMNs) and complement. IgG binds to bacteria with the Fab end, allowing interaction between the Fc portion and Fc receptors on the phagocytic cell. Interaction of IgG and Fc receptors opens calcium-dependent channels that may be important in regulation of phagocytosis and post-phagocytic events (Young *et al.*, 1985). Complement fixation can occur by the classical or alternative pathway,

either of which results in production of a C3 esterase essential for cleavage of C3 to C3a and C3b (Fig.7). C3a is a vasoactive component, C3b is an opsonic fragment. C3b binds to the CR1 receptor and can also undergo hydrolysis to C3bi, which binds to CR3 receptor on PMNs. Lew *et al.*, (1985) have shown that phagocytosis of C3b - and C3bi-coated particles differs from phagocytosis of IgG-coated particles in that the former is calcium-independent. Cell wall components of Gram-positive bacteria (Wilkinson *et al.*, 1978) and outer-membrane components of Gram-negative bacteria (LPS) can combine with serum factors to form C3 esterase and to fix C3b to microbial surfaces (Morrison and Kline, 1977). Capsules of bacteria may prevent exposure of these outer-wall or -membrane components to the serum, and in such cases specific antibody to the capsule is required before complement activation can occur (Horwitz and Silverstein, 1980)

Phagocytosis occurs with sequential receptor-ligand binding between the PMN and the microorganism (Fig.8). The motive force of phagocytosis resides in actin, myosin and actin-binding proteins. Undergoes gelation and cross-linking of F actin. The mechanisms of phagocytosis are incompletely understood. It has been proposed that the cytoplasm near the site of particle-PMN contact undergoes gelation and cross linking of F actin. This causes puckering of the plasma membrane at the site of contact because of attachment of microfilaments to the membrane. Bulging of the membrane around the particle occurs, and new particle-membrane contacts result. The net result is the formation of pseudopodia and a phagocytic vacuole (Yin and Stosfel, 1982).

Fig. 6

A highly simplified schema of PMN activation after FMLP stimulation. Coupling of FMLP to its cell-surface receptor leads to G protein-regulated activation of phospholipase C. Phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate in the cell membrane to produce two second messenger molecules, inositol 1,4,5-trisphosphate ( $IP_3$ ) and 1,2-diacylglycerol (DAG).  $IP_3$  mobilizes calcium ( $Ca^{++}$ ) from intracellular stores, thereby increasing concentrations of cytosolic calcium, which may also be elevated by influx of calcium from the extracellular space. DAG is a direct stimulus of protein kinase C. These processes together lead to activation of a variety of intracellular processes.

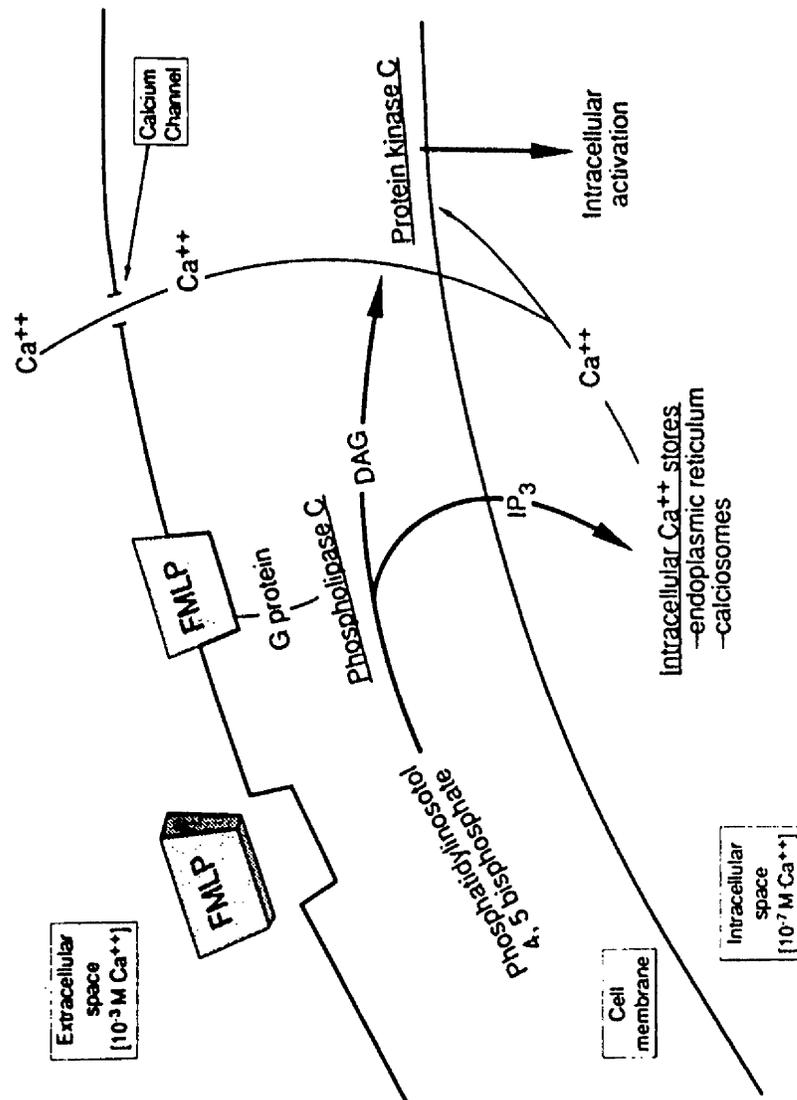
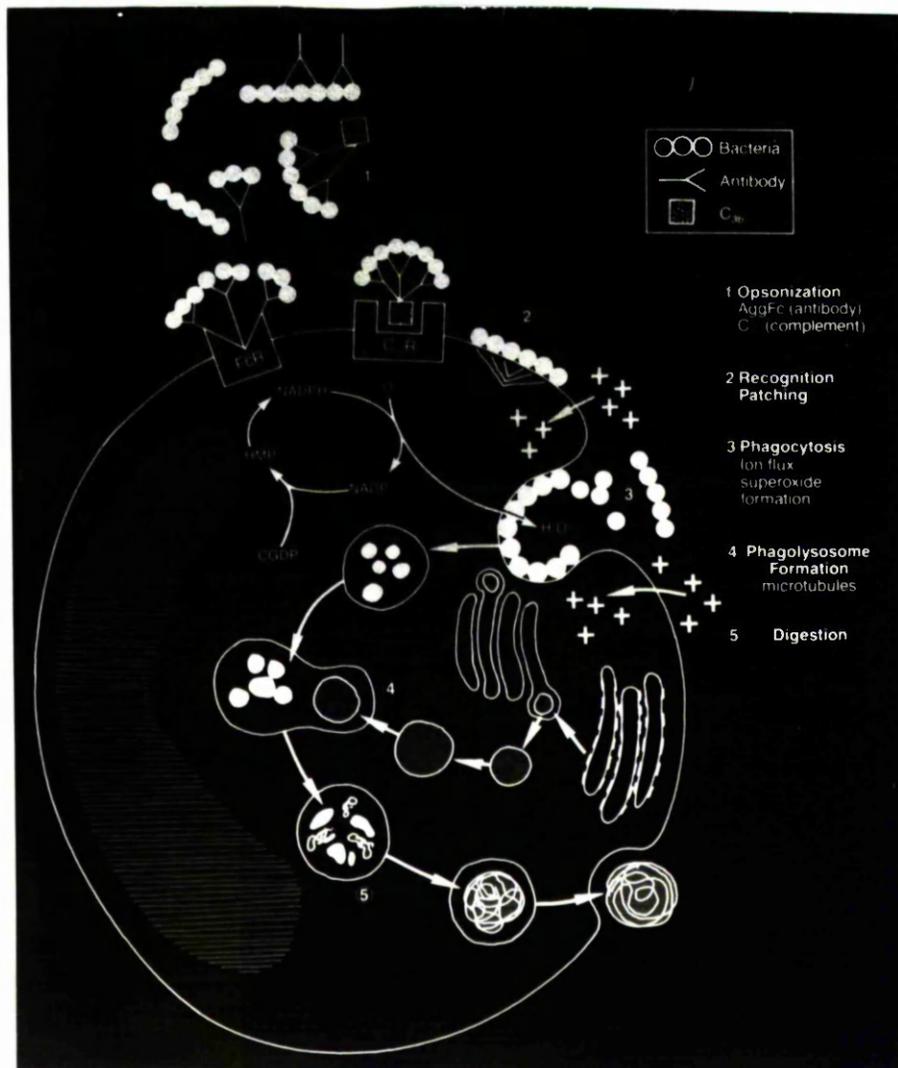
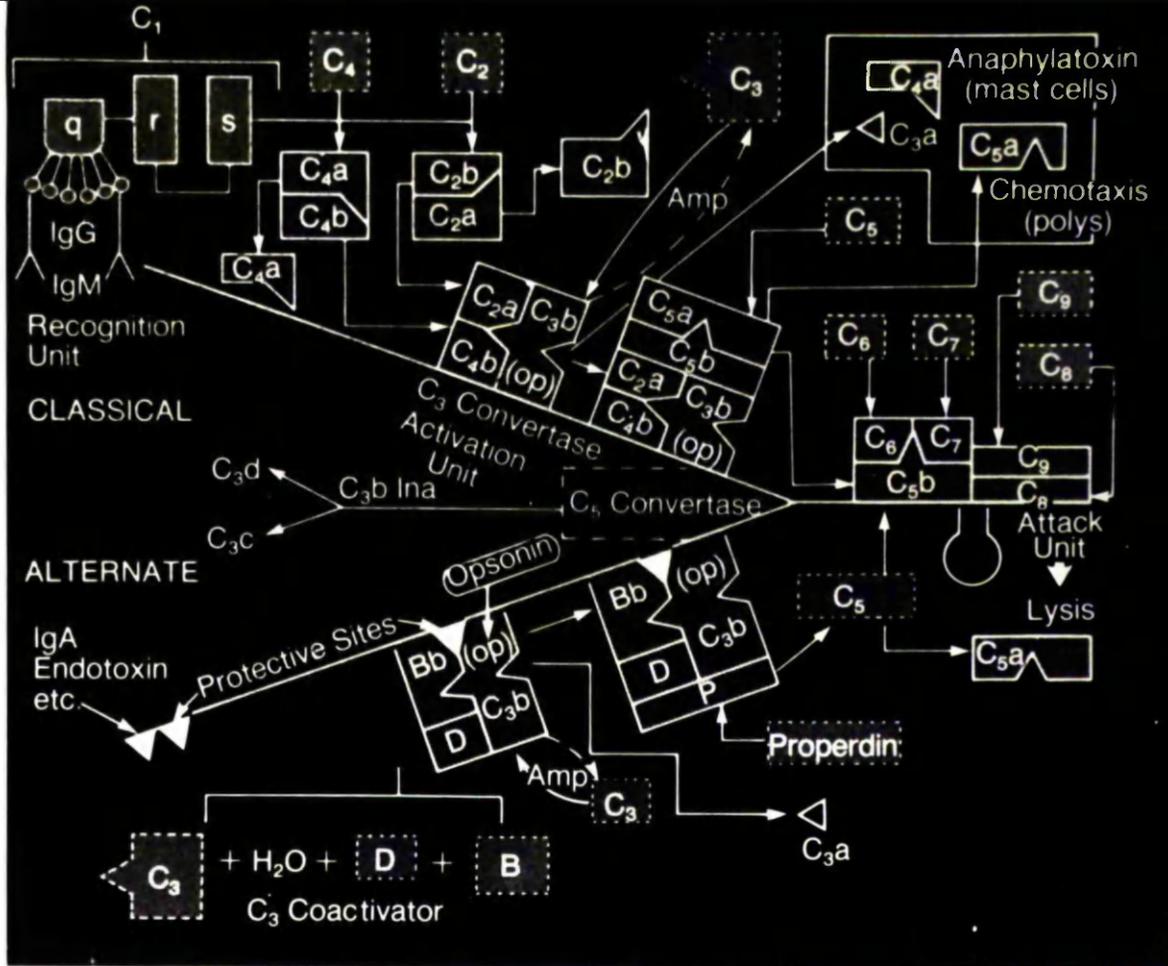


Fig. 7

Details of classical and alternate pathways of complement activation .

Fig. 8

Schematic drawing of steps in phagocytosis.



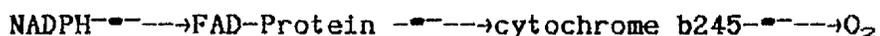
Observations suggest that the concentration of free cytoplasmic  $Ca^{++}$  is involved in either activation of NADPH oxidase (the enzyme responsible for superoxide production) or degranulation of primary granules into the phagosome (Sawyer *et al.*, 1985) or in both processes.

#### 4.6. Microbiocidal activity

There are two main arms of the microbiocidal activity of the PMNs: oxygen-dependent mechanisms and oxygen-independent mechanisms. Oxygen-dependent mechanisms rely on toxic molecules produced as a result of the respiratory burst. Resting PMNs consume only small amounts of oxygen, since most of their energy comes from anaerobic glycolysis. Oxygen-independent mechanisms are responsible for microbial killing in anaerobic environments such as the gastrointestinal tract, gingival crevices, and vaginal mucosa. Oxygen-independent mechanisms utilize lysozyme, lactoferrin, and cationic proteins (Leffell and Spitznagel, 1972). Six small cationic peptides called defensins, with molecular weights between 3,000 and 3,900 daltons, are localized in the azurophilic granules and possess antimicrobial activity against bacteria, fungi, and certain enveloped viruses (Ganz *et al.*, 1985). Another cationic protein with microbicidal activity in PMN azurophilic granules is the bactericidal/permeability-increasing protein (BPI). BPI is membrane-associated, and the intracellular presence of bacteria is required for its bactericidal activity (Weiss *et al.*, 1982).

The respiratory burst consists of a marked increase in oxygen consumption that occurs during and subsequent to ingestion of

microbes by PMNs. This increased oxygen consumption results from activation of an NADPH oxidase, which accepts two electrons from NADPH and transfers them to an FAD-flavoprotein associated with cytochrome b245 and from there to oxygen.



NADPH oxidase is not expressed in the resting PMNS. Part of the cytochrome b245 is translocated from the cytoplasm (perhaps from the secondary granules) to the plasma membrane following stimulation. In an acidic milieu, superoxide anion is converted to  $\text{H}_2\text{O}_2$  by superoxide dismutase. Hydrogen peroxide with myeloperoxidase (released from primary granules) and a chloride ion produces hypochlorous acid, a potent microbicidal agent. Other halides, such as iodide, can be used in the myeloperoxidase-hydrogen peroxide reaction. Bacterial iodination is closely correlated with bacterial death.

The hexose monophosphate shunt is activated during the respiratory burst and provides reduced pyridine nucleotide and reduced glutathione, which protects the PMN from oxidative damage by removal of excess  $\text{H}_2\text{O}_2$ .

##### 5. Iron in man and microbes

It is well established that iron is an essential constituent (element) of the body necessary for haemoglobin formation and certain chemical processes in living cells. The body of an adult man contains an average 4g of iron, over half of which is contained in haemoglobin in the red cells, the rest being distributed between myoglobin in muscles, cytochromes and iron stores in the form of ferritin and haemosiderin (Conrad and Barton, 1981, Messenger, and Rätledge, 1986),

Microbes, except *Lactobacillus plantarum* (Archibald, 1983,

Verstraete,<sup>etal.,</sup> 1989), *S. faecalis*, *S. avium*, and *S. faecium* (Verstraete, <sup>etal.,</sup> 1989), as for other living tissues, require iron for their cellular functions. This essential micro-nutrient is involved in many biochemical processes in micro-organisms serving as catalysts, or enzymes or engaged in electron transport processes of one sort or another (Messenger<sup>and Rattledge</sup> 1986) as shown in Table 4.

Abnormalities like anaemia and haemochromatosis occur in man in the presence of an unbalanced amount of iron. Similarly, iron deficiency can cause a plethora of effects on microbial structure and function. The concentration of some enzymes (or haeme-proteins), such as cytochromes, peroxidase and catalase are diminished in iron-limitation. *Torulopsis utilis* (*Torulautitis*, *Torula yeast* or *Candida utilis*), grown under iron-limiting conditions, produced cells containing between 45 and 59 nmoles cytochromes per gram dry weight whereas the cytochrome content under iron-replete conditions was 155 nmoles /gm dry weight of cells or still more (Clegg and Garland, 1971).

The activity of membrane-bound enzyme, NADH dehydrogenase, responsible for NADH oxidation in terminal electron transport system of aerobes, is altered in *T. utilis* by iron limited growth (Clegg and Garland, 1971). Other non-haem iron proteins like succinate dehydrogenase and ferredoxins are also affected by the iron-limitation (Knight and Hardly, 1968).

Aconitase (Dixon and Webb, 1958), aldolase (Kauppinen, 1963, alcohol dehydrogenase (Kauppinen, 1968), enzymes of flavin biosynthesis (Tanner *et al.*, 1945), enzymes involved in the biosynthesis of iron transporting compounds (O'Brien *et al.*, 1969), and enzymes of

porphyrin and haem synthesis are directly or indirectly affected with an increase in the activity of such enzymes under iron-stress conditions.

Moreover, reports on *Mycobacterium smegmatis* (Winder and O'Hara, 1962 ), *Clostridium perfringens* (Bard and Gunsalus, 1950), and *Torulopsis utilis* (Davison, et al., 1974), indicate that morphological changes appear in microorganisms grown under iron-limitation. It was also reported that length of *Mycobacterium smegmatis* was increased several fold as compared with cells grown in iron-replete medium. Explanations suggested by Winder and O'Hara, (1962) for this phenomenon were :

- 1) Either inhibition of DNA synthesis without proportional inhibition of cell growth
- 2) Or that iron deficiency had a direct effect on cell division or cell separation .

In summary several effects including changes in growth rate, growth efficiency (mitochondrial functions, cytochromes etc), and growth yields are associated with growth of microorganisms under iron-limiting conditions.

It is generally accepted that iron is essential for microbial growth and it is also clear that microbial multiplication is essential to virulence. That animals injected with iron in various forms were much more susceptible to infection than the untreated controls has already been reported (Table 5). This enhanced susceptibility of iron-treated animals to infection is now well-recognized and can be seen after challenge with a number of different

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TABLE 4                      ROLE OF IRON IN MICROORGANISMS

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<u>CELL COMPOSITION</u>	Iron deficiency can cause : growth inhibition, decrease in RNA and DNA synthesis, inhibition of sporulation, Changes in cell morphology .
<u>INTERMEDIARY METABOLISM</u>	Processes requiring iron : Tricarboxylic cycle (aconitase ), electron transport, oxidative phosphorylation, nitrogen fixation, aromatic biosynthesis, photosynthesis .
<u>METABOLIC PRODUCTS</u>	Biosynthesis of the following products regulated by iron : porphyrins, toxins, vitamins, antibiotics, hydroxamates, cytochromes, pigments, siderophores, aromatic compounds, DNA and RNA .
<u>PROTEIN AND ENZYMES</u>	Peroxidase, superoxide dismutase,
<u>REQUIRING IRON</u>	nitrogenase, glutamate synthase, ribonucleotide diphosphate reductase, aconitase, cytochromes, ferredoxin , flavoproteins, ferritin or ferritin-like iron storage compounds, iron-sulphur proteins .

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organism in numerous animals species (Bullen and Griffiths, 1987).

However, it is also apparent that *S. pyogenes* and related organisms appear to be the exceptions to this general rule.

### 5.1. Availability of iron

Iron is generally biologically unavailable, because in an oxidizing atmosphere, surface iron exists as an insoluble oxyhydroxide polymer (Carrano and Raymond , 1979). Various means of solubilizing iron, and holding it in a utilizable form, have therefore evolved in all organisms. In microorganisms, low molecular weight chelate compounds are employed (Griffiths , 1987) but in higher animals a family of iron-binding proteins, the glycoproteins, fill this role , controlling iron levels and playing a crucial role in iron metabolism. As mentioned earlier, the majority of iron in humans is bound to iron-binding proteins, such as transferrin, lactoferrin, ferritin, haemosiderin, myoglobin and haemoglobin. To be more specific, the total iron concentration in mammalian body fluids is high (20  $\mu\text{M}$ ), yet almost all of the iron is tightly bound to specialized iron-binding glycoproteins. This limitation in the concentration of free iron in the body fluid creates bacteriostatic conditions for many microorganisms and is an important nonspecific defence mechanism against invading bacteria. This results in a free-iron concentration of  $10^{-12}$  to  $10^{-18}\text{M}$ , far below the bacterial requirement of 50nM to 4- $\mu\text{M}$ . Before discussing how potential pathogens overcome this lack of available iron, it is necessary to consider briefly the glycoproteins involved in iron-binding .

5.1.1 Transferrin: Transferrin is a monomeric glycoprotein of molecular weight 80,000, with the capacity to bind reversibly two  $\text{Fe}^{3+}$  ions per molecule. Transferrin is responsible for the transport of iron in blood around the body and binds to specific receptors found

Table 5

Bacteria whose virulence in experimental infections is enhanced by injecting iron compounds

<i>Aeromonas hydrophila</i>	Miles, Khimji and Maskell, 1979
<i>Clostridium perfringens</i>	Bullen, Cushnie and Rogers, 1967
<i>Cl. oedematiens</i>	Miles, Khimji and Maskell, 1979
<i>Corynebacterium renale</i>	Henderson, Kadis and Chapman, 1978
<i>Escherichia coli</i>	Bullen, Leigh and Rogers, 1968
<i>Klebsiella pneumoniae</i>	Miles, Khimji and Maskell, 1979
<i>Listeria monocytogenes</i>	Sword, 1966
<i>Neisseria gonorrhoeae</i>	Payne and Finkelstein, 1975
<i>N. meningitidis</i>	Calver, Kenny and Lavergne, 1976
<i>Pasteurella multocida</i>	Bullen <i>et al.</i> , 1968
<i>P. haemolytica</i>	Chengappa, 1983
<i>Pseudomonas aeruginosa</i>	Forsberg and Bullen, 1972
<i>Salmonella typhimurium</i>	Kaye, Merselis and Hook, 1965
<i>Staphylococcus aureus</i>	Gladstone and Walton, 1971
<i>Vibrio anguillarum</i>	Crosa, 1980
<i>V. cholerae</i>	Ford and Hayhoe, 1976
<i>V. vulnificus</i>	Wright, Simpson and Oliver, 1981
<i>Yersinia pestis</i>	Jackson and Burrows, 1956
<i>Y. enterocolitica</i>	Robins-Browne and Prpic, 1985

on proliferating cells or those involved in haemoglobin synthesis. Its presence and function is of great importance in almost every aspect of iron metabolism as is clear from its specificity for iron and its high stability constant, ( $10^{24}M^{-1}$ ) as the iron complex (Asien and Brown, 1975). This protein transports iron through the body via the serum and binds to mammalian cells via specific surface receptors (Iacopetta *et al.*, 1982). (Tf+Iron) is transported into and out of cells where it donates its iron without being destroyed (Awai and Bron 1963). By what mechanism the iron is released intracellularly from transferrin is not clearly understood. Konopka *et al.*, (1980) proposed that release of iron from Tf may be mediated primarily by pyrophosphate, although other organic phosphates and even ascorbate also may mediate in this reduction. At this gate-way of metabolism, iron exist as a "labile iron pool" (White *et al.*, 1976) destined for storage in the form of ferritin (Messenger and Rattledge, 1986). It is this iron pool that is subject to chelation therapy; that is, the pool may be depleted of iron by treatment of cells with certain chelating agents. It is important to note here that most microbial siderophores are of a molecular weight of <1,000, and it is possible that they might easily diffuse into the cell and attack the mammalian iron system intracellularly at the point where Tf iron is exchanged. Other possible mechanisms utilised by microbial siderophores are discussed under "Acquisition of Iron by Bacteria" (below). Tf has two binding sites for iron, and these may differ in their ability to accept and donate iron (Marx *et al.*, 1982). It is also interesting to note the dual role of glycoproteins i.e., they serve either to withhold iron from bacteria, or as a source of iron to them. This

largely depends upon the experimental approaches to the same problem. Some organisms are able to compete successfully, and perhaps, directly with Tf to acquire iron whereas other microbes have alternative mechanisms.

5.1.2. Lactoferrin: The sources of lactoferrin are mammalian milk and other secretions, plasma, and neutrophils (Table 6) (Mason *et al.*, 1969). Unlike transferrin, lactoferrin has a high isoelectric point and does not readily release its iron at low pH. Most previous studies attribute the bacteriostatic effect of milk to its lactoferrin content because the effect is reversible by addition of free iron. Arnold *et al.*, (1977) reported that *S. mutans* and *Vibrio cholerae*, but not an enteropathogenic serotype of *Escherichia coli*, were killed by incubation with purified human apo-lactoferrin (Iron-free lactoferrin) but not by iron-saturated lactoferrin. This antibacterial effect of lactoferrin presumably was due to its ability to chelate and withhold iron, an essential material for these organisms. Unsaturated lactoferrin was shown by Finkelstein *et al.*, (1983) to inhibit growth in numerous bacteria including *E. coli*, *Vibrio cholerae*, *Salmonella typhimurium*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Neisseria meningitidis*, *Neisseria gonorrhoeae* and *Neisseria sicca*. From these observations, Finkelstein suggested that most of the organisms tested were able to multiply for only a few divisions in the presence of apo-lactoferrin, presumably utilising endogenous iron stores.

5.1.3. Haemoglobin: One of the basic prerequisites for normal haemoglobin synthesis is a regulated supply of iron into a system

producing haem, i.e., mitochondria. Iron forms part of haem which is coupled to globin synthesis. As the majority of body iron is haemoglobin-associated, haemoglobin is considered to be of relatively little importance to microbes. It is only accessible to bacteria when it is released from the red cell. Haemoglobin consists of four haeme complexes, each linked to a polypeptide chain. Each haeme complex can bind one molecule of oxygen and contains one atom of iron. Although haemoglobin contains 0.34% iron, it does not readily donate this iron to microbes. Although microbes utilize haeme or haemin as an iron source *in vitro* it does not necessarily follow that they do so *in vivo*. In serum, breakdown products of haemoglobin rapidly form complexes with other circulating proteins such as haptoglobin. Eaton *et al.*, (1982) demonstrated that haptoglobin prolongs the survival of rats inoculated i.p. with *E. coli*. But with the production of haemolysins, microbes may promote release of considerable quantities of haem *in vivo*. Interestingly, the production of haemolysin by *Listeria monocytogenes* was shown to be inversely related to the iron level in the medium (Coward *et al.*, 1981).

5.1.4. Ferritin: Ferritin consists of an apoprotein shell (molecular mass 480 KDa) enclosing a core of iron in the form of ferric hydroxy-phosphate, which may contain up to 4500 iron atoms. Ferritin is the main soluble iron-storage protein in mammals and provides the major non-toxic store of iron in the liver, spleen, and other organs which can be mobilized when needed.

5.1.5. Bacterioferrin: It is important to note that ferritin-like molecules have been found in several bacterial species (Harrison,

Table 6

## Locations of host iron-binding glycoproteins

Name	Sources
Lactoferrin	Saliva Tears Nasal secretion Intra <sup>o</sup> cular fluids Intestinal fluids Seminal fluids Cervical mucus Colostrum Milk (Human)
Transferrin	Polymorphonuclear leucocytes Tears Nasal secretion Intra <sup>a</sup> ocular fluids seminal fluids
Ovotransferrin	Serum Lymph Avian egg white

1979). What advantage is gained by storage of iron by bacteria is not clear. This bacterioferrin, first reported by Stiefel and Watt, was isolated from the nitrogen-fixing bacterium *Azotobacter vinelandii*. *Proteus mirabilis* and *Mycoplasma capricolum* have also been reported to possess similar proteins as has *E. coli* (Harrison, 1979).

## 5.2. Acquisition of iron

Pathogens assimilate iron by one of the following methods.

5.2.1. *Through proteolytic cleavage*: The pathogens degrade glycoproteins, and remove iron from iron-binding proteins. The prominent example in this category is *Bacteroides* species (Carlsson, 1984).

5.2.2. *By reduction of Fe<sup>3+</sup> complex to Fe<sup>2+</sup>*: Cowart and Foster (1985) showed that *in vitro*, *Listeria monocytogenes* secretes a soluble reductant that effectively removes iron from the Fe<sup>3+</sup>-transferrin complex. It is not yet clear whether such a reduction process can actually operate *in vivo*.

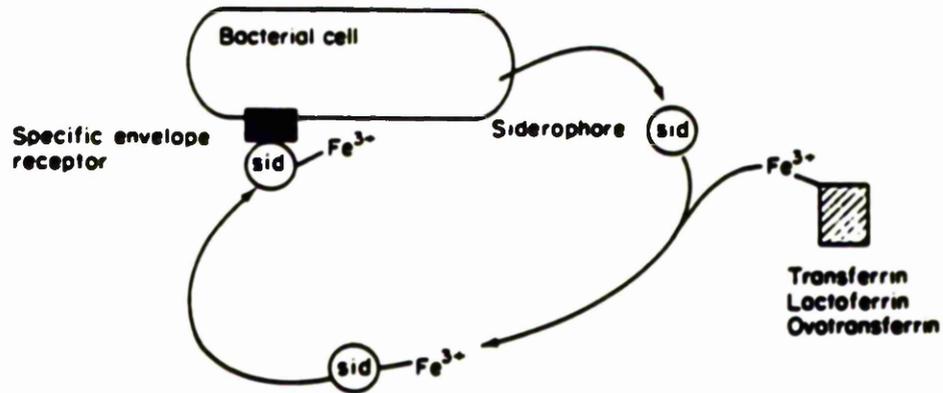
5.2.3. *Direct interaction (at cellular level)*: Until now only *Neisseria* and *Bordetella* species are reported to acquire iron by direct interaction with iron-binding proteins although this is not universally accepted. P<sub>y</sub>ne and Finkelstein (1978) were the first to report that siderophores are produced by certain strains of gonococci and in 1981 they classified these siderophores as hydroxamates. These siderophores were detected in the culture supernates of both gonococcus and meningococcus. However, several other investigators (Norrod and Williams, 1978; Mickelson and Sparling; 1981, Simonson *et al.*, 1982) failed to confirm the observations, although West and

Sparling (1985) reported siderophore production by both *N. gonorrhoeae*, and *N. meningitidis*. Generally it is now accepted that neither the gonococcus nor the meningococcus synthesize siderophores, but they can utilize siderophores provided exogenously. In 1981, Yancy and Finkelstein showed that *N. meningitidis* and *N. gonorrhoeae* could use ferric citrate as an iron source and West and Sparling (1985) showed that aerobactin (hydroxamate-type) siderophore can be used by gonococcus. Isolates of *Neisseria* were able to use haemin as a sole source of iron and could also remove iron from haemoglobin (Mickelson and Sparling, 1981). Schematic representation of iron-uptake by direct interaction with host iron-binding proteins is shown in the Fig. 9b.

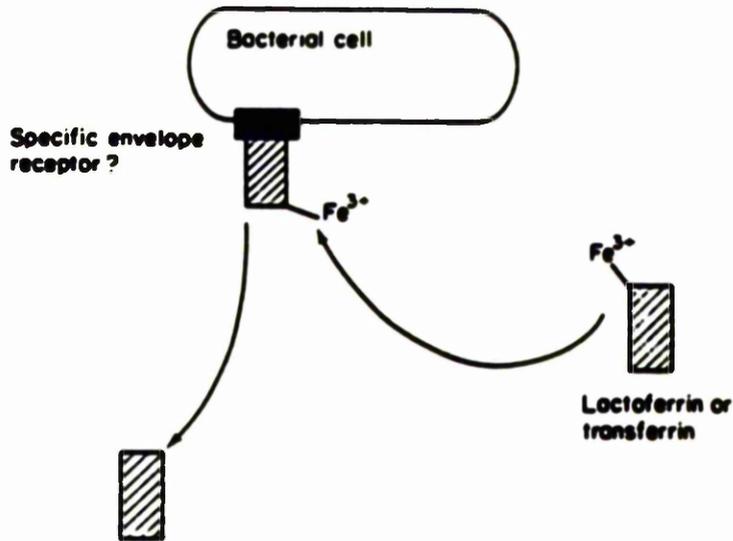
5.2.4. *Siderophores as iron transporters*: To combat the scarcity of the essential growth element iron, microbes have evolved a very sophisticated iron-uptake mechanism. This involves the production of powerful iron-binding factors known as siderophores (Fig. 9a). Genetically determined, these siderophores are synthesized and exported into the medium where they capture iron and are then internalized through the mediation of specific membrane protein receptors that are also genetically determined and iron responsive (Neilands, 1984) as shown in Fig. 10.

Siderophores belong to two distinct classes: the phenolates (or catechols) and the hydroxamates (Lankford, 1973). A given organism may produce siderophores of either one or both classes: (The catecholamides typically bind iron more tightly than do the hydroxamates). Some siderophores may be plasmid-encoded. These low

Fig. 9



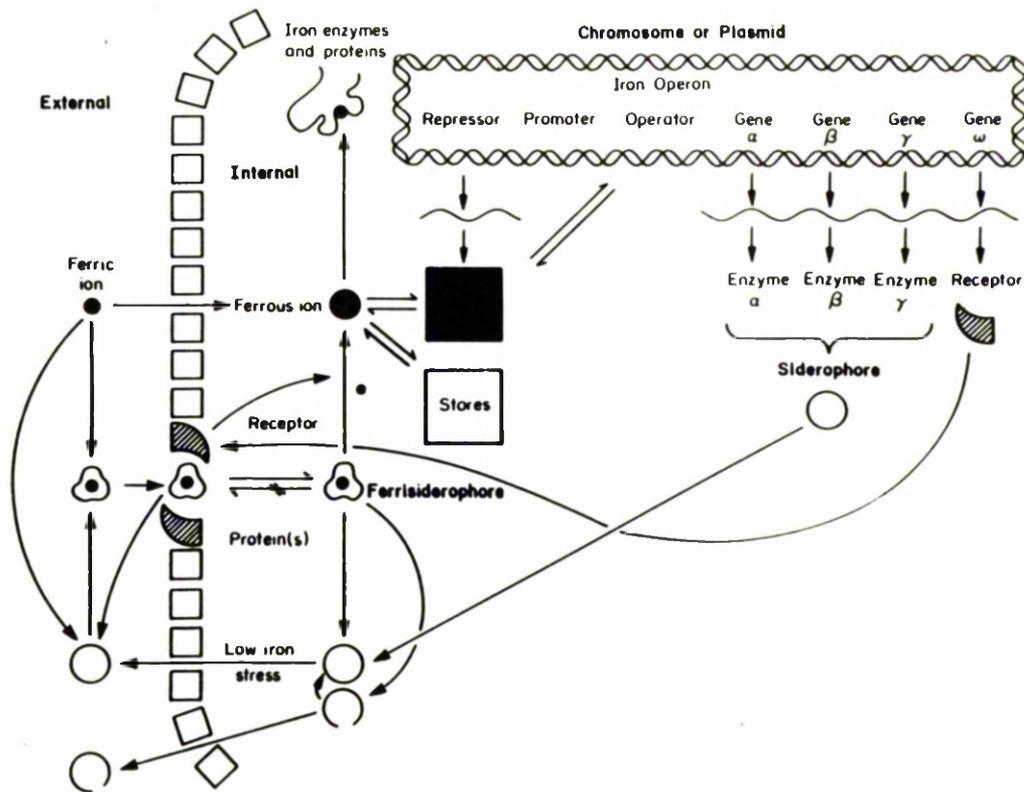
(a)



(b)

Schematic representation of two ways by which pathogenic bacteria obtain iron from iron-binding proteins: (a) siderophore-mediated iron uptake; (b) iron uptake by direct interaction with host iron-binding glycoproteins

Fig. 10



*Schematic model of low and high affinity iron assimilation pathways in aerobic and facultative anaerobic microorganisms (slightly modified from a scheme presented earlier).<sup>16</sup>*

molecular weight chelators are part of what is termed "the high affinity" iron transport system. This pathway, which has been identified in virtually all aerobic, and facultative anaerobic microbes is comprised of two parts:

- 1) Relatively low-molecular-weight (55-1000) ferric ion-specific ligands generally termed siderophores (Greek for "iron bearer") (Neilands, 1984).
- 2) The membrane-bound system for transport and utilization of the chelated iron. "A low affinity" iron transport system is thought to operate when iron is freely available, but little is known about the mechanism involved. This process is relatively insensitive and non-specific and does not require specific carriers. This pathway has played a vital role, however, in the development of knowledge of the high-affinity process. Moreover, the low affinity process enables the cell to survive genetic elimination of all or part of the high-affinity system. The schematic representation of low and high-affinity systems is shown in Fig. 10.

A) Phenolates (Catecholamides) are derivatives of catechol (O-dihydroxybenzene). An example is enterobactin (=enterochelin): a cyclic trimer of 2,3-dihydroxyl-L-serine produced by various enterobacteria; in *E. coli* it is synthesized by the condensation of 2,3-dihydroxybenzoic acid (formed via chorismic acid) with L-serine—the synthesis involving products of genes *ent-entG*. In *E. coli* the cell surface receptor for enterobactin is the Fep A protein. *Vibrio anguillarum* forms a novel catechol-type siderophore, anguibactin (Neilands, 1984).

B) Hydroxamates are derivatives of hydroxamic acid (R.CO.NHOH). They

include aerobactin, a siderophore formed by various enterobacteria ; genes encoding the aerobactin system i. e., the siderophore (genes iucA-iucD) and its outer membrane receptor protein (gene iutA)- are carried by many ColV plasmids, although the system may also be chromosomally encoded (Neilands, 1984). Other hydroxamate siderophores include coprogen (produced by many fungi) ; ferrichrome (formed by species of *Aspergillus*, *Neurospora* and *Ustilago*) ; Ferrioxamines and Desferrioxamine (formed by certain bacteria).

Recently Staphyloferrin A, a structurally novel siderophore from staphylococci has been reported by Rapp *et al.*, 1990.

### 5.3. The role of iron in the growth and production of SLS in

#### *S. pyogenes* Gr A

Haemolysins are virulence factors for many microorganisms (Martinez, *et al.*, 1990). It has been suggested that stimulation of bacterial growth might be due to the increase of available iron produced by the lysis of erythrocytes by haemolysins (Martinez *et al.*, 1990). It has been reported that the combination of *Escherichia coli* with haemoglobin in the peritoneal cavity is lethal (Bornside *et al.*, (1968). In addition toxins and other virulence factors are often made in optimal yields only during bacterial growth in low-iron media. Among such toxins include diphtheria toxin from *Corynebacterium diphtheriae* (Russell and Holmes, 1983), Toxin A from *Pseudomonas aeruginosa* (Bjorn *et al.*, 1978), Shiga toxin from *Shigella dysenteriae* (van Heyningen *et al.*, 1953), and tetanus toxin from *Clostridium tetani* (Mueller and Miller, 1945).

Francis *et al.*, (1985) reported "it seems unclear whether or

not *Streptococcus pyogenes* Gr A even uses haemoproteins for aerobic respiration. Although haemin has been observed to be necessary for the aerobic existence of streptococci such as *S. faecalis* and *S. sanguis* (Whittenbury, 1978), *S. pyogenes* was reported in one study to lack haemin-induced NADH oxidase activity, thereby suggesting a lack of a cytochrome system (Ritchey and Seeley, 1976). However, that same study indicated *S. sanguis* to be devoid of NADH oxidation. If the studies of *S. sanguis* are accurate, it appears that although this bacterial species does not require a cytochrome system for aerobic respiration, it still requires haemin for growth (probably to synthesize a catalase-like protein for protection against  $H_2O_2$  formation". In the interpretation of the ability to remove iron from haemoglobin and the haptoglobin-haemoglobin complex of *S. pyogenes* gr A, Francis, *et al.*, (1985) suggested that *S. pyogenes* might secrete a high haem affinity protein such as haemopexin which would be bound by a membrane receptor when carrying haem. The haem could then be transferred into the cell interior.

Griffths and McClain (1987) reported for the first time that streptolysin S produced by *S. pyogenes* was affected by the levels of iron. They showed that streptococcal growth was stimulated by iron and that a low iron concentration (1.2  $\mu\text{g/ml}$ ) in the medium was conducive to high haemolysin production while an increase of iron up to 5.0  $\mu\text{g/ml}$  offered no correlation.

**OBJECT OF RESEARCH**

Objects of research

In the last decade considerable advances have been made in knowledge of streptolysin S (SLS), namely

- 1) *In vivo* production of SLS (Duncan, 1983)
- 2) Influence of SLS on T lymphocytes subpopulations (Hryniewicz, *et al.*, 1984)
- 3) Insertional inactivation of SLS expression in *Streptococcus pyogenes* (Nida and Cleary, 1983)
- 4) Purification of RNA-core-induced SLS, and isolation and haemolytic characteristics of the carrier-free toxin (Alouf and Loridan 1986)

However, there is much uncertainty about other aspects which are largely unexplored and less developed, for example, the mechanism of production of SLS, and its interaction with cells of the defence system.

The objects of this research were :

- (a) To investigate the role of iron on the growth of *Streptococcus pyogenes* group A and its possible role in SLS production.
- (b) To purify RNA-core-induced SLS and characterize the carrier-free toxin.
- (c) To examine the effect of homogeneous SLS on the chemiluminescent response of rabbit peritoneal neutrophils.
- (d) To examine the effect of SLS on the opsonization process.

## MATERIALS AND METHODS

## 6. Toxin production

### 6.1. Strains

Two strains of *Streptococcus pyogenes* GrA were examined for streptolysin S (SLS) production. Strain C203S was obtained from Professor Joseph E. Alouf, Institut Pasteur, Paris, and strain 55903M from the Department of Bacteriology, Royal Infirmary, Glasgow. Strain C203S was selected because it produces a high level of SLS whereas strain 55903M had not been tested for SLS production. The strains were stored lyophilised or as broth cultures supplemented with 20% glycerol at -20°C.

### 6.2. Media

The culture medium (BHI-BM) used for toxin production was that of Alouf and Loridan (1986). Details of the preparation were as follows : 50 ml of Brain heart infusion broth (Oxoid or Difco) was supplemented with 1%(w/v) maltose (BDH) and 2% (w/v) sodium bicarbonate (May and Baker) and was abbreviated as BHI-BM. Stock solutions of sodium bicarbonate (10% w/v) and maltose (10% w/v) were sterilized by membrane filtration (0.45 µm pore size), and stored at 4°C until used. Volumes of BHI-BM sterilized by autoclaving (15 lb/15-min) were supplemented with sterile maltose (1% final conc) and sterile sodium bicarbonate (2% final conc) before inoculation . Inocula were grown on agar plates consisting 200 ml of BHI broth (Difco) solidified with 0.7 % w/v technical agar (Oxoid) and sterilised by autoclaving at 121°C. After pouring, solidifying and drying the plates were stored at 4°C until used .

### 6.3. Cultural conditions

BHI agar plates were inoculated from a loopful of thawed glycerinated broth culture and incubated at 37°C for 10 h. Simultaneously, plates of Sheep blood agar (Oxoid Blood Agar Base No.2, Oxoid Ltd, London, England) plus BHI Blood agar were also inoculated to confirm the presence of  $\beta$ -haemolytic phenotype of the colonies. After incubation, 50 ml of BHI.BM (Oxoid or Difco) was inoculated as a starter culture from the growth harvested from one BHI agar plate. After incubation for 10 h at 37°C without shaking, the starter culture was added to 2 l of BHI-BM in a 2 l Erlenmayer flask which was then incubated at 37°C for 6 to 7 h without shaking.

### 6.4. Measurement of bacterial growth

Growth was estimated by measuring the  $A_{600nm}$  of samples of culture withdrawn at 1 h intervals from 5 to 9 h. When the  $A_{600nm}$  exceeded 0.3 the sample was diluted with BHI-BM to bring the absorbance value to within measuring range. Absorbance values were measured on a Shimadzu recording spectrophotometer (Graphicord-UV 240) using cells with a 1cm light path.

### 6.5. Induction of streptolysin S

The method used was essentially that of Alouf and Loridan (1986) except that centrifugation time was reduced to 10 min. A 50 ml volume of overnight culture grown statically was inoculated into 2.5l (or 20 ml in 1 l or 10 ml in 500 ml) and grown unshaken for 6 to 7h at 37°C. The culture was centrifuged at 12000g (Sorval RC-5B) for 20 min at 4°C and the cell pellet was washed in 100 mM potassium phosphate buffer, pH 7.0 before resuspension to a final volume of 40- ml (or 16

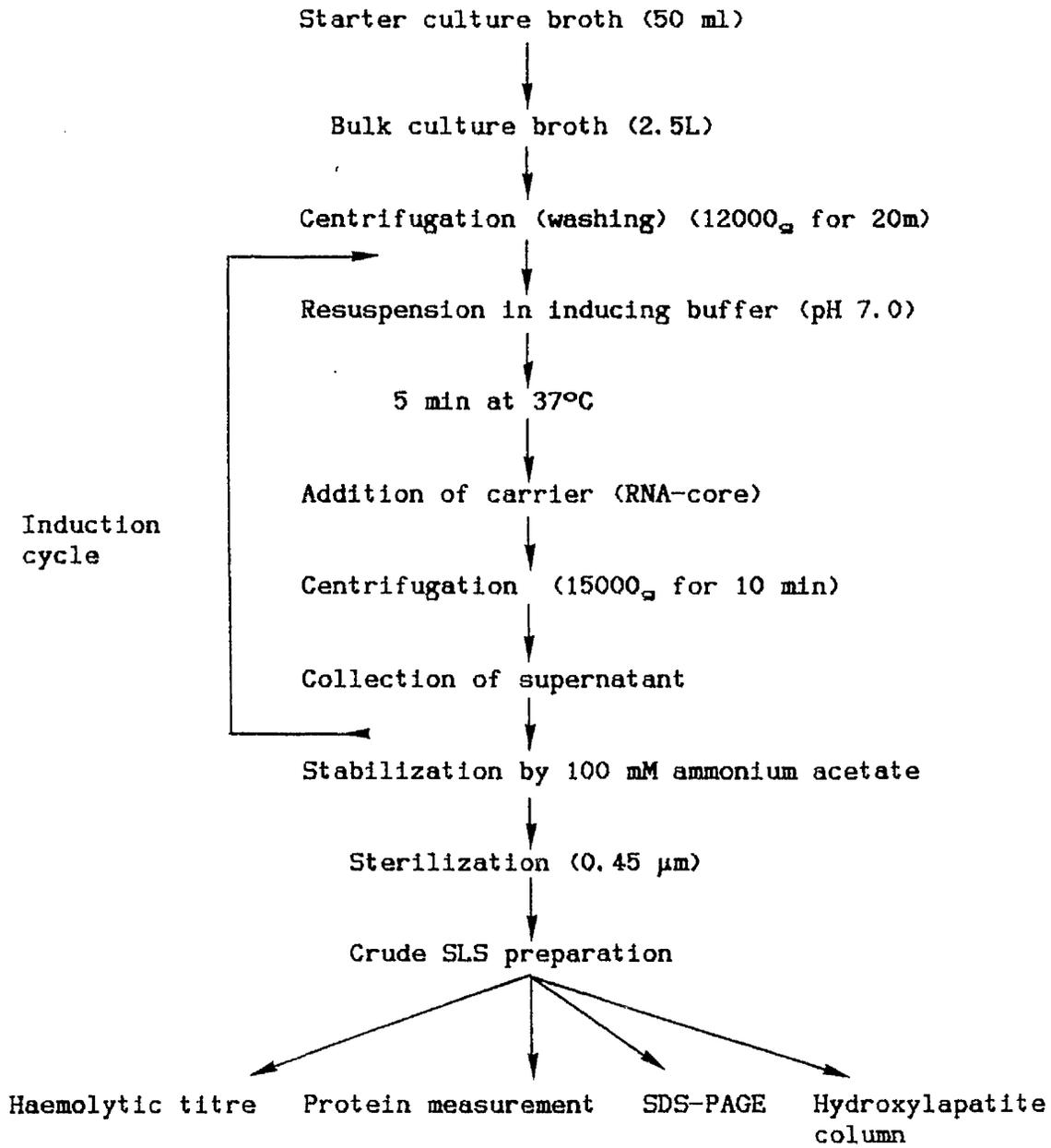
ml in case of 1 l , 8 ml in case of 500 ml) of induction buffer (IB) (see Appendix 15.3. ; ) supplemented with 30 mM-maltose. The cell suspension was incubated for 5 min at 37°C and then induced by adding 0.5 mg/ml RNA-core (Sigma) (see Flow sheet 1). After 5 min the cell suspension was centrifuged (Sorval RC-5B) at 15000g for 10 min at 4°C. The supernate (crude SLS) was collected and made 100 mM with ammonium acetate (Sigma) to stabilize SLS (Lai *et al.*, 1978). The pellet was then resuspended in 40 ml (or 16 or 8 ml) of induction buffer supplemented with maltose (15 mM final concentration ) and induced as described above. Ten to 14 inductions could be made on the same pellet. The supernates from inductions were kept at 4°C.

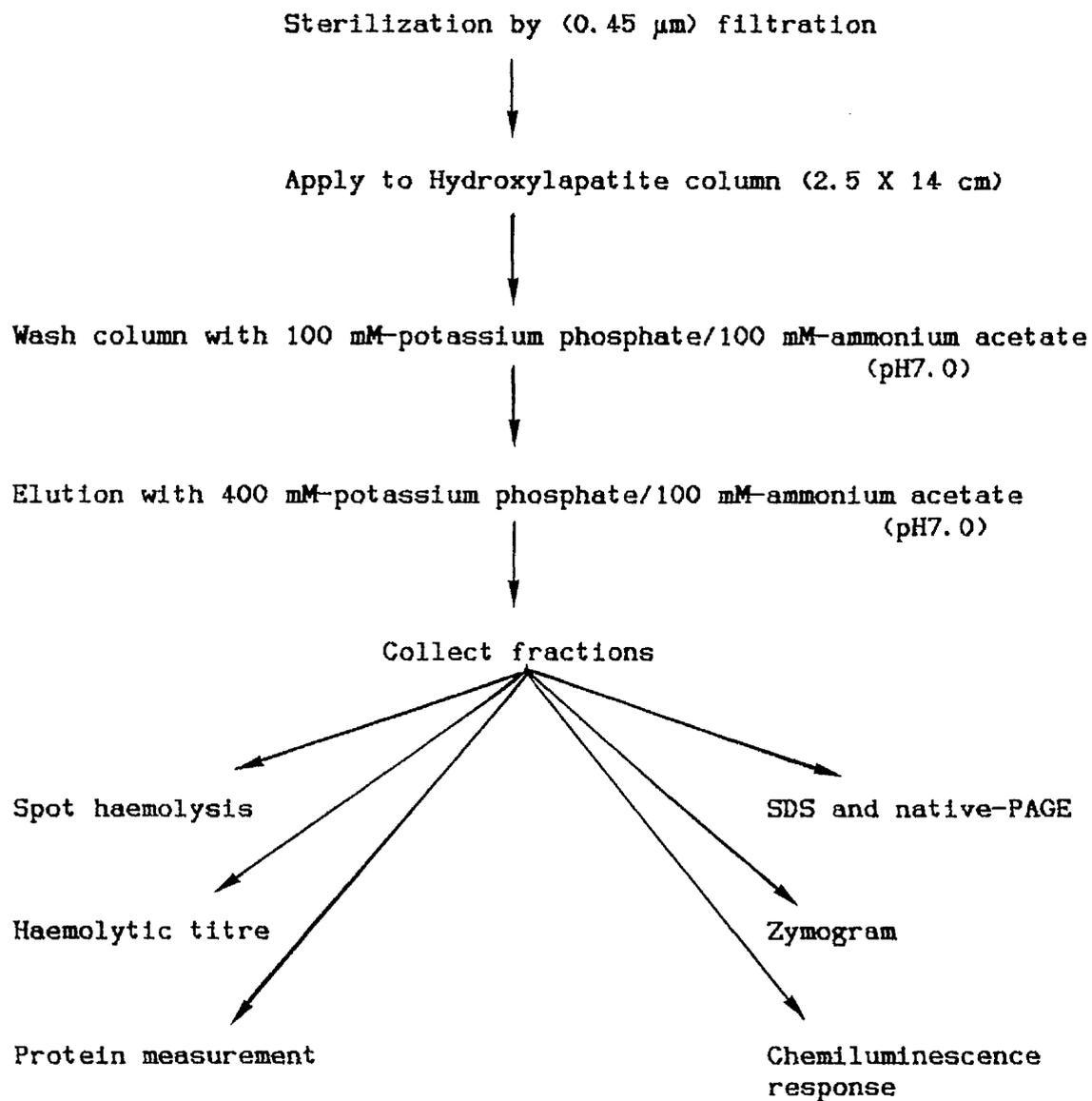
#### 6.6. Purification of Streptolysin S

All steps were done at 4°C . Crude SLS preparation ; i. e. , pooled supernates from 14 induction cycles (see Materials and Methods 6.5) was applied to a column (2.5x14cm) of hydroxylapatite (Bio-gel HTP, from Bio-Rad) equilibrated with 100 mM potassium acetate /100 mM ammonium acetate (pH 7.0). After application of the crude toxin the column was washed with 1 column volume (100 ml) of this buffer and then the toxin was eluted with 400 mM-potassium phosphate/100 mM ammonium acetate buffer (pH 7.0). The flow rate was 30 ml h<sup>-1</sup> and 3.0 ml fractions were collected and analysed by A<sub>280</sub> and spot haemolysis. Haemolytic fractions were then further characterized by haemolytic titre , molecular weight estimation on SDS-PAGE and haemolytic activity on native gels (see Flow sheet 2) .

Flow sheet 1

INDUCTION OF STREPTOLYSIN S



Flow sheet 2 PURIFICATION OF STREPTOLYSIN S (All steps 4°C)

## 6.7. Assays

### 6.7.1. Haemolytic assay

Haemolytic activity was determined using serial two-fold or ten-fold dilutions of SLS in 150 mM sodium phosphate buffer (pH 6.8) in tubes or in microtitre trays (Sterilin). Defibrinated sheep blood (Becton Dickinson) was centrifuged at 5000g for 5 min and the sedimented erythrocytes washed three times in 150 mM sodium phosphate buffer (pH 6.8). Sheep red blood cells (SRBC) were suspended (about 2% v/v in 150 mM sodium phosphate buffer) such that a 30 fold dilution of this suspension in distilled water gave an  $A_{541}$  of 0.16. This standard SRBC suspension (about  $4.8 \times 10^7$  cells / ml) was kept at 4°C and used within 4 days. For the tube assay, 0.9 ml diluent (150 mM PBS) was placed in tubes and 0.1 ml of original sample toxin (crude or purified) was added to the first tube ( $10^{-1}$ ) and was mixed gently. Diluted toxin (0.1 ml) was transferred to tube No 2 ( $10^{-2}$ ) and mixed well and in this way a sample was transferred to tubes 3 ( $10^{-3}$ ) and 4 ( $10^{-4}$ ). To all these tubes 0.5 ml of 2% SRBC was added. The tubes were incubated at 37°C for 45 min and then briefly centrifuged at 5000g (Biofuge A, Heraeus Sepatech or Mistral 6L) for 5 min. The percentage of released haemoglobin was estimated by the  $A_{541}$  of the supernatant fluid. One haemolytic unit (HU) was defined as the amount of test material which caused release of 50% of the haemoglobin. Values were estimated graphically. Controls were done by mixing the test material with cholesterol (10 µg / ml) which inhibits the haemolytic activity of SLO and not that of SLS (Hewit and Todd, 1944), or with trypan blue (13 µg / ml) which inhibits SLS (Ito, 1940). The RNA-core alone, and mixtures with 30% maltose plus 100 mM ammonium acetate did not exhibit

any haemolysis up to  $10\text{mg ml}^{-1}$ .

#### 6.7.2. Protein estimation

Protein estimation was carried out by the method of Bradford (1976) using bovine serum albumin (Sigma) as standard. Protein concentration was also estimated by  $A_{280\text{nm}}$  (Shimadzu recording spectrophotometer). The protein concentration in the eluant from the hydroxylapatite column was monitored by measuring  $A_{279\text{nm}}$  using an LKB UVICORD 1 linked to an automatic LKB fraction collector and LKB chart recorder.

#### 6.8. Polyacrylamide gel electrophoresis of protein

##### 6.8.1. Molecular weight estimation

SDS-PAGE was performed according to the method of Laemmli, (1970). 15% resolving gels were used throughout this study. Stacking gels contained 4.5% (w/v) acrylamide. Samples were solubilised in an equal vol of sample buffer (see Appendix 15.2.vi) in a water bath at  $100^{\circ}\text{C}$  for 90 seconds. The same procedure was applied to the molecular weight standards (SDS-7 Kit), obtained from Sigma. Loading volume of samples was adjusted according to the capacity of respective gel i.e., 50ul for 1.5 mm thick gel and 25-30 ul for 0.8mm thick gel. Samples were electrophoresed into the stacking gel at 20 mA and at 25 mA in the separating gel for 0.8 mm thick gels. For 1.5 mm thick gel, the current for the stacking gel was increased to 30 mA and for the separating gel to 45 mA. Gels were run until the tracking dye reached the bottom of the gel, after which gels were fixed and stained either by Coomassie blue or by the silver staining method. This silver staining method was adapted and modified from the procedures of

Oakley *et al.*, (1980).

Whenever analytical SDS-PAGE was used, a mixture of polypeptides of known molecular weight (SDS-7, Sigma), was included in the gel. This contained the following seven proteins :

$\alpha$ -lactalbumin (14.2 kDa)

Trypsin Inhibitor (20.1 kDa)

Trypsinogen (24 kDa)

Carbonic anhydrase (29 kDa)

Glyceraldehyde-3-Phosphate Dehydrogenase (36 kDa)

Egg Albumin (45 kDa)

Bovine Albumin (66 kDa)

#### 6.8.2. Native polyacrylamide gel

The procedure described in (6.8.1) was modified for native polyacrylamide gel as follows. The two denaturants, 2-mercaptoethanol and SDS were omitted from the sample, resolving, stacking and running buffers. Samples were not heated and gels were run at 4°C. These gels were used for zymogram assay and for transferring SLS to nitrocellulose for blotting experiments.

#### 6.8.3. Immunoblotting / Western blotting

Immunoblotting was performed according to the method of Towbin and Gordon (1984) in a Bio-rad "Transblot" transfer apparatus. A sandwich of nitrocellulose paper (Schleicher and Schuell) or Hyband-C (Amersham) and gel was prepared between two sheets of Whatman paper (3mm), with one Scotch bright pad on each side. This sandwich was loaded into a sandwich assembly and placed in a tank of transfer buffer (see Appendix 15.3. v) with the nitrocellulose facing the

anode. Protein was transferred by applying 30V across the sandwich for 18h followed by 50V for 1hr in order to transfer the higher molecular weight proteins. After completion of the run, the nitrocellulose membrane was fixed and stained with 0.5% w/v Ponceau S in 0.1% acetic acid for 5 min and then de-stained with water for 10 min. Transferred protein bands were then recorded.

#### 6.9. Blood agar overlay (zymogram)

The zymogram technique was used to locate haemolytic activity of SLS on native gels. After electrophoresis, the gel was washed briefly with 150 mM sodium phosphate buffer (pH 6.8). Defibrinated sheep blood (Becton Dickinson) agar consisting of 2% thrice washed erythrocytes and purified agar (1%) in 150 mM sodium phosphate buffer (pH 6.8) was prepared, cooled to 40°C and poured onto the gel to a thickness of 1 mm. The electrophoresis gel plus the blood agar overlay was supported by a glass plate, and was left undisturbed for five min in order to solidify. A second glass plate was placed onto the blood agar overlay with maximum care to avoid trapping air bubbles between the sandwich. The sandwich was kept at 37°C in a humid atmosphere and inspected for haemolysis each hour for 8 h.

#### 6.10. Blood agar diffusion assay

Washed SRBCs were added to blood agar base (Oxoid) to give a concentration of 7% v/v blood agar. After pouring the blood agar (45°C), into the plastic tray, wells were cut 1.5 cm apart in the solid gel with a sterile cork borer (3 mm). Samples were diluted by 10-fold serial dilution in 150 mM sodium phosphate buffer (pH 6.8).

After addition of samples to the wells, the tray was incubated at 37°C for 12 h (see Fig. 20).

#### 6.11. Stability and storage study of SLS

After every step of the purification procedure, the stability and storage properties of SLS were determined. For this purpose small aliquots of purified SLS were stored at room temperature, 4°C, -20°C, and -70°C or in a freeze-dried state and with or without different stabilizing agents. These agents included glycerol (10% , 20% and 40%) or addition of an extraneous protein such as bovine serum albumin (BSA, Sigma) in different concentrations (0.1% 0.5% and 1%). A mixture consisting of 10% glycerol plus either 0.1% 0.5% or 1% BSA was used to stabilize the SLS. In this way 20% and 40% of glycerol were tested with BSA.

#### 6.12 Effect of chloramphenicol on the synthesis of SLS

SLS released by successive inductions with carriers could originate from either (a) presynthesized SLS associated with cell surface layers or in the cytoplasm or (b) *de novo* synthesized SLS during the period of each induction. In order to test which of these mechanisms was operating the following experiment was done. C203S was grown in BHIB-BM and after 6h growth, the culture was divided into two aliquotes of 250 ml each and centrifuged and washed (see Materials and Methods 6.5). These two preparations of washed cells were called "cells A" and "cells B" and after each being suspended in 8 ml induction buffer, both were induced with RNA-core for SLS release. The presence of SLS was determined by haemolytic assay using trypan blue and cholesterol as controls (Materials and Methods 6.5). The "cells A" were suspended in induction buffer containing 200 µg

chloramphenicol / ml and "cells B" were induced with induction buffer alone. After induction and centrifugation, supernates were collected and analysed for haemolytic activity .

#### 7. Neutrophil preparation

Glycogen-induced rabbit peritoneal neutrophils were obtained from female New Zealand white albino rabbits. Normal sterile saline (500 ml) containing 0.1% (w/v) oyster glycogen (Sigma) was injected intraperitoneally via a large bore, blunt ended 18 gauge needle and the peritoneal exudate recovered by aspiration 4h later (Lackie, 1977). The exudate was stored at 4°C and used within 2 days of isolation. PMNs were recovered from the fluid by centrifugation at 200 x g for 10 min and washed once in divalent-cation-free HBS-EDTA (see Appendix 15.3.iii) then in HBS (see Appendix 15.3.iii). Contaminating erythrocytes were removed from the resulting neutrophil pellet by hypotonic lysis (5 ml distilled water for 5 sec). Washed cells were used within 2 h of preparation. The chemiluminescence response was measured on a monodisperse population of cells obtained by passing the cells through a 10 µm Nitex filter (Plastok Associates, Birkenhead). This technique produced PMN suspension of >95% purity and viability as determined by the Trypan blue exclusion test (Hryniewicz and Pryjma 1977). Cell numbers were standardized after counting in a haemocytometer.

##### 7.1. Chemiluminescence Assay (CL assay)

The CL-assay was performed using an automated luminometer (Wallac LKB 1251 luminometer) connected to an Acorn BBC 'B micro computer. Chemiluminescence emission was measured in millivolts (mV) at 37°C. The number of neutrophils per assay tube was  $10^6$  and the final volume of sample per tube was 500 µl. Luminol (Sigma) was added

(after adding PMNs+buffer) by a micro-pipette to a final concentration of  $10^{-5}$  M. Luminol was first prepared as a  $10^{-2}$  M solution in dimethyl sulphoxide, diluted 100 fold in HBS, then stored at  $-20^{\circ}\text{C}$  until use. The stimulus used was either the soluble synthetic peptide FMLP (Sigma) or suspensions of zymosan (1mg / ml). The final concentration of FMLP per assay tube was  $10^{-7}$  M. The zymosan was suspended in sterile normal saline (1mg / ml) and boiled for 2h. After centrifugation at 2500xg for 15 min, the zymosan was washed three times in cold sterile saline, resuspended in normal saline at a final concentration of 1mg / 1 ml and stored at  $-70^{\circ}\text{C}$  ( Fine, *et al.*, 1972). Zymosan suspension (100  $\mu\text{l}$ ) was used in the opsono-phagocytosis assay. If the FMLP was used as the stimulus then it was added just before putting sample tubes in the luminometer. Before addition of a stimulus, the neutrophils were always pre-warmed for 30 min at  $37^{\circ}\text{C}$ .

#### 7.1.1. Opsonization procedures

Whole rabbit blood was allowed to clot at  $4^{\circ}\text{C}$  for 8h, then centrifuged at  $4^{\circ}\text{C}$  and the serum was stored at  $-70^{\circ}\text{C}$  in aliquots of 0.5 ml. Just before use, the opsonin was thawed and kept at  $0^{\circ}\text{C}$ . For complete complement inactivation, serum was heated at  $56^{\circ}\text{C}$  for 30 min (Mathay *et al.*, 1981). For inactivation of the classical pathway (CP) only, serum was chelated with MgEGTA (see Appendix 15.3.v) for 10 min at ambient temperature (Prez *et al.*, 1975, Mathay *et al.*, 1981) in a tightly capped sterile Eppendorf tube and was kept at  $37^{\circ}\text{C}$  for 30 min. Zymosan (100  $\mu\text{l}$ ) was added to each tube except unopsonized control and the tubes were tumbled at 10 rpm at  $37^{\circ}\text{C}$  for 30 min. After opsonization, the micro tubes were centrifuged at 5000 rpm for 5 min and resultant zymosan was washed twice with PBS (see Appendix 15.3.).

The supernates were always assayed for haemolytic activity and washed zymosan was used for the CL response.

#### 7.1.2. Effect of SLS on opsonization of zymosan

Fresh rabbit serum (100  $\mu$ l of neat, 1/2, 1/4 and 1/8 dilutions) and 100  $\mu$ l of a ten-fold dilution of purified SLS were mixed in Eppendorf microfuge tubes in duplicate and were incubated at 37°C for 30 min. Zymosan (100  $\mu$ l) was added, and the tubes were again incubated for 30 min at 37°C. The Eppendorf tubes were divided into two sets; i.e., set A and set B. After 30 min the tubes of set A were centrifuged at 5000 rpm for 5 min and the tube contents were washed twice with PBS (pH 7.4). The supernates from opsonized tubes were tested for haemolytic activity. The washed zymosan was used in the chemiluminescence assay. The tubes of set B were centrifuged and the pellet was directly used in the CL response assay after resuspending in HBSS. Immediately after the sequential addition of 500  $\mu$ l of neutrophils (prewarmed at 37°C), buffer and luminol, chemiluminescence was measured at 5 sec intervals for the first 10 min and at 5 min intervals for remaining 50 min. Zymosan plus serum, both washed and unwashed, were treated as opsonized controls and zymosan plus PBS was treated as the unopsonized control.

#### 7.1.3. Effect of 56°C for 30 min and Mg<sup>++</sup>EGTA on the ability of rabbit serum to opsonize zymosan

For complete complement inactivation, fresh rabbit serum was inactivated by heating at 56°C for 30 min. Mg<sup>++</sup>EGTA buffer (see Appendix 15.3.v) was used to inactivate the classical pathway only. The sequential scheme of tubes and mixtures is given in Table 7. Each

100  $\mu$ l of rabbit serum (whole, heated or +Mg<sup>++</sup>EGTA chelated) and PBS was mixed and incubated for 30 min at 37°C. Zymosan (100  $\mu$ l) was then added and again the mixture was incubated for 30 min at 37°C. After 30 min the tubes were centrifuged at 5000 rpm, the zymosan was washed twice in PBS and the pellet was used in the chemiluminescence assay. Prewarmed neutrophils at 37°C were used in the assay. Buffer and Luminol, kept at room temperature, were also used.

Table 7 EFFECT OF 56°C FOR 30 MIN AND Mg<sup>++</sup>EGTA ON RABBIT SERUM

Tube no	Zymosan	Rabbit serum	Heated rabbit serum	Mg <sup>++</sup> EGTA	PBS
1	100 $\mu$ l	100 $\mu$ l (1/2)	-	-	100 $\mu$ l
2	-do-	-	100 $\mu$ l (1/2)	-	100 $\mu$ l
3	-do-	100 $\mu$ l (1/2)	-	100 $\mu$ l	-
4	-do-	-	-	-	200 $\mu$ l

#### 7.1.4. Agglutination of rabbit serum and zymosan

In order to determine whether rabbit serum agglutinated zymosan (particulate), the following procedure was developed. For 100  $\mu$ l each of buffer, serum or zymosan, the following additions were made in wells 1-5 of a microtitre tray and gently mixed as shown in Table 8. The tray was incubated at 37°C with the lid on for 2 h after which the contents of each well was examined for agglutination

of zymosan by light microscopy. This process was repeated after a further period of 16 h incubation at 37°C.

Table 8 AGGLUTINATION OF RABBIT SERUM AND ZYMOSAN

Well No	Buffer	Serum	Zymosan
1	+	+	-
2	+	+	+
3	+	-	+
4	+	-	-
5	-	+	+

#### 7.1.5. Ability of SLS to bind zymosan

This assay was used to follow SLS haemolytic activity after incubation with zymosan at 37°C for 30 min. Ten fold dilutions of SLS (100 µl) were mixed with 100 µl of zymosan in Eppendorf micro-centrifuge tubes and incubated at 37°C for 30 min. Zymosan (100 µl) and 100 µl of PBS (pH 7.0) were mixed, incubated and served as a negative control. After 30 min, tubes were centrifuged at 5000 x g for 5 min and the supernates removed and tested for haemolytic activity to detect free SLS. The zymosan was resuspended and opsonized as described (Materials and Methods) under the caption opsonization procedure (7.1.1).

#### 7.1.6. Effect of SLS on bound serum opsonin

Zymosan (100 µl) was opsonized with a 1 in 2 dilution of

fresh rabbit serum for 30 min at 37°C in micro-centrifuge tubes. Neat and dilutions (1/2, 1/5, 1/10) of SLS were mixed with both opsonized and non-opsonized zymosan and incubated for 30 min at 37°C.

Table 9 EFFECT OF SLS ON BOUND SERUM OPSONIN

TUBE NO	SERUM <sup>§</sup>	ZYMOSAN <sup>+</sup>	SLS <sup>*</sup>	PMNs <sup>*</sup>	BUFFER	LUMONOL
1	1/2	0.1	-	0.5	0.2	0.1
2	1/2	-	-	0.5	0.3	0.1
3	1/2	0.1	Neat	0.5	0.1	0.1
4	1/2	0.1	1/2	0.5	0.1	0.1
5	1/2	0.1	1/5	0.5	0.1	0.1
6	1/2	0.1	1/10	0.5	0.1	0.1
7	-	0.1	Neat	0.5	0.2	0.1
8	-	0.1	-	0.5	0.3	0.1

Volumes in  $\mu$ l

§ Serum was diluted 1/2 and 100  $\mu$ l was used in each tube

+ For each tube 100  $\mu$ l of zymosan was used

\* SLS was diluted 1/2 , 1/5 and 1/10 and each tube contained 100  $\mu$ l of each dilutions

\* Each tube contained 500  $\mu$ l of PMNs

The tube contents were washed twice with sterile PBS at 5000 rpm for 3 min and the washed zymosan was then used in the CL response assay (Table 9).

#### 8. Effect of iron on growth of *Streptococcus pyogenes* Group A

All glassware was immersed in 1M HCl for 24 h and rinsed thoroughly six times in deionized water before being sterilized by dry heat at 160°C for 2h before use . All solutions were made with

glass distilled and deionized water .

### 8.1. Bacteria

The strains used are given in Table 10. Before use bacterial strains were cultured in either Brain Heart Infusion Broth (BHIB, from Oxoid), Chemical Defined Medium (CDM, Rijn and Kessler 1980, see Appendix 15.1.B) or Todd Hewit Broth (THB, from Gibco) for 12 h at 37°C. After 12h of incubation, bacteria were harvested by centrifugation at 5000 rpm, washed twice with sterile saline before being used as inoculum.

### 8.2. Measurement of bacterial growth

The organisms were grown in either 50 mls BHIB, THB or chemical defined medium with or without the following chelating agents:  $\alpha, \alpha$ -dipyridyl, (2-2-Bipyridine, Sigma), Transferrin (Sigma), Desferal (Ciba-Geigy, Horsham), or ethylenediamine-di-O-hydroxyphenyl acetic acid (EDDA) (Sigma). The effect of the respiratory inhibitors potassium cyanide (KCN) and sodium azide ( $\text{NaN}_3$ ) on the growth of strains of Gr A streptococci was determined. Growth was estimated by measuring  $A_{600\text{nm}}$  of samples withdrawn at 1h or 2h intervals ; when  $A_{600\text{nm}}$  exceeded 2, the culture was diluted with the appropriate sterile medium until the absorbance value was brought into the range of 1. Absorption values were measured on an SP6-550UV/VIS spectrophotometer (Pye Unicam). The appropriate uninoculated medium served as a blank and the growth curves were plotted graphically.

### 8.3. Biochemical assays

#### 8.3.1. Estimation of $\text{Fe}^{3+}$ present in culture medium

Growth in 50 ml of either THB, BHIB, NB (Nutrient Broth, Oxoid) and CDM was monitored in these media in the presence and

Table 10

## Bacterial Strains - Designation and origin

<u>HOSPITAL NO:</u>	<u>ORGANISM</u>	<u>SITE</u>	<u>SOURCE:</u>
<i>S. pyogenes</i>	GrA		
1) 54359V	"		GRI
2) 55903M	"		"
3) 60343X	"		"
4) 88/00657	"		RH
5) 88/00657	"		"
6) 52114	"		CL
7) 45713/87	"		"
8) 52011	"	TS	"
9) 45543/87	"		"
10) 47061	"	SS	"
11) 48749	"	ES	"
12) 48137	"	S	"
13) 05790	"		"
14) 11324	"	WS	"
15) 13151	"	TS	"
16) 09242	"	DS	"
17) 12757	"	SV	"
18) 12997	"	TS	"
19) 10132	"	"	"
20) 05790	"	P	"
21) 52986	"	NI	"
22) 53141	"	PSW	"
23) 52942	"	OM	"
24) 01537	"	"	RH
25) 01594	"	NK	"
26) 02750	"	NK	"
27) 02750	"	NK	"
<i>Strep. milleri</i>			
28) 505		NK	NK
29) 586		NK	NK
30) 591		NK	NK
31) <i>E. coli</i> MW		NK	NK
32) <i>Staph. aureus</i>		NK	NK

TS Throat swab    SS Swab from scalp    ER Ear swab    S swab  
 WS wound swab    DS Discharge from sore    P Pus    NI Nail infection  
 OM Otitis media    PSW Pus from Skin wound    SV Swab from vulva  
 NK Not known

GRI : Department of Bacteriology, Royal Infirmary, Glasgow.

RH : Department Laboratory Medicine, Ruchill Hospital, Glasgow

CL : Bacteriology South Lannarkshire Laboratory, Law Hospital, Carluke

absence of chelating agents (namely EDDA,  $\alpha, \alpha$ -dipyridyl, Desferal or human apo-Transferrin). The Fe content (nmol/ ml) of THB, BHIB, NB, and CDM was 627, 32.22, 59.08 and 0.089 respectively, as measured by the Iron Binding Capacity Kit (Sigma). Stock aqueous solutions of chelating agents were sterilized by membrane filtration (0.4  $\mu$ m pore size Millipore). After determination of the concentration of Fe in each of the media, each chelating agent was added to each medium at concentrations of equimolar, or 5, 10, and 20 fold molar excess of Fe. The broths were stored at 4°C for 24 h in order to allow further binding of Fe in the medium.

#### 8.3.2. Assays for detection of Phenolate- and Hydroxamate-type siderophores

The Arnow colorimetric assay (1937) was used to detect phenolate-type siderophores in spent medium. To 1 ml of bacterial culture supernate or lyophilized culture supernate resuspended to 1/10 of the original volume, the following were added sequentially. 1 ml 0.5M hydrochloric acid, 1 ml nitrite molybdate reagent (10g sodium nitrite and 10g sodium molybdate in 100 ml distilled water), 1 ml 1N sodium hydroxide and 1 ml distilled water. After each addition, the samples were mixed thoroughly. Any positive red colour was measured at  $A_{515nm}$ . The minimum amount which can be detected with this assay is 5nmol of diphenol (Barnum, 1977). 3,4 dihydroxy benzoic acid (Sigma) was used as a positive control and fresh culture medium as a blank .

#### 8.3.3. The ferric perchlorate test for Hydroxamate-type siderophore

Hydroxamate siderophores were detected by the addition of 1-ml ferric perchlorate (2.5 mg/ ml) and 1 ml perchloric acid (0.14g/-

ml) to 1 ml culture supernate or lyophilized culture supernate resuspended to 1/10 of the original volume. A red/brown colour was indicative of a positive hydroxamate reaction (Atkin and Neilands 1966). Desferal was used as a positive hydroxamate standard and fresh culture medium as a negative control.

#### 8.3.4. Csaky Test for Hydroxamate-type siderophore

This was a modification of the original Csaky test (Csaky, 1948). To 0.5 ml bacterial culture supernate, 0.5 ml 6 M sulphuric acid was added and the sample was hydrolysed in a sealed tube by autoclaving at 122°C for 18 h. Sodium acetate (35% w/v) was added to the hydrolysed sample to adjust the pH to 5.5. After the sequential addition of 1 ml sulphanic acid (1% w/v in 30% acetic acid) and 0.5 ml iodine (1.3% w/v in 30% acetic acid, the solution was mixed and allowed to stand for 5 min at room temperature. To decolorize the solution, 0.5 ml sodium thiosulphate (2.5% w/v) was added. After mixing the solution, 0.5 ml 3N HCl and 1 ml of  $\alpha$ -naphthylamine (0.3% w/v in 30% acetic acid, Sigma) was added and the volume adjusted to 10 ml with distilled water. The pink colour, indicative of a positive reaction, was allowed to develop for 30 min at room temperature. Desferal was used as a positive hydroxamate standard and fresh uninoculated culture medium as a negative control. With all samples, the assay was carried out with and without the hydrolysis step as described earlier.

#### 8.3.5. Universal chemical assay for the detection and determination of siderophores (Blue plate assay)

The method described by Schwyn and Neilands (1987) was employed to detect siderophores by using their high affinity for iron

(III). The method is based on the formation of the ternary complex, chrome azurol/ S/ iron (III) / hexadecyltrimethylammonium bromide with an extinction coefficient of approximately  $100,000 \text{ M}^{-1} \text{ cm}^{-1}$  at 630nm which serves as an indicator. When a strong chelator removes the iron from the dye, its colour turns from blue to orange. To prepare 1 litre of blue agar, 60.5 mg CAS (chrome azurol S) was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 10 mM HCl). With stirring, this solution was slowly added to 72.9 mg HDTMA (hexadecyltrimethylammonium bromide, Sigma) dissolved in 40 ml water. The resultant dark blue liquid was autoclaved. Chemically defined medium (see Appendix 15.1.B) was simultaneously autoclaved. After cooling the CDM solution to  $50^\circ\text{C}$ , filter sterilized solution of casamino acids and other required supplement-like vitamins (see Appendix 15.1.B) were added. The dye solution was finally added to a sterile solution of CDM along the glasswall, with enough agitation to achieve mixing without generation of foam in the medium after cooling to  $50^\circ\text{C}$ . Each plate received 30 ml of blue agar. With a bacteriological loop, a heavy point inoculation of each of the test strains was made. The inoculated plates were incubated at  $37^\circ\text{C}$  for 24 h before inspection of the plates for growth and colour changes around the colonies. An orange 'halo' was indicative of siderophore production.

#### 8.3.6. Thin layer chromatography

##### Preparation of samples for analysis:

The following methods for the preparation of samples of both hydroxamate- and phenolate -type siderophores were adapted from those of Andrus *et al.*, (1983) and Rogers (1973 ).

Cultures were grown in iron-limiting media (BHIB containing either EDDA, apotransferrin, or  $\alpha, \alpha$ -dipyridyl) for 18 h and 24 h at 37°C on an orbital shaker operating at 200 rpm. For phenolate-type siderophores, lyophilised culture supernates of 18 h and 24 h cultures were resuspended to 1/10 the original volume in sterile distilled water and phenolates were extracted with ethyl acetate by the following procedure. To 5 ml of redissolved lyophilised supernate, 5 ml 0.5 M HCl was added to adjust the pH to 1.5. Ethyl acetate (5 ml) was then added and the resulting emulsion was shaken and allowed to stand for 2h. The aqueous layer and ethyl acetate layer were separated by centrifugation at 3000 x g for 20 min at room temperature and the ethyl acetate layer recovered and evaporated to dryness under a stream of nitrogen before redissolving in 0.5 ml ethanol.

For hydroxamate-type siderophores, lyophilized supernate of cultures grown in iron-limiting media (BHIB containing either EDDA, Transferrin, or  $\alpha, \alpha$ -dipyridyl) for 18h and 24h at 37°C shaken at 200rpm, was resuspended to 1/10 the original volume in sterile distilled water.

Spots (10  $\mu$ l) of each concentrate were applied to an activated (100°C for 15 min) silica gel plate (20x20cm coated with 0.25mm of silica gel, Camlab) with a micropipette and dried with a hair dryer.

#### 8.3.7. Detection of phenolate-type siderophores by thin layer chromatography

Samples in ethanol were spotted onto activated TLC plates and ascending chromatography was carried out at room temperature for

2h with 5% (w/v) ammonium formate, 0.5 % (v/v) formic acid as solvent.

#### 8.3.8. Detection of Hydroxamate-type siderophores by thin layer chromatography

The method was adapted from that of Gibson and Magrath (1969). Samples (10  $\mu$ l) were spotted onto activated TLC plates and ascending chromatography was carried out at room temperature with N-butanol-acetic acid-water (60:25:15 v/v) as solvent. The plates were air-dried and examined as described in (i) of this section.

#### 8.3.9. Detection of siderophores on TLC plates

The dried plates were sprayed with 1% FeCl<sub>3</sub> in order to detect iron-binding compounds. For the specific detection of phenolates, 3,4-dihydroxybenzoic acid (sigma) was used as a positive standard for mobility ( $R_f$ ). Desferal was used as a mobility ( $R_f$ ) standard for hydroxamate-type siderophore.

To compare the spots which appeared on the stained plates, their  $R_f$  value was calculated as follows :

$$R_f = \frac{\text{Distance travelled by the iron binding Compounds (cm)}}{\text{Distance travelled by the solvent front (cm)}}$$

#### 8.3.10 Inhibition of growth by EDDA

The method used was essentially that of Marcelis *et al.*, (1978) except that the medium was changed to nutrient agar. Nutrient agar (15 ml) was poured into plastic petridishes (6.8 cm diameter), resulting in an agar layer of 5 mm thickness. Wells of (3 mm) were cut out of the agar and the plates were then flooded with 3 ml of a 10<sup>-2</sup> dilution of an 18h culture of bacteria in nutrient broth. After

removal of the excess fluid, especially from the cups, the surface of the agar plates contained approximate  $10^{-2}$  bacteria, sufficient to produce confluent growth. After drying the plates (1h at  $45^{\circ}\text{C}$ ), 5  $\mu\text{l}$  of solutions containing EDDA (1.5 , 4.4 ,15, and 44 mM) were pipetted into the wells. Zones of growth inhibition in the lawn were observed after 10 to 18 h at  $37^{\circ}\text{C}$ .

#### 8.4. Chemical defined medium

The chemically defined medium (CDM) used was essentially that of Rijn and Kessler (1980) except that the mixture of individual amino acids was replaced by casamino acids (Difco) at appropriate concentration (see Appendix 15.1.B). The CDM (final concentration mg/l) was as follows :  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 5.0;  $\text{K}_2\text{HPO}_4$ , 200;  $\text{KH}_2\text{PO}_4$ , 1000;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 700;  $\text{MnSO}_4$ , 5.0;

Amino acids. 20% casamino acids (Difco) together with tryptophan (200- mg/l) final concentration.

Vitamins. The following vitamins (Sigma) were used (final concentration, mg/l): p-aminobenzoic acid, 0.2; biotin, 0.2; folic acid, 0.8; nicotinamide, 1.0;  $\beta$ -nicotinamide adenine dinucleotide, 2.5; pantothenate calcium salt, 2.0; pyridoxal, 1.0; pyridoxamine dihydrochloride, 1.0; pyridoxamine dichloride, 1.0; riboflavin, 2.0; thiamine hydrochloride, 1.0; and vitamin  $\text{B}_{12}$  0.1.

Other chemicals . Glucose, 10g/l; adenine, 20 mg/l; guanine- hydrochloride, 20 mg/l; uracil, 20 mg/l;  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 10 mg/l;  $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ , 4.5g/l; L-cystein, 500 mg/l;  $\text{NaHCO}_3$ , 2.5g/l;  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 3.195g/l;  $\text{Na}_2\text{HPO}_4$  7.350g/l .

Each constituent was treated in Chelex 100 resin , except  $\text{MgSO}_4$ ,  $\text{FeSO}_4$ ,  $\text{MnSO}_4$ ,  $\text{CaCl}_2$  and vitamins. The final pH was adjusted to 7.4. Iron

levels present in CDM after Chelex-100 treatment were determined by the Sigma Iron Binding Capacity Kit.

#### 8.4.1. Ion-exchange chromatography on Chelex 100 resin

##### Preparation and regeneration of resin

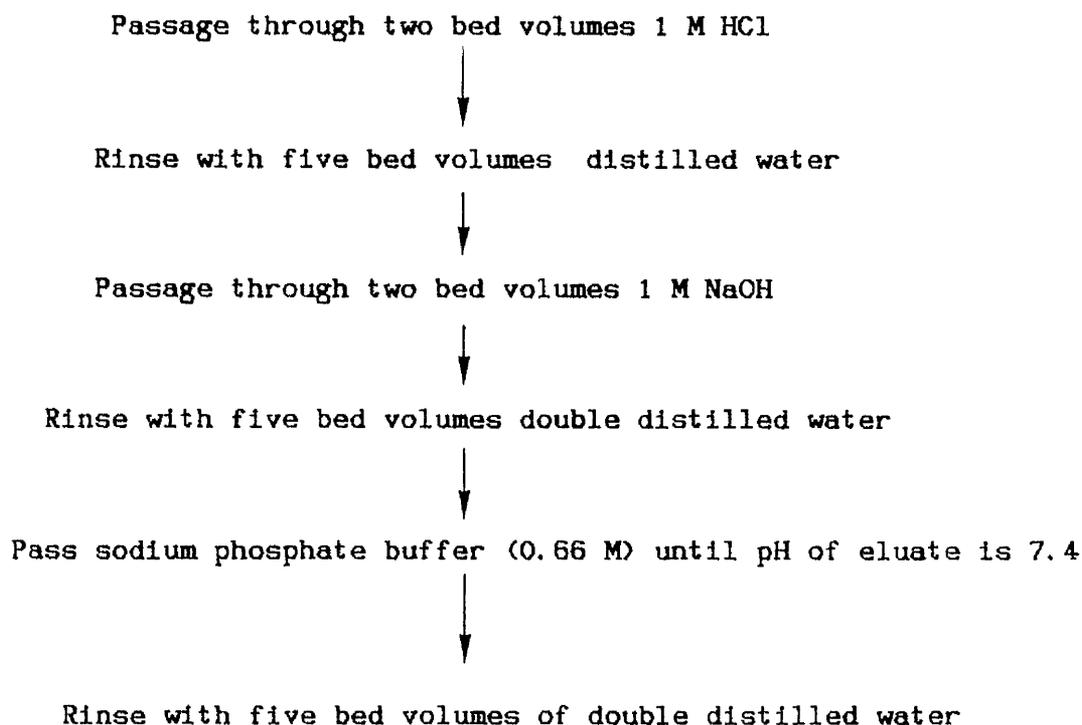
The sequence used to condition and regenerate the column (2.5X14cm) was as follows : Passage of 2 bed volume 1 M HCl followed by 5 bed volumes of double distilled water ; then 2 bed volumes 1 M NaOH followed by 5 bed volumes of double distilled water. Finally sodium phosphate buffer (0.66 M, pH 7.4) was passed through the column until the pH of the eluate was 7.4. and the column was finally rinsed with 5 bed volumes of double glass distilled water before it was used to deionise culture medium (Kadurugamuwa *et al.*, 1987) (see flow sheet 3). Removal of cations was carried out by passing media through a column of resin (Chelex-100; Bio-rad) in the sodium form with a flow rate of 2 ml /min.

#### 8.5. Effect of Potassium cyanide (KCN) and Sodium azide (NaN<sub>3</sub>) on the growth of *Streptococcus pyogenes* Gr A

A number of streptococcal isolates, *S. milleri*, *E. coli* MW, and *S. aureus* were tested for the effect on growth of KCN and NaN<sub>3</sub>. Generally, KCN, a respiratory inhibitor, binds to and inhibits both the oxidized and reduced forms of cytochrome oxidase of the a<sub>3</sub>-type (Smith, 1954). Azide (N<sub>3</sub><sup>-</sup>) acts as a respiratory inhibitor by combining with, and preventing the reduction of oxidized cytochrome oxidases of the a<sub>3</sub>-type. The final concentrations of these respiratory inhibitors in BHIB were 1 mM, 10 mM, 50 mM, and 100 mM

Flow sheet 3

Preparation and regeneration of Chelex 100 resin column



and they were added after 2h of initial growth at 37°C in shaken , unshaken or unshaken anerobic cultures . Growth curves were determined by measuring  $A_{600nm}$  on a SP6-550UV/VIS spectrophotometer at intervals of 30 min for 6 h .

8.6. Effect of KCN or  $NaN_3$  plus iron chelator EDDA on the growth of *Streptococcus pyogenes*

*S. pyogenes* was grown under iron-restricted conditions either

shaken, or unshaken or unshaken anaerobically at 37°C with chelators to Fe molar ratios of 1:1, 10:1 and 20:1 EDDA in BHI broth. Potassium cyanide (KCN) or sodium azide ( $\text{NaN}_3$ ) at final concentrations (1 mM, 10 mM, 50 mM, and 100 mM) were added after 2 h of growth. The growth pattern was determined by measuring  $A_{600\text{nm}}$  ( SP6-550UV/VIS spectrophotometer) at 30 min intervals for 6 h.

#### 8.7. Crude membrane preparation and SDS-PAGE

Bacteria grown under iron-limited conditions were harvested by centrifugation at 5,000 g for 10 min at 4°C and washed with 0.1 M tris buffer. The bacterial pellet was resuspended in 20 ml distilled water and broken by five cycles of (each cycle consisted of 1 min of ultrasound followed by 30s cooling in an ice bath) 60s pulses of sonication 10 times in an ice bath, with a 30s interval for cooling. Unbroken cells were removed by centrifugation at 5,000 x g for 10 min. The supernate obtained was then centrifuged at 38,000 x g for 45 min. The resultant wall membrane pellets were washed twice with distilled water and added to sample buffer. After boiling for 2 min, the preparation was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using the system described by Laemmli (1970) with 12.5% acrylamide separating gels.

## RESULTS

## 9. Production of SLS

### 9.1. Inductions of SLS

Production of SLS by strain C203S was examined. SLS was obtained by induction of a 1 litre culture in BHIB (Difco) grown statically with RNA-core. RNA-core has been used previously as an inducer and a carrier molecule for SLS (Ginsburg, 1970, Duncan *et al.*, 1981, Alouf and Loridan, 1986). Fourteen consecutive inductions were carried out on the same pellet of bacteria, which was repeatedly resuspended in 16 ml induction buffer and induced each time with RNA-core. The combined haemolytic material (about 220 ml) contained 220000 haemolytic units (HU) and this served as the crude preparation for further purification.

### 9.2. SLS production in relation to growth rate

The relationship between optimum production of SLS and growth phase was studied in two strains (C203S and 55903M) of *S. pyogenes* Gr A in static conical flask cultures (500 ml of BHI-BM, Difco) grown at 37°C. The inoculum was 10 ml of overnight broth culture (see Materials and Methods 6.5). Aliquots of 100 ml of culture were withdrawn at 1h intervals during the growth period and centrifuged to yield a cell pellet. Each pellet was induced 5 times (each induction was of 8 ml) and the induction fluid was assayed for haemolytic activity. The results showed that:

- (i) the amount of SLS released in both strains was maximal in cells taken between early and late exponential growth phase (Figs. 11 and 12).
- (ii) the yield of SLS was 1000 HU/ ml and was maximal after 6 h growth

in strain C203S (Fig.11) whereas strain 55903M (Fig.12) gave a maximal titre was 316 HU/ ml after 6 h of growth

(iii) although the  $A_{600}$  of both strains continued to increase even after 9 h incubation when the last samples were taken, the yield of SLS declined as estimated by haemolytic assay.

### 9.3. Effect of growth medium on SLS levels

In a number of other studies on cytolytic toxin production, the growth medium has been shown to have a major influence on toxin production (Bernheimer, 1972). It was therefore decided to test the effects of two different sources of BHI medium on the levels of SLS produced.

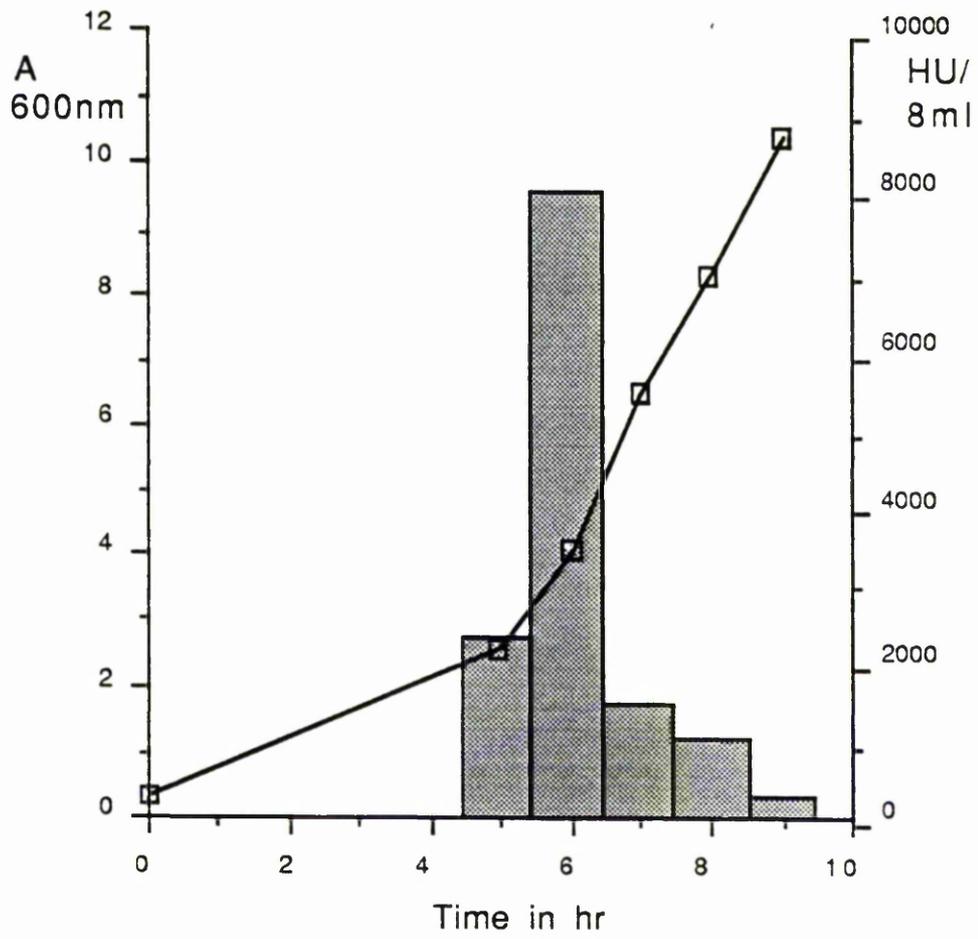
The yield of SLS after induction from strains 55903M and C203S grown in Oxoid BHI-BM and Difco BHI-BM under identical conditions was compared. Strain 55903M grew profusely in both Oxoid and Difco BHI-BM. After 6 h of growth, five inductions (each induction was of 8 ml) were made from the culture grown in Oxoid BHIB but only the first induction yielded haemolytic material after 6 h growth. Eleven inductions were made from the culture Difco BHIB and all yielded haemolytic material (Fig.13. a).

Five inductions were also made from the cell pellets of strain C203S after 6 h of growth in Oxoid BHIB and only the first induction yielded haemolytic material whereas all eleven inductions made from the cells grown in Difco BHIB were haemolytic (Fig.13. b). The finding that both strain C203S and strain 55903M produced higher yield of SLS in Difco BHIB corresponded with the findings of previous workers (Calandra and Oginsky 1975, Lai et al., 1978 and Alouf and Loridan, 1986). It was interesting to note that there was no obvious

Fig: 11. SLS production in relation to growth rate (C203S)

The line shows the absorbance of culture at 600nm. The histograms show the HU/8 ml.

SLS production in relation to growth rate  
strain C203S



---

Fig: 12. SLS production in relation to growth rate (55903M)

The line shows the absorbance<sup>b</sup> of culture at 600nm. The histograms show the HU/ 8 ml.

SLS production in relation to growth rate  
strain 55903M

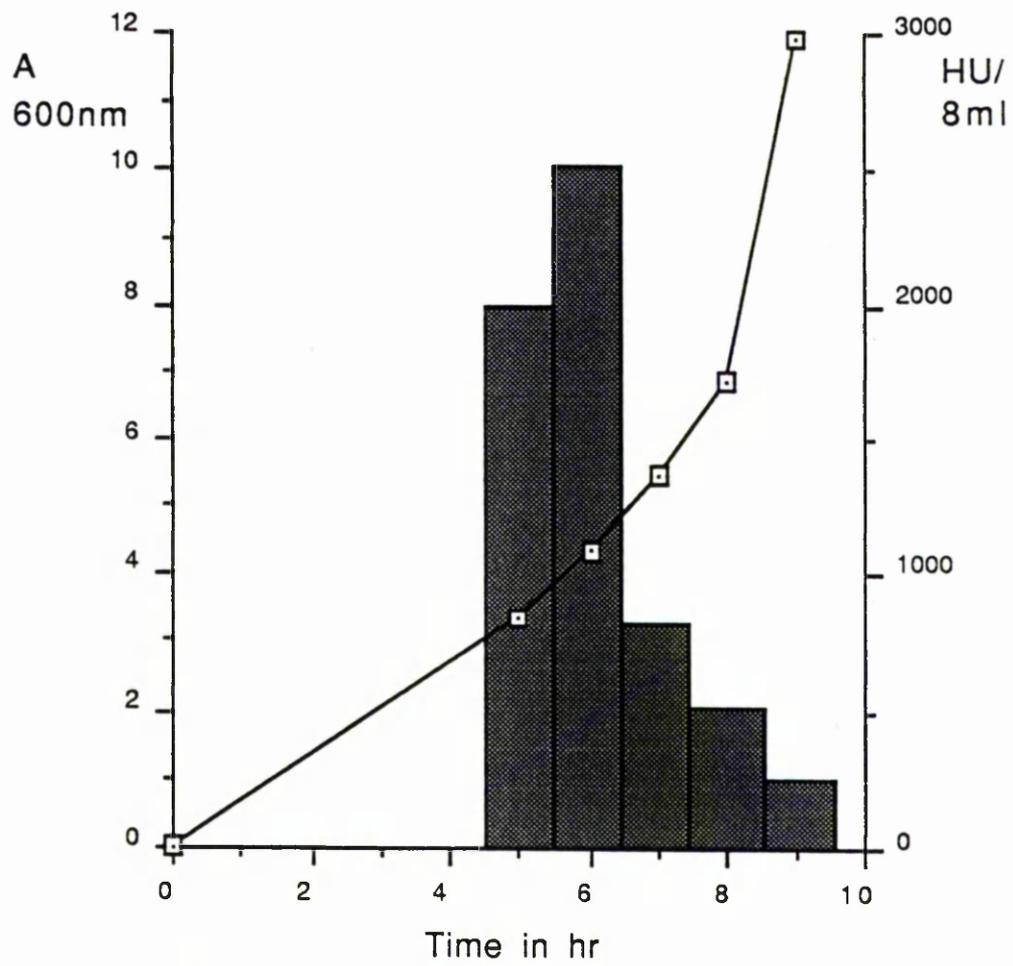


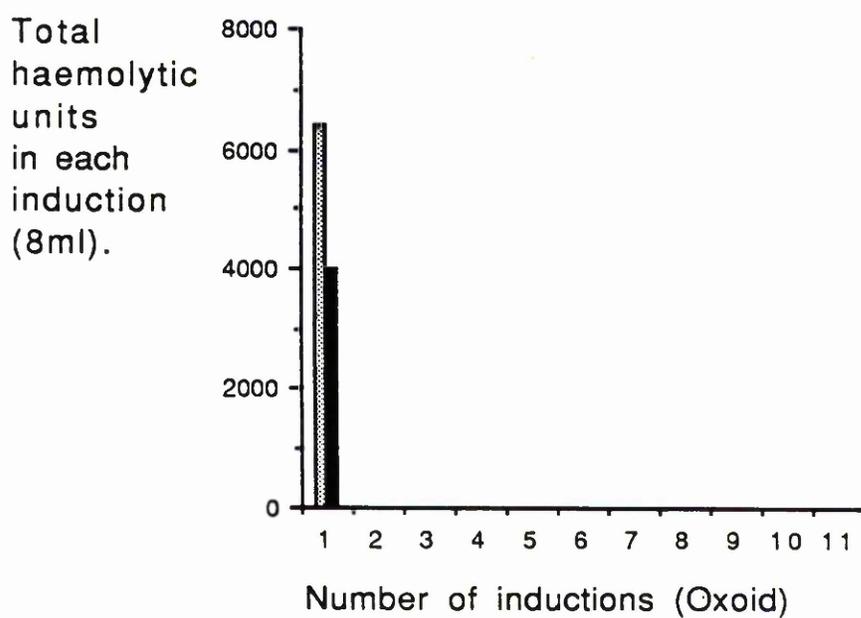
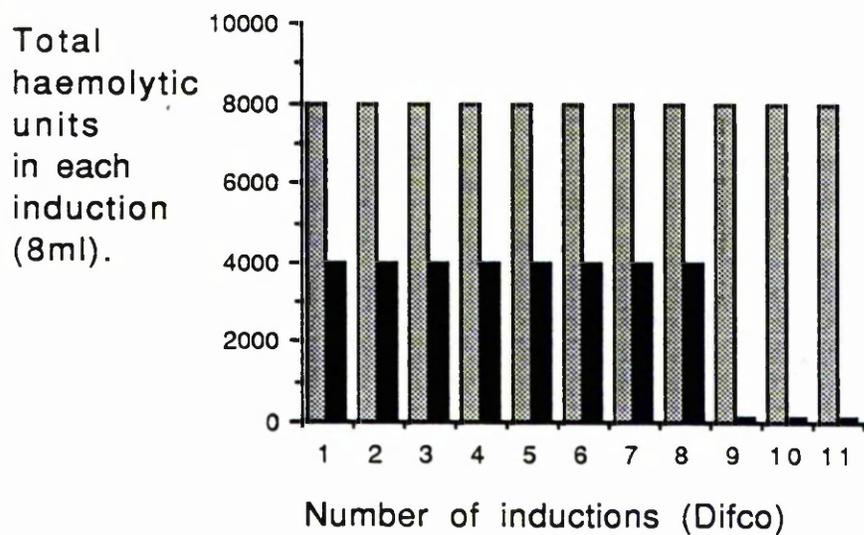
Fig: 13 a and b

Comparative studies of SLS yield in Difco and Oxoid media

The histograms show HU from strains C203S (stippled bars) and strain 55903M (solid bars).

Comparative studies of SLS yield in  
Difco (upper figure) and Oxoid (lower figure) media

■ Strain C203S  
■ Strain 55903M



correlation between amount of growth and SLS released upon induction. Both strains under investigation grew to high cell density in either Oxoid or Difco BHIB but only cells grown in Difco culture medium gave high yields of SLS. The nutritional basis for this is unclear.

#### 9.4. Purification of SLS

The method of purification used was that described by Alouf and Loridan (1986). A summary of purification data is presented in Table 11. The elution profile shown in Fig. 14 revealed the haemolytic activity of the crude material was recovered as a single sharp peak in fractions 19-22 (each fraction of 3 ml) from the hydroxylapatite column. Fraction 19 gave the highest haemolytic activity (8000 HU/ml) and contained approximately 0.11 mg of purified SLS with a specific activity of approximately  $3.5 \times 10^5$  HU/mg protein. The peak haemolytic fractions were pooled (about 9 ml total volume) and were assessed by SDS PAGE, native PAGE and by their inhibitory activity in the chemiluminescence response of PMNs.

#### 9.5. Criteria of purity

##### 9.5.1. SDS-Polyacrylamide gel electrophoresis

The degree of purity of the peak fractions from hydroxylapatite chromatography was assessed by 15% SDS-polyacrylamide gel electrophoresis. The peak haemolytic fractions contained a low molecular weight peptide which proved difficult to visualize by Coomassie blue staining of SDS-PAGE gels but was detected by the silver staining technique (Oakley *et al.*, 1980). The low molecular weight peptide in crude and purified preparation (Fig. 15 and 16) always ran at the dye front of the gel as visualized through silver

Table 11.

Purification and recovery of SLS

Purification and Recovery of Streptolysin S

Purification step	Volume (ml)	Total activity (Hu)	Total Protein (mg)	Specific activity	Purification Factor	Recovery (%)
Starting material	220	220000	2.5	$9 \times 10^4$	1	-
Hydroxylapatite column	9 (18, 19, 20 Three fractions)	49152	0.13	$3.5 \times 10^5$	4.7	22
Fraction 19	3	36864	0.11	$3.5 \times 10^5$	2.5	11

Fig. 14

Elution profile from hydroxylapatite column

Elution profile from hydroxylapatite column.

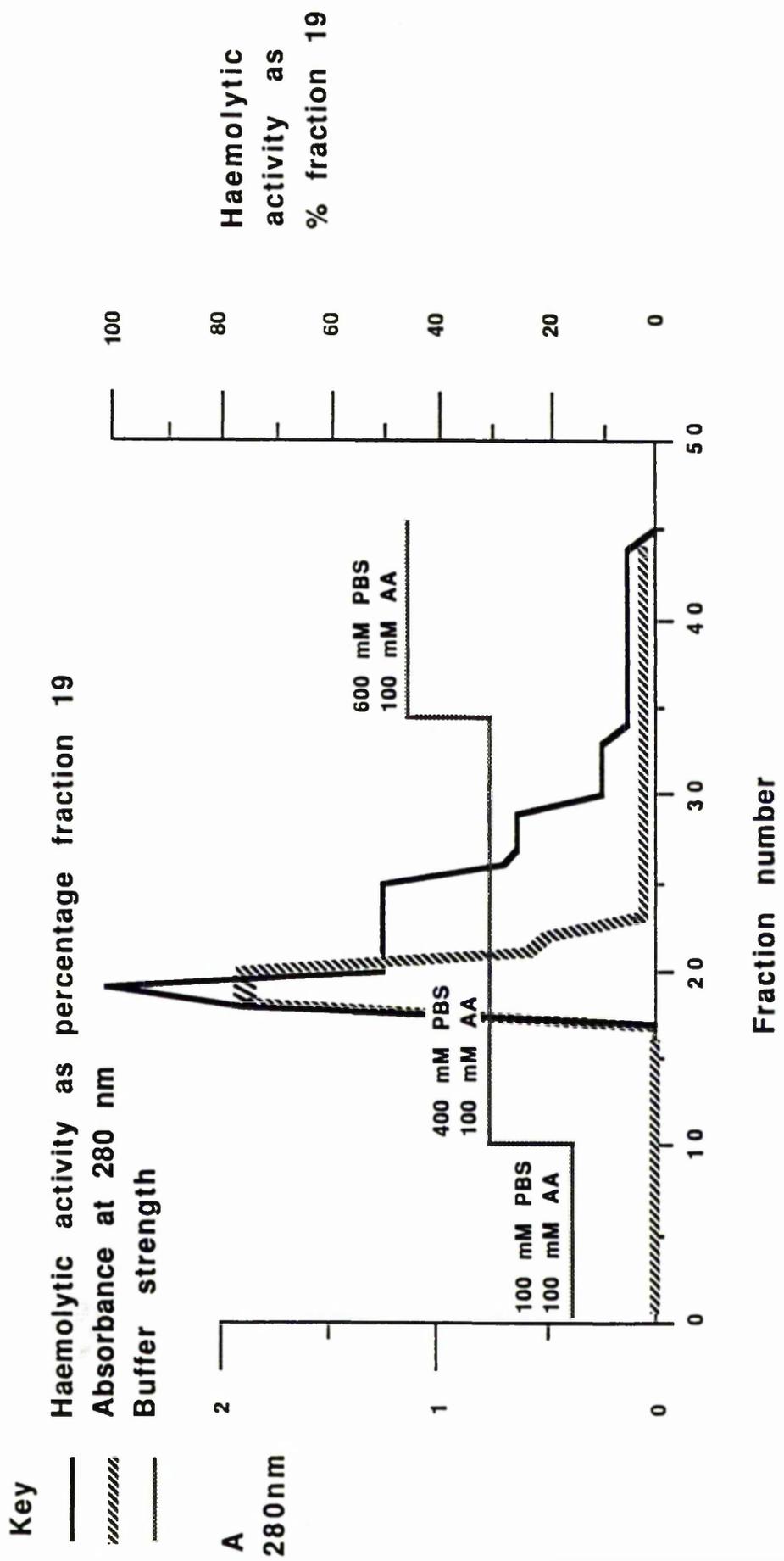
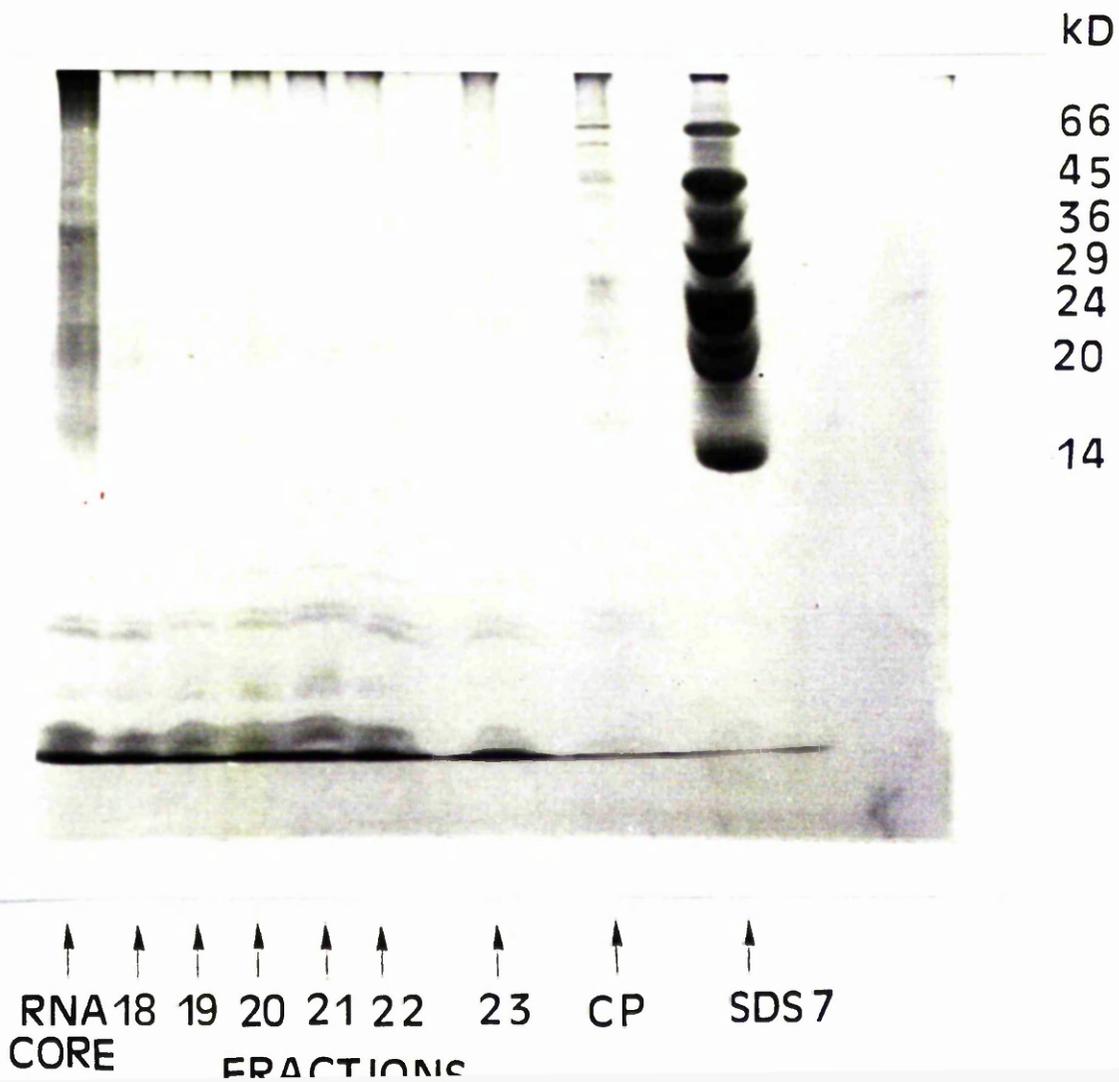
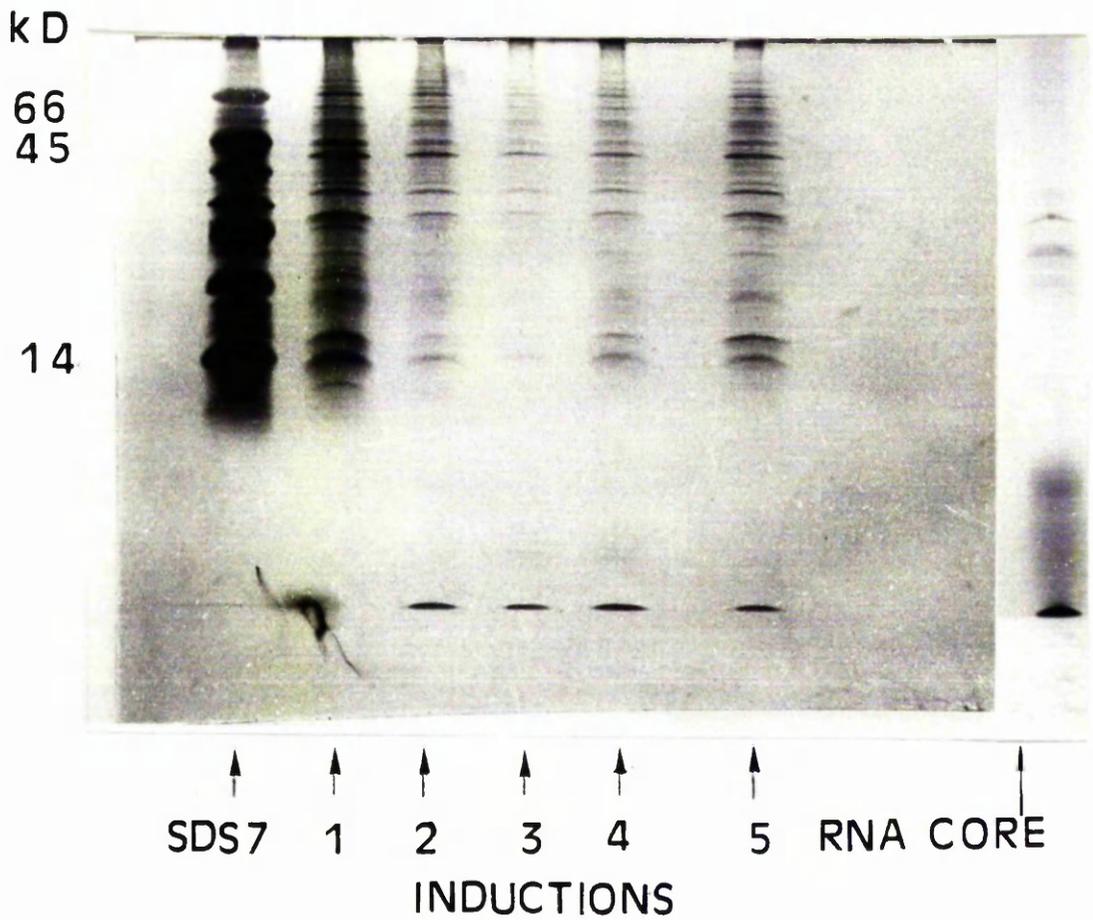


Fig. 15. Analysis of crude preparation of SLS by SDS Polyacrylamide gel

Samples from five inductions (tracks 1-5) were taken and analysed on 15% SDS polyacrylamide gel. The standard was SDS-70L (Sigma) and the gel was silver stained .

Fig. 16. Analysis of purified preparation of SLS by SDS Polyacrylamide gel

Samples from 6 hydroxylapatite column fractions were run on 15% SDS-polyacrylamide gel. The standard was SDS-70L (Sigma) and the gel was visualized by silver staining. CP indiccates crude preparation of SLS.



staining whereas appearance of other extra bands were all components of yeast RNA-core, the carrier molecule.

When RNA-core was run as a control under the same conditions, it was observed that the RNA-core profile was similar to that of purified SLS. Almost the same pattern of bands was observed and it was difficult to determine the possible molecular weight of SLS as both RNA-core and SLS migrated at the dye front. Alouf and Loridan (1986) reported that SLS migrated in SDS-PAGE gels with the dye front, although the RNA-core profile in the gel was not reported. The molecular weight of SLS was reported to be below 4000.

#### 9.5.2. Native-Polyacrylamide gel electrophoresis

In order to detect the biological activity of SLS, native PAGE was first run and developed by the silver staining technique of Oakley, *et al.*, (1980). It was found that the crude material from the induction buffer (Fig.17) and the purified preparation (Fig.18) both contained a band which was not present in the sample of RNA-core which was included as a control.

#### 9.5.3. Zymogram

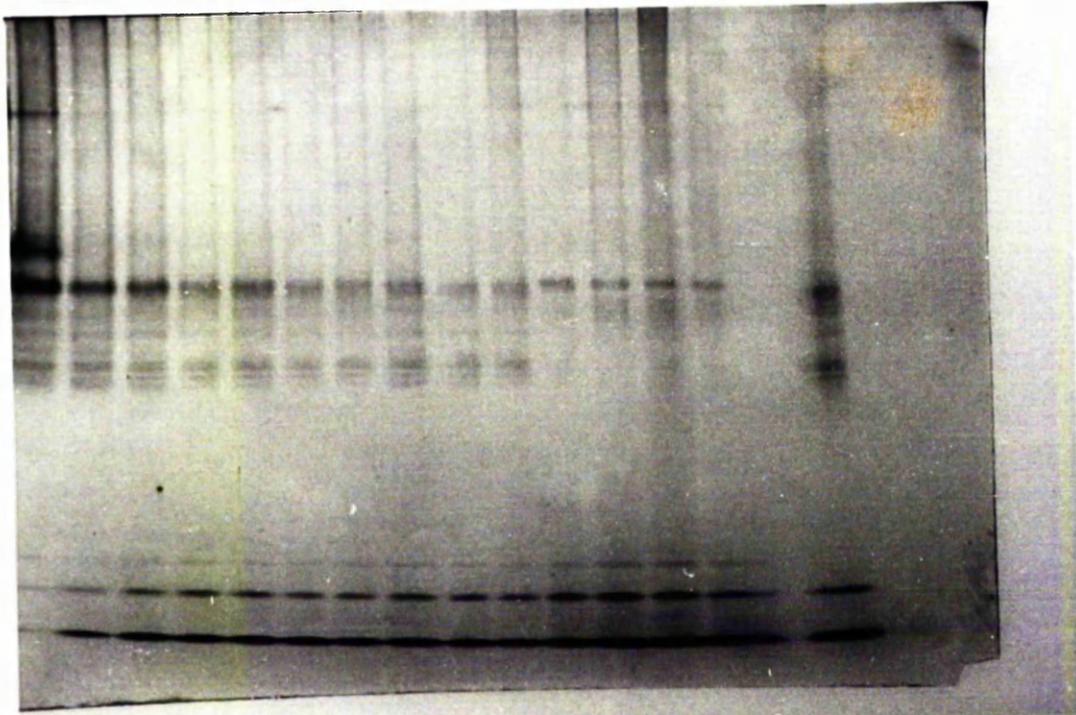
Since the molecular weight and identity of the SLS peptide was not clear from the SDS and native PAGE analysis, a zymogram technique was used to locate SLS by its haemolytic activity on native-PAGE. A native SDS PAGE gel was run with a duplicate set of bands at side of the gel. After electrophoresis was completed, the gel was cut into two halves, one was stained by the silver technique, (Fig.19. b) and the duplicate half was used for the zymogram assay (Fig.19. a). A suspension of washed sheep RBCs and agar was mixed and overlaid on the gel. After incubation, an area of lysed RBCs appeared in a position on

Fig. 17. Analysis of crude preparation of SLS by Native Polyacrylamide gel

Samples from fourteen (14) inductions were analyzed on 15% native polyacrylamide gel. RNA-core (Sigma) was also analyzed as control. The gel was silver stained. Arrow indicates the suspected band of SLS.

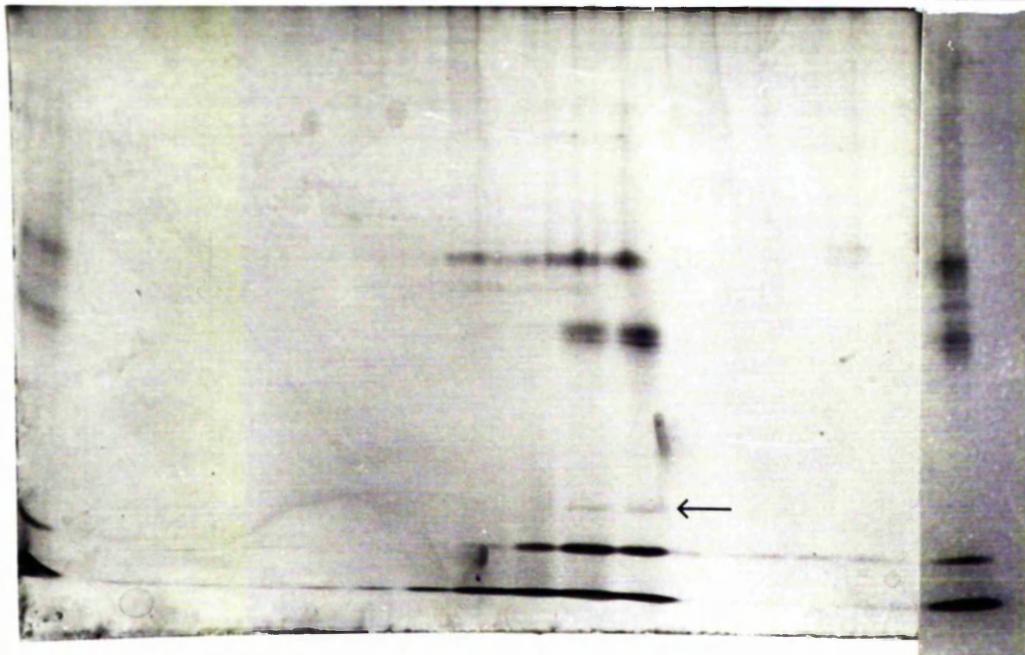
Fig. 18. Analysis of purified preparation of SLS by Native Polyacrylamide gel

Different fractions of purified SLS were analyzed on 15% native PAGE. RNA-core was also run. The gel was silver stained. Arrow indicates the suspected band of SLS.



↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ RNA CORE

1 2 3 4 5 6 7 8 9 10 11 12 13 14



↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑

RNA CORE 1 2 5 8 11 14 17 18 19 20 22 24 26 RNA CORE

the gel equivalent to that occupied by the stained band, tentatively identified as SLS. Three fractions were assayed by this method and fraction 19 produced a larger haemolytic zone than fractions 18 and 20, which also corresponded to the result of the assay for total haemolytic activity.

#### 9.5.4. Agar diffusion assay

A more direct assay of haemolytic activity than the doubling dilution titration assay was the measurement of haemolytic zone diameter in blood agar. Three fractions were tested in this diffusion assay in blood agar. The wells in the last horizontal row served as negative controls. Fraction 19 in column no 2 of Fig.20 exhibited marked haemolysis surrounding the wells. This result supports the observation from the zymogram in which the same fraction exhibited the largest haemolytic zone. In the fourth horizontal row there was no haemolysis at all suggesting the absence of SLS and it was clear that haemolysis was not the feature of RNA-core or ammonium acetate but it was due to SLS.

#### 9.6. Western blotting of SLS

Attempts were made to transfer SLS onto nitrocellulose and Hybond C membranes for further characterization by amino acid sequencing and animal inoculation for antigenicity. Purified SLS, in its native state was run on non-denaturing PAGE. Keeping in mind the low molecular weight of SLS, nitrocellulose membranes of two different pore-sizes (0.45  $\mu\text{m}$  and 0.22  $\mu\text{m}$ ) were used. As Fig 21 indicated, neither of the two membranes showed any transferred band except one, that was of carbonic anhydrase (mol.wt. standard) after an overnight

Fig. 19.                      Localization of SLS by zymogram

- (a)            Three haemolytic fractions of purified SLS (18, 19, 20) were run on 15% native polyacrylamide gel. 1% purified agar containing 2% v/v washed sheep erythrocytes was overlaid on the gel to detect haemolytic activity after electrophoretic separation had occurred.

Native polyacrylamide gel (other portion of gel)

- (b)            The fractions (18, 19 and 20) loaded as duplicate of zymogram gel with mol:wt standard markers (Carbonic anhydrase and  $\alpha$ -lactalbumin and were run in 15% native polyacrylamide gel. RNA-core was run as control. The gel was silver stained. Lane 1 and 2 represent  $\alpha$ -Lactalbumin and Carbonic anhydrase respectively.

a



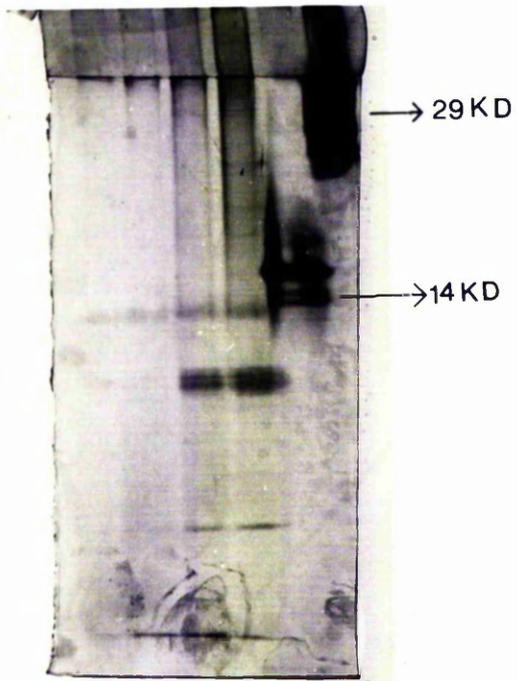
↑  
RNA CORE

↑ 18

↑ 19

↑ 20

b



↑

↑ RNA CORE

↑ 18

↑ 19

↑ 20

↑ 1

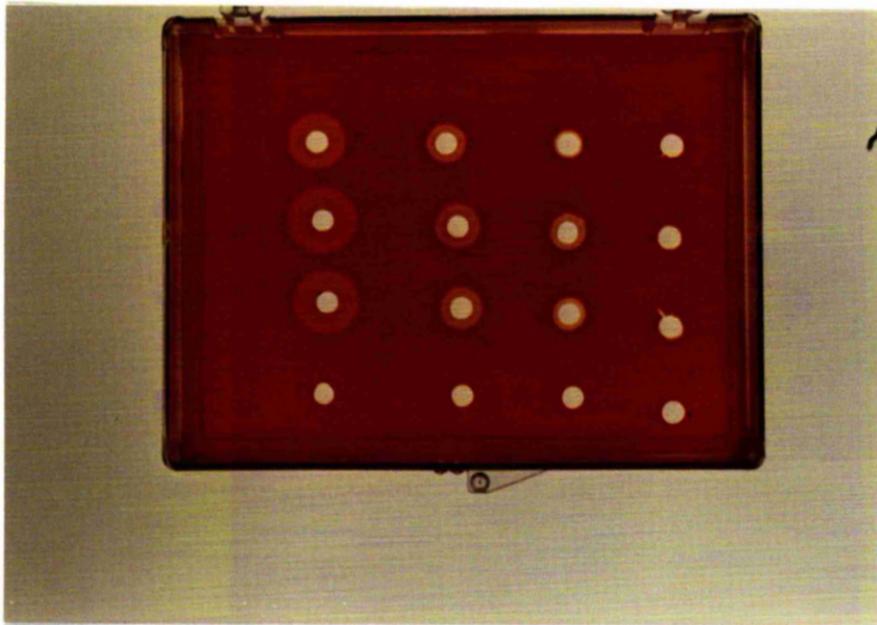
↑ 2

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Figure 20.            Blood agar diffusion assay of purified SLS

Ten-fold dilution of SLS were assayed on 7% washed SRBCs blood agar. Rows 1, 2, 3 and 4 correpond to 10  $\mu$ l vol of neat,  $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$  dilutions. Row A = fraction 18, B = fraction 19, C = fraction 20, D = fraction 21. The diameter of the haemolytic zone is proportional to haemolytic activity.

A  
B  
C  
D



transfer at 30 V. The same result was obtained when:

(a) SLS was transferred for different lengths of time i.e., 10, 20, 30, and 60 min at 30 V.

(b) SLS was transferred over-night at different voltage (10, 15, and 20 V).

Both wet and semi-dry (Nova blot) techniques were tried with 0.45  $\mu\text{m}$  and 0.22  $\mu\text{m}$  membranes but in all cases SLS proved difficult to visualize by Ponceau S and Coomassie-blue staining. This observation suggests that Ponceau S staining may not be sensitive enough to detect SLS or that SLS may pass through the membranes during transfer.

#### 9.7. Stability and storage study

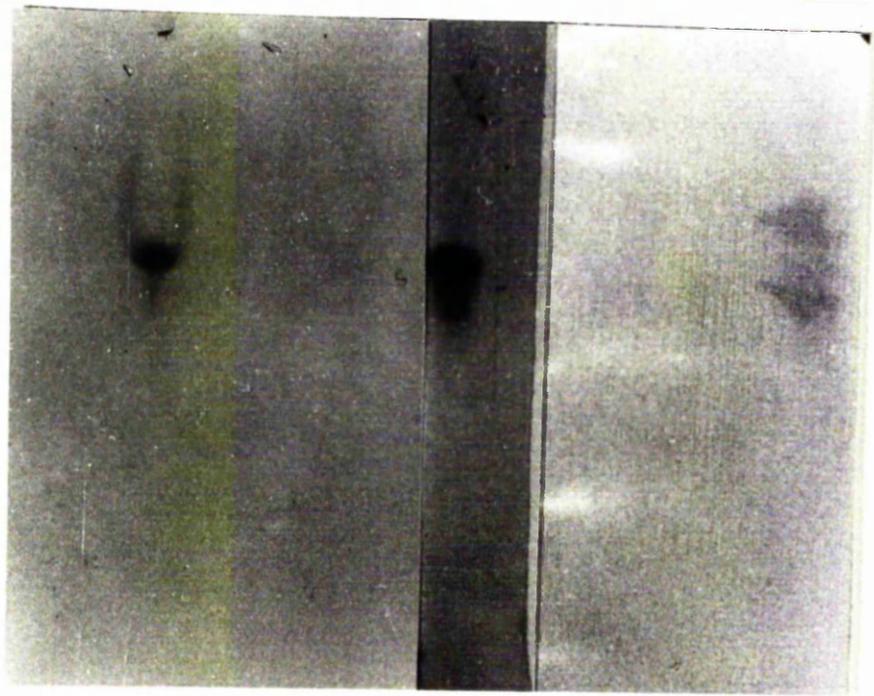
SLS is unusually labile (Bernheimer 1983) and its instability through manipulative steps, probably due to its hydrophobicity, has hampered its purification and characterization (Ginsburg, 1970; Lai *et al.*, 1978, Alouf and Loidan, 1986). Aliquots of SLS were stored at a range of different temperatures with or without a supplement of glycerol or bovine serum albumin (Table 12). The results suggested that SLS is completely stable for more than six (6) months when stored with a supplement of 0.1% bovine serum albumin plus 20% glycerol at  $-20^{\circ}\text{C}$ . When bovine serum albumin alone (0.1% or 0.5%) was used for the storage of SLS without glycerol, there was 99% loss of SLS activity within 12 h. (Table 12) SLS in buffer (400 mM-potassium phosphate /100 mM ammonium acetate (pH 7.0) alone, it lost 99% of its activity in 12 h at  $37^{\circ}\text{C}$ ,  $4^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$ .

#### 9.8. Effect of Chloramphenicol on the synthesis of SLS

Weak haemolysis was observed at 1/10 dilution of material obtained from "cells A" (see Materials and Methods 6.12) but at no

Fig. 21.      Western blotting of SLS

Purified SLS was run on native-PAGE and attempted to transfer on nitrocellulose and Hybond C membranes at 30 V for 12 h. Lane A represents carbonic anhydrase (mol. wt. standard), lane B represents SLS (fraction 19) on nitrocellulose membrane. Lane C represents carbonic anhydrase, lane D represents SLS (fraction 19) on Coomassie-stained gel. Lane E represents SLS (fraction 19), and lane F represents  $\alpha$ -lactalbumin (mol. wt. standard) on Hybond-C membrane.



A



B



C



D



E



F

higher dilutions, whereas a titre of 1000 HU /ml was found in material obtained from "cells B" which was suspended in induction buffer without added chloramphenicol (Table 13). On resuspension and induction for a second time, no haemolysis was observed in material obtained from "cells A" (induction buffer containing chloramphenicol) whereas "cells B" gave a titre of 1000 HU/ml. Induction 3 gave the similiar results. In order to study the reversibility of SLS synthesis, "cells A" were suspended and induced in induction buffer alone on the 4th induction cycle, following 3 cycles of induction in the presence of chloramphenicol. On removal of chloramphenicol, the titre of induction cycle 4 was 100 HU/ml. This was also the case after a further induction cycle in the absence of chloramphenicol (in induction 5). Upon induction in the presence of an increased concentration of chloramphenicol (400 µg/ml, in induction 6), no haemolytic material was released thereafter. Removal of chloramphenicol from the subsequent induction cycles (induction 7 and 8) yielded no further haemolytic material. After 8 cycles of induction in control induction buffer (cells B), the titre was 1000 HU/ml. The loss of reversibility in the effects of chloramphenicol after induction 6 in "cells A" indicated an irreversible loss of protein synthetic ability (possibly cell death). These findings are in keeping with those of Bernheimer (1949), who demonstrated by using variety of enzymes poisons that SLS is formed only when the cocci are actively metabolizing and that SLS was not released from a preformed pool.

#### 9.9. Iron and haemolysin (SLS) production in *Streptococcus pyogenes*

Many different phenotypic changes occur in bacteria during growth under iron-limiting conditions (Weinberg, 1978). The activity

Table 12. LOSS OF SLS ACTIVITY AT DIFFERENT CONDITIONS

TEMPERATURE	TIME IN HOURS	SLS ACTIVITY HU/ml
<u>Immediate after passing through column.</u>	12 hours	16384
<u>Room temperature</u>	-do-	128
37°C	-do-	128
+4°C	-do-	128
-20°C	-do-	128
<u>With Glycerol</u>		
5%---- +4°C	-do-	16
5%---- -20°C	-do-	16
10%--- +4°C	-do-	16
10%--- -20°C	-do-	16
50%----+4°C	-do-	16
50%--- -20°C	-do-	16
<u>In Ice Box</u>	-do-	256
<u>With Bovine Serum Albumin (BSA)</u>		
0. 1%BSA+20glycerol (-20°C)	6 (six) month	100% stable
0. 1%BSA (-20°C)	12 h	128
0. 5%BSA (-20°C)	12 h	128

Table 13. EFFECT OF CHLORAMPHENICOL ON SLS PRODUCTION

Induction no:	Cells A* (250 ml)	HU/ml	Cells B* (250 ml)	HU/ml
	<u>IB+ 200 µg/ml Cm</u>		<u>Only IB</u>	
1		10		1000
2		-		1000
3		-		1000
	<u>Only IB</u>			
4		100		1000
5		100		1000
	<u>IB+400 µg/ml Cm</u>			
6		-		1000
	<u>Only IB</u>			
7		-		1000
8		-		1000
9		-		1000
10				1000
14				1000
15				100
16				100
17				100

C203S was grown in 500 ml BHI-BM . \* Culture was divided into two samples of 250 ml named A and B . IB= Induction Buffer .  
Cm= Chloramphenicol

of membrane transport systems for iron and the production of extracellular or cell associated iron-complexing compounds called siderophores are usually increased dramatically by iron deprivation (Neilands, 1981). In addition, bacterial toxins such as diphtheria toxin from *Corynebacterium diphtheriae* (Russell and Holmes, 1985), Toxin A from *Pseudomonas aeruginosa* (Bjorn *et al.*, 1978), Shiga toxin from *Shigella dysenteriae* (van Heyningen and Gladstone, 1953) and tetanus toxin from *Clostridium tetani* (Mueller *et al.*, 1945) are produced in optimal yields only in low-iron medium.

Two strains of *S. pyogenes* used in this study C203S and 55903M were grown in iron-restricted and iron-replete medium. The medium used in this study was a complex medium BHI-BM (Difco) as discussed in Materials and Methods . The iron content of this medium was 39 nmole /ml as measured by the Iron Binding Capacity Kit (Sigma). EDDA was added at a concentration giving a 20-fold molar excess over Fe and broth was stored at 4°C for 24 h in order to allow further binding of Fe in the medium . After 6 h growth, the cells (grown in iron-restricted and iron-replete medium) were harvested, washed and suspended in induction buffer. After induction with RNA-core, the supernate material was collected and examined for haemolytic activity. The supernates obtained from both induced cells (grown in iron-restricted and iron-replete medium) gave the same haemolytic titre (1000 HU/ml). It was observed from the experiments on growth in the presence of (see Result 11 and 11.1 and 11.2) various chelators, that there was no absolute requirement for iron in the growth medium of any strains of *Streptococcus pyogenes* group A. These findings also showed

that SLS is not regulated by iron nor was there any dependence of SLS production on iron.

10. The influence of purified SLS on the chemiluminescence response of rabbit peritoneal neutrophils to FMLP

Chemiluminescence is the process of emission of light as the result of an exergonic chemical reaction, when the vibrationally excited product of a reaction relaxes back to the ground state. The emitter of light is the product of the reaction and is structurally different from the initial substrate (Anthony, 1986).

Alteration of the chemiluminescence response of PMNs, which itself results from stimulation by chemotactic agents, provides a uniquely sensitive assay for measuring biological activity of many bacterial toxins including pertussis toxins, streptolysin O and adenyl cyclase toxins.

Polymorphonuclear leucocytes (PMNs) in their resting state consume little oxygen. Perturbation or stimulation via the plasma membrane can initiate a burst of metabolic activity with an accompanying dramatic increase in oxygen consumption, superoxide production and the formation of hydrogen peroxide and singlet oxygen. When this free radical returns from its excited state to its ground state there is emission of a photon. Such chemiluminescence is measurable during stimulation by chemotactic peptides such as FMet-Leu-Phe or by phagocytosis. This chemiluminescent response can be affected by a number of bacterial toxins which interact with cell membranes and in some cases, may be a major mechanism in the virulence of the toxigenic bacterium.

FMLP-induced PMN activation responses include directed

chemotaxis, degranulation, oxygen radical generation, and arachidonate metabolite release (Steven *et al.*, 1988).

In this section, the aim was to investigate the effects of purified SLS on PMNs function using a sensitive chemiluminescence assay. Previous studies suggest that most strains of *Streptococcus pyogenes* (group A, beta-haemolytic) contain a potent leukotoxic factor (Ofek, *et al.*, 1970). When bacteria containing relatively large quantities of this factor are ingested by or come into contact with PMNs, the neutrophils are destroyed (Wilson and Salt, 1957). Death of PMNs is associated with intracytoplasmic rupture of PMN granules; this release of granule contents is thought to be the leukocidal killing mechanism (Bernheimer and Schwartz, 1960). Ofek, *et al.* (1970) concluded that the factor inducing granule rupture was cell-bound streptolysin S (SLS).

SLS, lyses erythrocytes and other types of eukaryotic cells and membrane-bound organelles (Hryniewicz, *et al.*, 1980; Wannamaker, 1983; Alouf, 1986). Ofek, *et al.*, (1970) reported that cell-bound haemolysin killed mouse peritoneal macrophages within 30 min whereas RNA-SLS or serum-SLS required 60-180 min for complete killing (as assessed by the trypan blue vital staining). However, such direct comparison between the killing effects of cell-bound haemolysin and RNA-core haemolysin is not meaningful, since the amounts of SLS in each case were not known. Thus, the observed lethal effects reported by Ofek could be due to SLS acting in concert with other exoproducts in the whole cell preparations or to products entirely unrelated to SLS. In order to further investigate the effects of purified SLS on phagocytic cells (rabbit peritoneal neutrophils), experiments were designed to detect whether this toxin could cause changes in the membrane-mediated

chemiluminescence response. Any membrane-associated events may alter the ability of PMNs to generate chemiluminescence when induced with FMLP in a luminol-amplified response. Toxin-dependent changes in chemiluminescent response of PMNs have been reported with SLO (Andersen, and Duncan, 1980) *P. haemolytica* leucotoxin (Czuprynski and Noel, 1990) adenylate cyclase toxin (Kharazmi, *et al.*, 1984) and several others (Alouf, 1986).

Purified SLS, at concentrations of between 8000 HU/ml and 256 HU/ml, when added directly to FMLP-induced rabbit PMNs, caused inhibition of the PMNs chemiluminescence response (Fig. 22). PMNs without FMLP (i.e. PMNs not induced), served as back-ground controls, and gave no significant endogenous CL response compared to cells induced with FMLP, which served as a positive control.

The inhibition of the chemiluminescence response could be a reflection of a lethal dose of SLS for PMNs or could reflect a more specific effect of the toxin on the membrane-mediated chemiluminescent response.

#### 10.1 Effect of SLS at sublethal doses on chemiluminescence response of peritoneal neutrophils to FMLP

It has previously been reported that at sublethal concentrations (i.e. 5 to 250 HU/ml), SLS can disturb specific functions in certain subpopulations of T cells (Hryniewicz, *et al.*, 1980, 1984). In particular, SLS was shown to reduce the number of T-rosettes and phytohemagglutinin (PHA) responsiveness *in vitro*. The experiments reported here were designed to investigate how potent was SLS as an inhibitor of the CL response. The effects of SLS at various concentrations (256 HU/ml and ten-fold dilutions) on the CL response

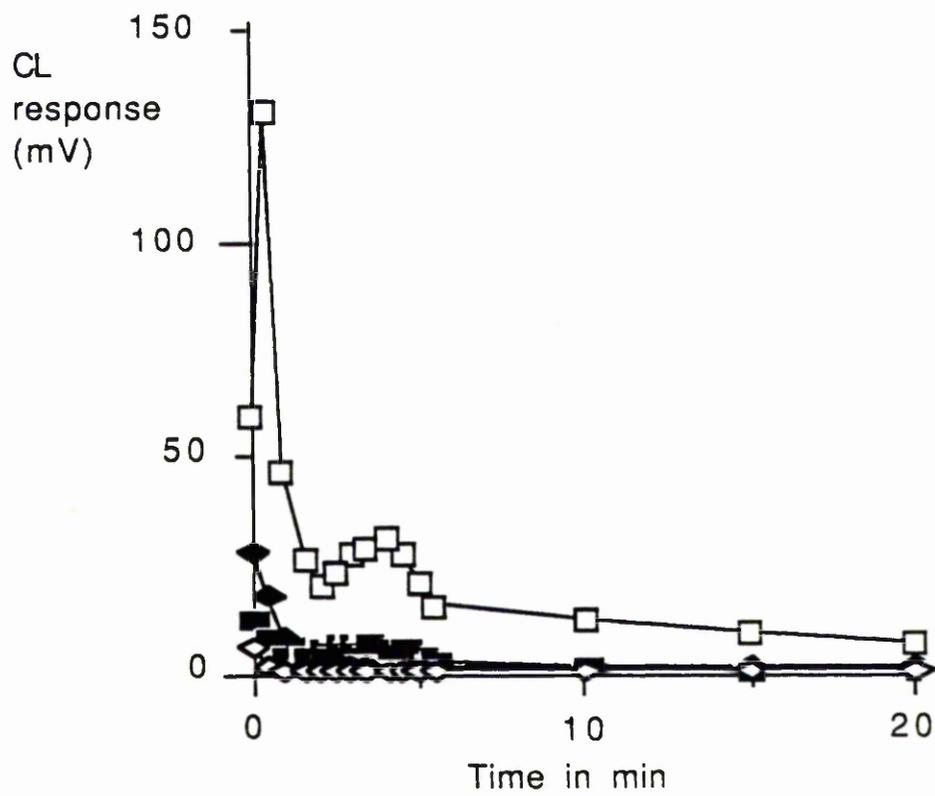
Fig. 22.Effect of SLS on rabbit peritoneal neutrophils: FMLP-induced CL response

SLS solution (100  $\mu$ l of 256 HU/ml) was directly added to PMNs (prewarmed at 37°C for 15 min) suspended in buffer. Luminol and FMLP were added immediately before the start of the measurement of chemiluminescence began (0 time). The figure shown is a mean of four observations and is representative of seven separate experiments.

Key:

- Positive control, cells plus FMLP
- ◆—◆ Negative control, cells minus FMLP
- Cells , plus FMLP, and SLS
- ◊—◊ Cells , minus FMLP , plus SLS

# Effect of SLS on FMLP-induced chemiluminescence response.



of PMNs stimulated by FMLP was assayed. The CL response was greater with increasing dilutions of SLS from  $10^{-1}$  (25.6 HU/ml) to  $10^{-4}$  (0.256 HU/ml) (Fig. 23). PMNs without FMLP gave no marked CL response compared with FMLP control. The result suggested that 50% inhibition of CL response of neutrophils occurred between neat and  $10^{-1}$ . The dilution  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  showed no inhibition. It is evident from these experiments that SLS acts in a dose dependent manner.

#### 10.2. Effect of SLS on opsonophagocytosis

##### Effect of SLS using different concentrations of normal rabbit serum for opsonization (Washed zymosan)

Phagocytes emit chemiluminescence when ingesting bacteria or other particles (Lofgren *et al.*, 1980). When PMNs are exposed to opsonized particles, the chemiluminescent response associated with phagocytosis is characterized by a single peak of emission.

Complement proteins interact in a precise sequence of reactions leading to the production of biologically active cleavage fragments capable of interacting with microbial cells, in some cases promoting opsonization.

First, the effect of SLS on opsonization of zymosan was examined. Fresh whole normal rabbit serum (100  $\mu$ l of neat, 1/2, 1/4, 1/8 and 1/16 dilutions) and 100  $\mu$ l of SLS (256 HU/ml) were each added to a series of Eppendorf tube (see Materials and Methods 7.1.1). Zymosan (100  $\mu$ l) was then added to all tubes, and tubes were again incubated at 37°C for 30 min and the tube contents then washed twice with PBS. The washed zymosan particles were used in CL assay.

A noteworthy observation was that SLS failed to inhibit opsonization of zymosan at neat but, at 1/2 dilution of serum SLS

Fig. 23.Effect of SLS at sub lethal doses : FMLP-induced CL response

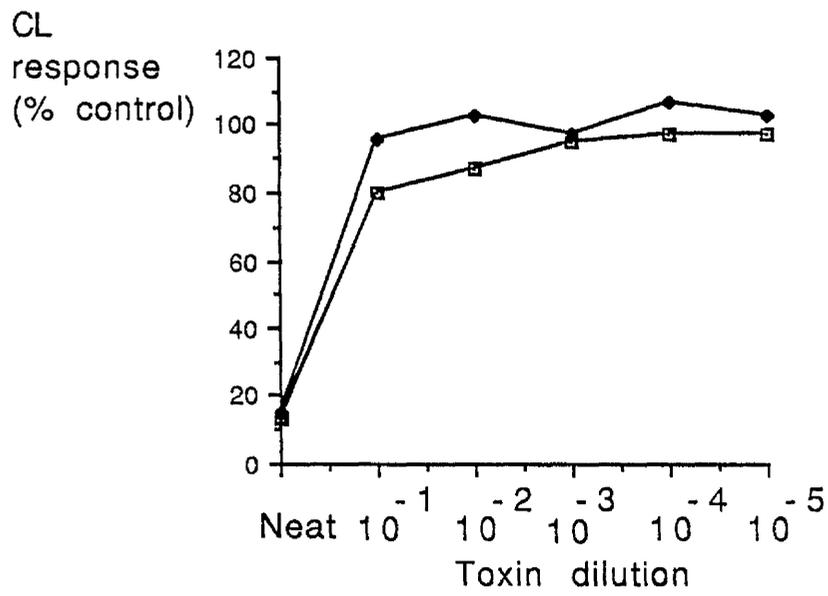
SLS (256 HU/ml) was diluted in PBS and various dilutions of SLS (100  $\mu$ l) were directly added to PMNs (prewarmed at 37°C for 30 min). Buffer, Luminol and FMLP were added just before putting samples into the luminometer. The figure shown is a mean of 3 observations and is representative of six separate experiments.

Key:

□—□ , Ten-fold concentration of SLS , cells plus FMLP

◆—◆ , Ten-fold concentration of SLS , cells minus FMLP

Effect of SLS on FMLP-induced chemiluminescence response: sub lethal doses.



induced a rapid and dramatically diminished opsonization of zymosan (Fig. 24). It was expected that SLS would be removed after 2 washes and that this washing should enable SLS-treated opsonized zymosan to behave like normal opsonized zymosan (SLS-untreated opsonized zymosan) in uptake by phagocytes. However, a result representative of 4 experiments (Fig 24) shows a marked decrease in the uptake of SLS-treated opsonized zymosan when compared to zymosan opsonized in the absence of SLS. From this result, it was difficult to assess whether the decreased uptake was due to the influence of SLS on the complement system or on some other serum factor. The effect of SLS may have been similar to M-protein which has been reported to inhibit the alternative pathway and thus cause failure of the opsonization process, which in turn resulted in resistance to phagocytosis.

The experiments were repeated several times with essentially identical results and in all future experiments involving opsonization and phagocytosis, serum was used at a 1 in 2 dilution.

### 10.3. Effect of SLS on opsonization of zymosan by rabbit serum

#### (unwashed zymosan)

After optimising the dilution of fresh normal serum (1/2) for opsonization of zymosan, ten-fold serial dilution of SLS were incubated with 1/2 dilution of fresh rabbit serum for 30 min at 37°C. The mixture was further incubated with zymosan for 30 min at 37°C. The suspension was centrifuged (see Materials and Methods 7.1.2) and the unwashed zymosan was used in the CL assay. Fig. 25 demonstrates the effect of SLS (256 HU/ml) and its ten-fold dilutions. It indicated that SLS inhibited opsonisation at 256 HU/ml (neat) and 50% inhibition occurred between neat and  $10^{-1}$  dilution (25.6 HU/ml). It is

Fig: 24

Effect of SLS on opsonization of zymosan by rabbit serum (washed zymosan)

The figure shown is a mean of four observations and is representative of seven separate experiments.

Key:

- Fresh rabbit serum (100  $\mu$ l of neat , 1/2 , 1/4 and 1/8 dilutions plus 100  $\mu$ l zymosan
  
- ◆—◆ Fresh rabbit serum (100  $\mu$ l of neat , 1/2 , 1/4 and 1/8 dilutions plus 100  $\mu$ l of ten fold dilution of purified plus Zymosan

**Effect of SLS on opsonization of zymosan  
by rabbit serum (washed).**

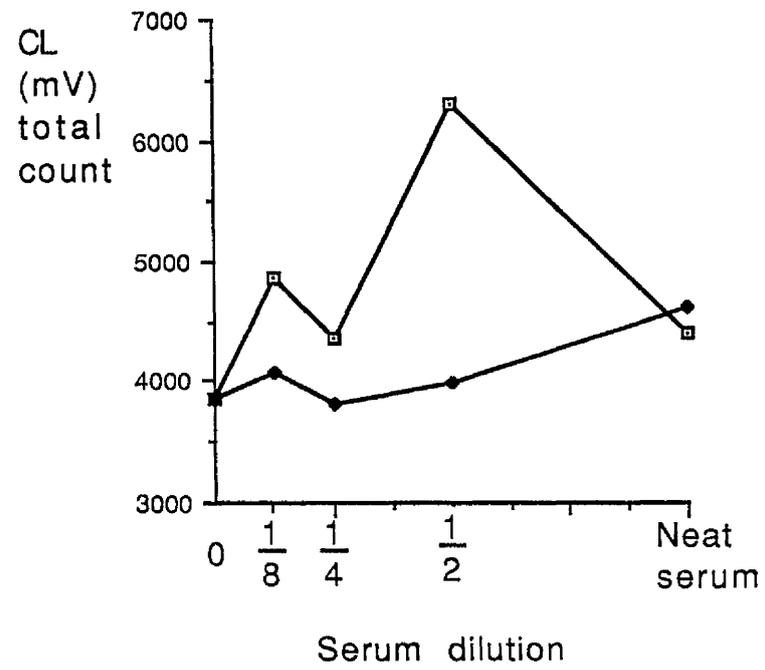


Fig: 25.

Effect of SLS on opsonization of zymosan by rabbit serum

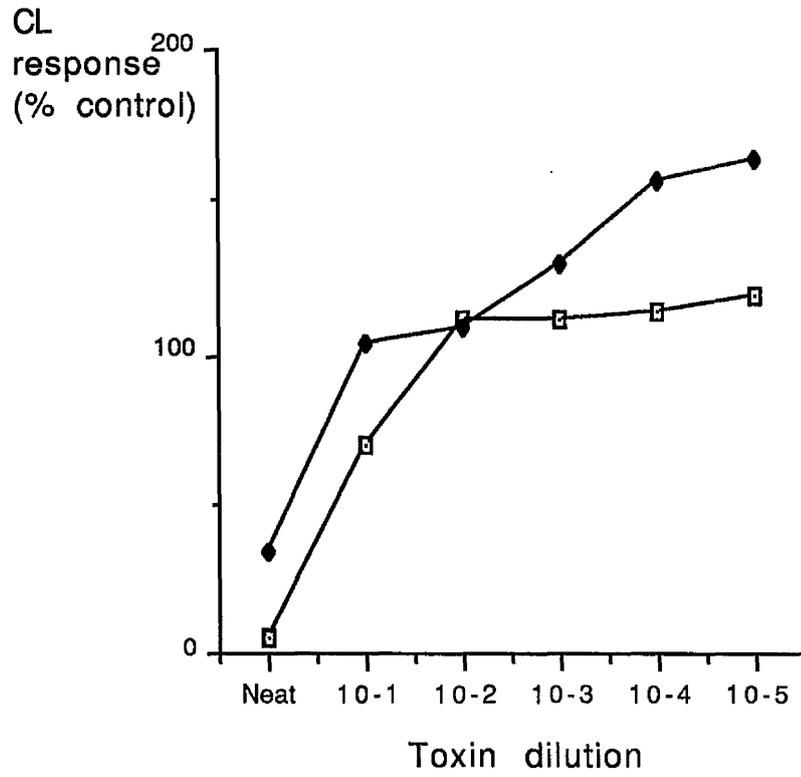
(unwashed zymosan)

The figure shown is a representative of 6 separate experiments.

Key:

- Fresh rabbit serum (100  $\mu$ l of, 1/2, plus 100  $\mu$ l of ten fold dilution of purified SLS plus Zymosan
- ◆—◆ Fresh rabbit serum (100  $\mu$ l of 1/2 dilution plus 100  $\mu$ l of ten-fold dilution of purified SLS

**Effect of SLS on opsonization of zymosan  
(unwashed)**



important to point out here that SLS behaved in a dose dependent manner, similar to its behaviour in the straightforward killing of PMNs-induced by FMLP. The exact mechanism of this blockade of opsonization was not clear and involvement of either the complement pathway or some other serum factor (s) could not be differentiated in this experiment.

#### 10.4. Effect of heating and Mg<sup>++</sup>EGTA on rabbit normal serum

In the light of the observation that SLS inhibited opsonization of zymosan, the study of opsonophagocytosis was subsequently extended to elucidate whether SLS could affect either of the Classical or Alternate complement pathways.

Experiments were designed to detect the pathways involved in opsonization. The CL produced by phagocytosis of zymosan preopsonized in whole normal rabbit serum (total complement pathway) was compared with the CL after opsonization with heated serum (no complement activity) or MgEGTA-chelated serum (alternate pathway, [AP] only) (Mathay *et al.*, 1981). Representative records of the CL responses in experiments following phagocytosis of zymosan after opsonization with whole or complement-depleted serum are shown in Fig. 26. Inactivation of complement by heating decreased CL to 43% of the whole serum level, showing that phagocytosis occurred less efficiently without complement. Also CL in the MgEGTA-chelated samples was significantly decreased compared to unheated serum. MgEGTA blocked the Classical pathway but did not completely block zymosan opsonization suggesting opsonization partly was a function of the classical pathway and partly involved the alternate pathway. These results suggest that SLS can inhibit opsonization, either by the Classical or Alternate pathways.

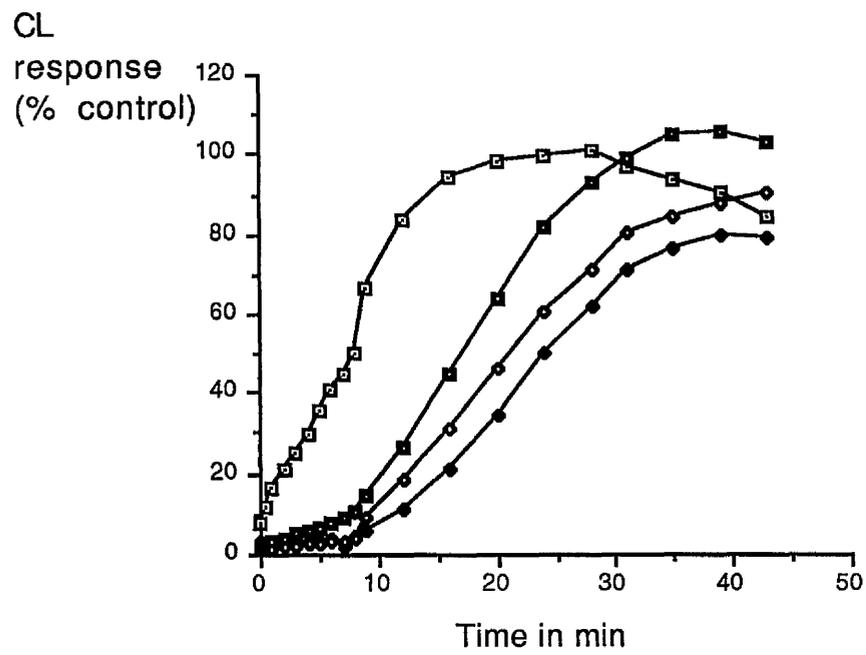
Fig: 26

Effect of heating and MgEGTA on rabbit normal serum

The figure shown is a representative of three separate experiments .

Key	Zymosan	Rabbit serum	Heated rabbit serum	Mg <sup>++</sup> EGTA	PBS
□—□	100 μl	100 μl (1/2)	-	-	100 μl
◆—◆	-do-	-	100 μl (1/2)	-	100 μl
■—■	-do-	100 μl (1/2)	-	100 μl	-
◇—◇	do-	-	-	-	200 μl

# Effect of heat and MgEGTA on opsonization



#### 10.5. Effect of SLS on bound opsonin (serum)

The effect of SLS on bound opsonin (serum) was determined where zymosan was preopsonized with fresh serum and then exposed to SLS (see Materials and Methods 7.1.6). SLS-treated opsonized zymosan induced a markedly reduced CL response as compared to CL response of SLS-untreated opsonized zymosan, which served as positive control (Table 14). The data presented suggests that SLS might affect bound opsonin in 3 possible ways :

- (i) causing C3b to dissociate from the zymosan
- (ii) inactivate the C3b, for example by binding to C3b to hide its receptor site recognized by PMNs
- (iii) binding to C3b or other complement proteins which may act as carriers releasing SLS later to kill PMNs

#### 10.6. Effect of SLS on responsiveness of PMNs

To determine whether these changes in CL induced by SLS (opsonized zymosan-treated SLS (256 HU/mL)) were irreversible and deleterious to the PMNs, the ability of the cells to respond to a second opsonized particle, namely opsonized zymosan (SLS-untreated), was studied. PMNs exposed to opsonized zymosan (SLS-treated) exhibited no CL response when a second dose of opsonized zymosan was added (Fig. 27). The degree of suppression of the zymosan-induced CL was directly related to the dose of SLS. PMNs challenged with opsonized zymosan (SLS-untreated) exhibited an insignificant response after addition of a second dose of opsonized zymosan after 5 min whereas PMNs challenged with unopsonised zymosan (control) exhibited a significant CL response.

Table 14      EFFECT OF SLS ON BOUND OPSONIN

The table shown is a mean of three observations and is representative of five separate experiments.

---

<u>Test particle</u>	<u>CL response (counts)</u>
Opsonin .    PBS	25
Opsonin .    zymosan	592
..            ..        Neat SLS	171
..            ..        1/2 SLS	316
..            ..        1/5 SLS	417
..            ..        1/10 SLS	421

---

Fig. 27.

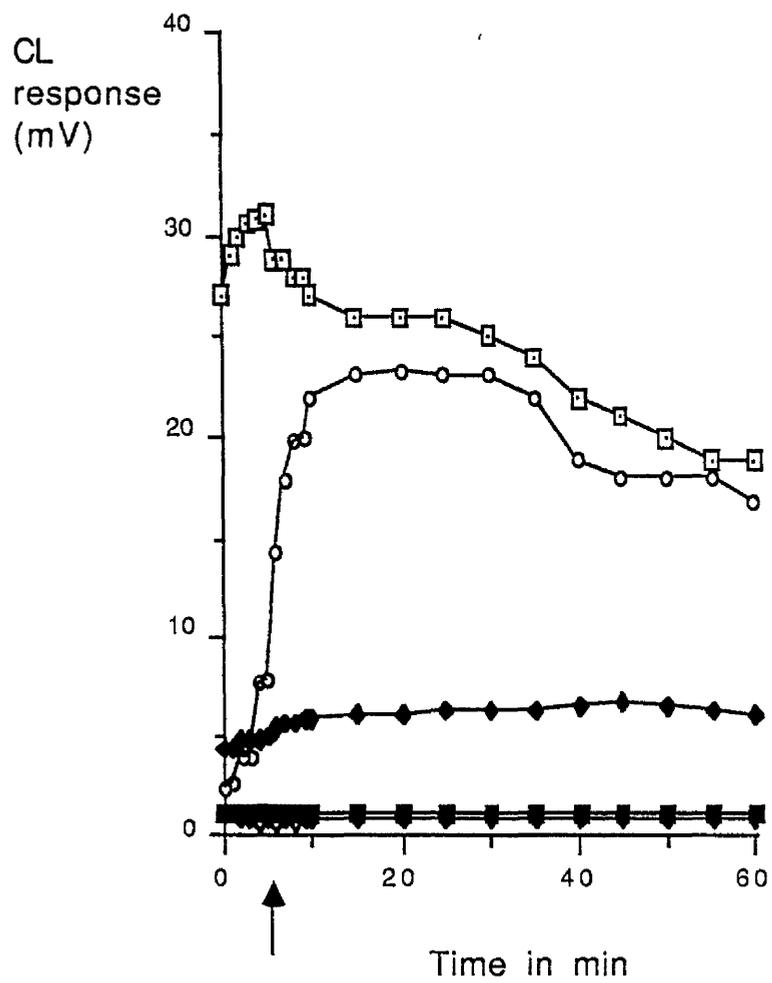
Effect of an additional opsonized zymosan

The figure shown is a mean of four observations and is representative of six experiments.

Key:

- 
- Control, (opsonized zymosan), after 5 min additional opsonized zymosan was added
  - ◆—◆ Unopsonized control
  - Unopsonized control, after 5 min opsonized zymosan was added
  - ◇—◇ SLS treated opsonized zymosan,
  - SLS treated opsonized zymosan, after 5 min additional opsonized zymosan was added
  - Time of addition of opsonized zymosan
-

# Effect of additional opsonized zymosan



### 10.7. Effect of SLS in lucigenin-amplified CL assay : FMLP induced CL response

The reactions of luminol and lucigenin with alkaline  $H_2O_2$  to yield CL appear superficially to be similar, and oxygenation is common to both reactions (Allen, 1982). However, the reaction mechanisms are fundamentally different. Luminol-CL is the net result of "oxidative" oxygenation. On the other hand, lucigenin-CL results from reductive oxygenation ; that is, generation of excited *N*-methylacridone involves the two-electron reduction of lucigenin and oxygenation. Hence, luminol is oxidised by products of <sup>the</sup> myeloperoxidase system (De<sup>l</sup>atelet *et al.*, 1982) whereas lucigenin dependent CL is almost entirely dependent on superoxide production (Allen, 1981).

The possible CL response of FMLP-induced rabbit neutrophils exposed to SLS in the presence of lucigenin, (bis-*N*-methylacridinium-nitrate) was determined. SLS inhibited completely the lucigenin-amplified CL response when compared to the cells not exposed to SLS (Fig. 28).

### 10.8. Sensitivity of cells towards SLS

In order to compare the relative sensitivity of different cells available for determination of SLS biological activity, an assay was performed where the sensitivity of sheep red blood cells (haemolytic assay) was compared to that of rabbit peritoneal neutrophils (CL assay).

#### Haemolytic Assay

0.5 ml 2% v/v SRBC  $\equiv$   $2.4 \times 10^8$  cells.

0.1 ml toxin added . End point detection = 1/256.

Fig. 28.

The figure shown is a mean of three observations and is representative of five separate experiments.

Effect of SLS in lucigenin CL assay on rabbit peritoneal neutrophils:  
FMLP-induced CL response

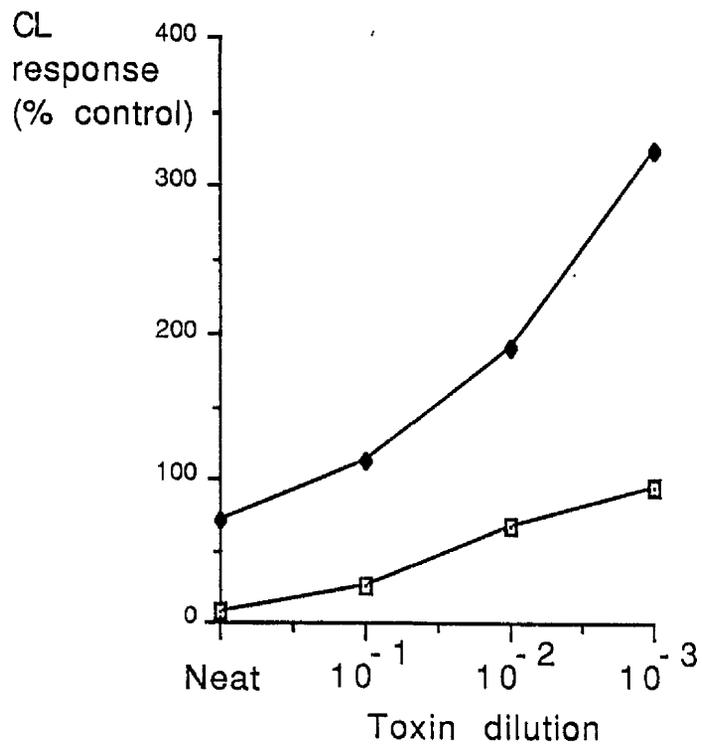
SLS (100  $\mu$ l of 256 HU/ml) was directly added to PMNs (prewarmed at 37°C for 15 min) suspended in buffer. Lucigenin and FMLP were added immediately before the start of the measurement of chemiluminescence (0 time).

Key:

□—□ , PMNs + Lucigenin + FMLP +SLS

◆—◆ , PMNs + Lucigenin -FMLP + SLS

# Effect of SLS : Lucigenin amplified CL Response



Neutrophil assay

$5 \times 10^5$  PMNs:

0.1 ml toxin

∴ Relative sensitivity of the haemolytic assay compared to the CL assay.

$2.4 \times 10^5$

————— 256 =  $1.28 \times 10^5$

$5 \times 10^5$

∴ On a cell basis, sheep RBC; are  $1.2 \times 10^5$  fold more sensitive to SLS than PMNs.

The effect of SLS on PMNs was tested by the chemiluminescence method.

10.9. Possible synergism between M-protein and SLS

Streptococcal survival in the host depends on the M-protein, which protects the organisms from phagocytosis by polymorphonuclear leucocytes (Lancefield, 1962). M-protein, which functions as an antiphagocytic substance, is a potent virulence factor for *S. pyogenes*. It was reported previously (Beachey, <sup>and Stollerman</sup> 1971) that M-protein attached to bacteria is not leucotoxic.

M-protein, lipoteichoic acid (LTA) and the glycoprotein have all been implicated as ligands in the adherence and colonization processes. However, the possible interactions of these molecules and their roles in mediating adherence and colonization are not clearly understood. More recently Caparon *et al.*, (1991) reported that M-protein was not the primary streptococcal adhesin, nor was it required

to orient streptococcal adhesin and fibronectin receptor. However, both M-protein and SLS appear to inhibit the same process - phagocytosis.

In an attempt to find out if there is any synergistic activity between SLS and M-protein, an assay was developed to examine the effect of M-protein on its own and together with SLS on opsonization of zymosan and uptake of opsonized zymosan by rabbit polymorphonuclear neutrophils.

(a) Influence of M-protein on opsonization and phagocytosis of zymosan by PMNs

The influence of M-protein on zymosan and phagocytosis of zymosan by PMNs was examined in a similar fashion to SLS (see Materials and Methods 7.1.1). Fresh rabbit serum (100  $\mu$ l of 1/2 dilution) and 100  $\mu$ l (200  $\mu$ g/ml) or 100  $\mu$ l of two-fold dilution of this concentration of M-protein were mixed and incubated at 37°C for 30 min. Zymosan (100  $\mu$ l) was added, and the tubes were again incubated at 37°C for 30 min. After 30 min, the tubes were centrifuged and tube contents were washed twice with PBS. The washed zymosan was used in the chemiluminescence assay.

Result of this experiment, as expected indicated that M-protein at concentration of 200  $\mu$ g/ml caused a reduction of phagocytosis of opsonized zymosan by PMNs. This finding also suggested that the decreased zymosan uptake and diminished chemiluminescence were manifestations of the influence of M-protein on the process of phagocytosis. 50% inhibition of the Cl response occurred in between 200  $\mu$ g/ml and 50  $\mu$ g/ml of M-protein. The results presented in Fig. 29 demonstrate that zymosan particles opsonized with normal serum

Fig: 29

Effect of M-protein, and both proteins on opsonization of zymosan by rabbit serum (washed zymosan)

The figure shown is a mean of 3 observations and is representative of four separate experiments.

Key:

a.

Fresh rabbit serum (100  $\mu$ l of 1/2 plus 100  $\mu$ l of neat and 1/2, 1/4, 1/8, dilution of pepsin extracted M- protein plus 100  $\mu$ l of Zymosan

b.

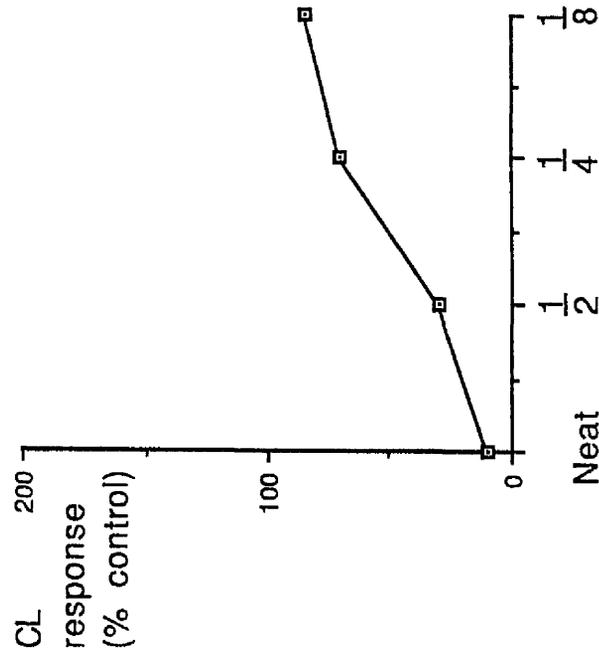
Fresh rabbit serum (100  $\mu$ l of 1/2 plus 100  $\mu$ l of neat and 1/10, 1/100, 1/1000 dilution of SLS plus 100  $\mu$ l of Zymosan

c.

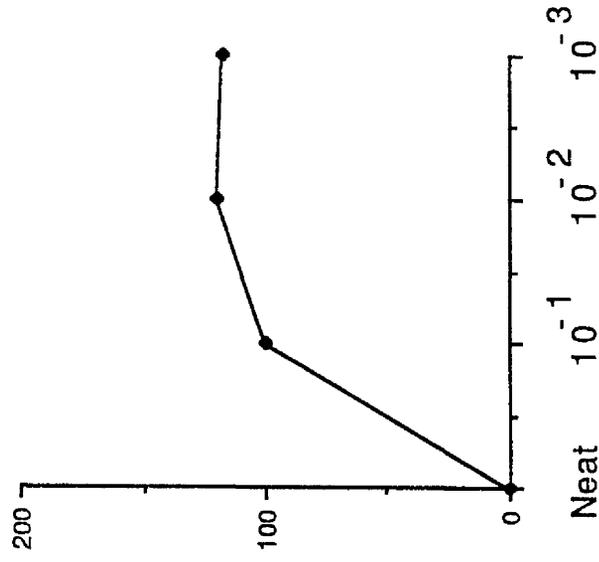
Fresh rabbit serum (100  $\mu$ l of 1/2 plus 50  $\mu$ l of M protien (200  $\mu$ g /ml) plus 50  $\mu$ l of SLS (256 HU /ml)  
50  $\mu$ l of each (two-fold dilutions of M-protein and ten-fold dilution of SLS) plus 100  $\mu$ l of 1/2 serum plus 100  $\mu$ l zymosan

**Effect of M-protein, SLS and both proteins on the opsonization of zymosan  
by rabbit serum**

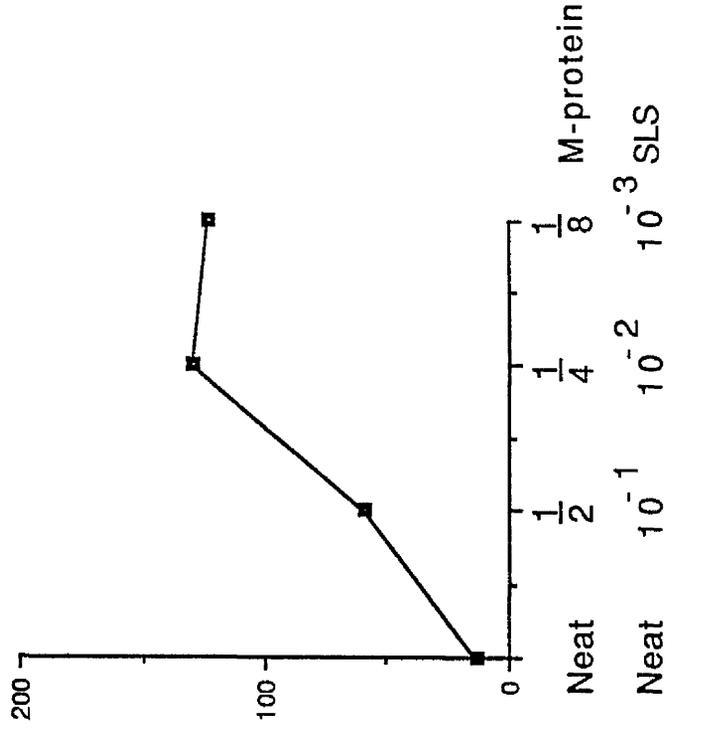
**a. M-protein**



**b. SLS**



**c. Combined proteins**



Dilution of protein

treated with M-protein failed to bind effectively to phagocytic cells, suggesting that C3b was either insufficiently deposited on the zymosan particles or inaccessible to C3b receptors on the phagocytic cell. This finding is not surprising as it is well established that M-protein exerts its antiphagocytic effect by interfering with the alternative complement pathway (Fischetti, 1991).

(b) Influence of mixture of both M-protein and SLS on opsonization and phagocytosis of zymosan by PMNs

As in the previous experiment (see Result 10.2 and 10.3) it was observed that SLS blocked opsonization of zymosan. The observation that M-protein blocked the opsonization and decreased zymosan uptake raised the possibility that synergism may exist between M-protein and SLS. In an attempt to find out if there was any synergistic activity between SLS and M-protein, 50  $\mu$ l of M-protein, (200  $\mu$ g /ml) + 50  $\mu$ l of SLS (256 HU /ml) were mixed with 100  $\mu$ l of 1/2 dilution of fresh serum. Similarly 50  $\mu$ l of each (two-fold dilutions of M-protein and ten-fold dilution of SLS) + 100  $\mu$ l of 1/2 serum were mixed and incubated at 37°C for 30 min. Zymosan (100  $\mu$ l) was added to each tube and incubated for 30 min at 37°C. After 30 min, the tubes were centrifuged and tube contents were washed twice with PBS. The washed zymosan was used in the chemiluminescence assay.

Fig. 29 demonstrates that there was a decreased CL response and decreased phagocytic activity by PMNs suggesting that at the highest concentrations of SLS and M-protein tested, inhibition of CL and uptake of zymosan particles occurred (Fig 29). It was not clear whether this was primarily due to M-protein or SLS. The result (Fig. 29) shows that 100  $\mu$ g M-protein and 25.6HU SLS did not induce any

inhibition of uptake of opsonized zymosan, suggesting that there was no synergism between M-protein and SLS. The greater the dilutions of M-protein 1/4, 1/8, (50, 25  $\mu$ g) and SLS (2.56, 0.256 HU), the greater the CL response was observed. However, there was no evidence of synergism between M-protein and SLS.

#### 11. Effect of transferrin on the growth of *S. pyogenes* Gr A

The ability of *S. pyogenes* Gr A to obtain iron bound to transferrin (Tf) was assayed by growth kinetics. The kinetics of growth of various strains, isolated from different sites of infection (Table 15), was examined when cultured in either BHIB, THB, and CDM (chelex-treated and chelex-untreated) containing apotransferrin. The Fe content (nmol/ml) of THB, BHIB, NB and CDM was 6.27, 32.22, 59.08 and 0.089 respectively, as measured by an iron binding capacity kit (Sigma). Representative results (see Fig. 30 & 31) show that apotransferrin, at a 10-fold molar excess over Fe, does not inhibit the growth of any of the streptococcal isolates under investigation. All isolates grew luxuriously in iron-restricted transferrin supplemented media, suggesting that they could tolerate the iron-restricted conditions by any of three possible mechanisms. Firstly, production of high affinity chelators (siderophore-mediated iron acquisition), secondly, a low iron-affinity mechanism or thirdly, the isolates did not display an absolute dependence on iron for growth.

##### 11.1. Effect of EDDA on the growth of *S. pyogenes* Gr A

The effect of EDDA (Ethylenediamine-di-o-hydroxyphenyl acetic acid), a synthetic chelator of high affinity for  $Fe^{3+}$ , was tested at increasing molar excesses (1, 5, 10, 20) over Fe on the growth of isolates of *S. pyogenes* group A, isolates of *S. milleri* and

Table 15

Growth\* of streptococci in presence of iron-chelators

Bacterial strain	Transferrin	EDDA	$\alpha$ - $\alpha$ -dipyridyl	Desferal
<u>Gr A streptococci</u>				
54359V	+	+	+	+
55903M	+	+	+	+
60343X	+	+	+	+
00657	+	+	+	+
00657	+	+	+	+
52114	+	+	+	+
52011	+	+	+	+
47061	+	+	+	+
48137	+	+	+	+
05790	+	+	+	+
12757	+	+	+	+
12997	+	+	+	+
10132	+	+	+	+
05790	+	+	+	+
52986	+	+	+	+
52942	+	+	+	+
01594	+	+	+	+
02750	+	+	+	+
<u>S. milleri</u>				
505	+	+	+	+
586	+	+	+	+
591	+	+	+	+
<u>E. coli MW</u>	-	-	-	NT
<u>Staph. aureus</u>	-	-	-	NT

NT = Not tested

\* Bacteria, in either B. H. I. Broth, C. D. Broth, and T. H. Broth were cultured under anaerobic conditions (Macintosh and Fildes jar) or under aerobic (either static or shaken culture) conditions at 37°C. The effect of chelators were tested at increasing molar excesses (1:1, 5:1, 10:1, 20:1) in over Fe in either B. H. I. Broth, C. D. Broth, and T. H. Broth.

Fig. 30.Growth kinetics of *S. pyogenes* strain 60343X in B.H.I. Broth in presence of apotransferrin

Bacteria were incubated at 37°C in shaken aerobic condition. Transferrin was added to the medium 12 hr before inoculation.

Key:

- control, no addition
- ◆—◆ addition of 1:1 apotransferrin to Fe
- addition of 2:1 molar excess of transferrin over Fe
- ◇—◇ addition of 10:1 molar excess of transferrin over Fe

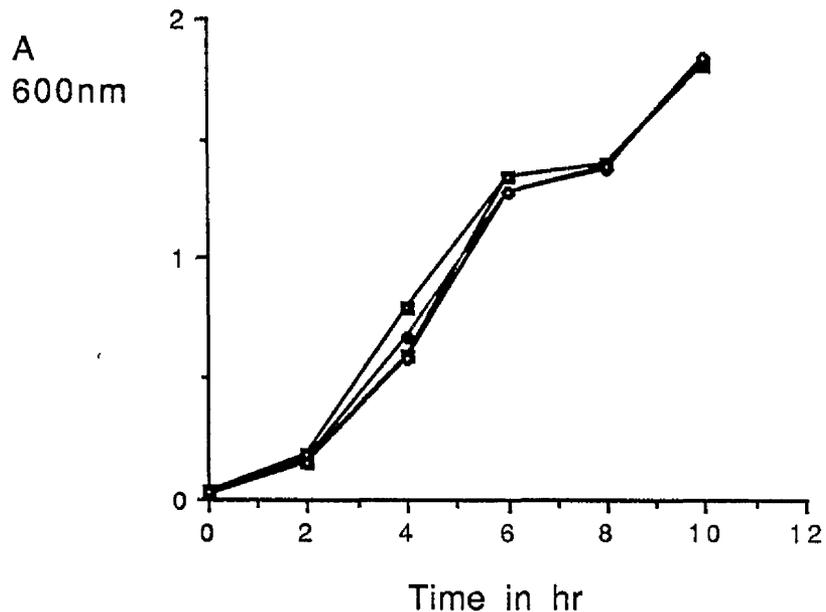
Fig. 31.Growth kinetics of *S. pyogenes* strain 54359V in C.D.M in presence of transferrin

Bacteria were incubated at 37°C in shaken aerobic condition. Transferrin was added to the medium 12 hr before inoculation.

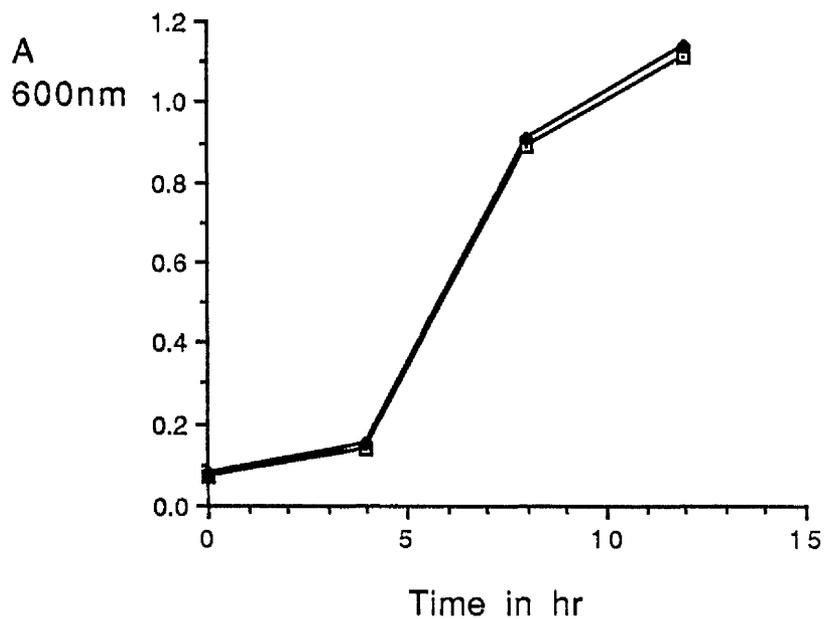
Key:

- control, no addition
- ◆—◆ addition of 10:1 molar excess of transferrin over Fe

**Growth pattern of strain 60343X  
in presence of transferrin**



**Growth pattern of strain 54359V  
in presence of transferrin**



*E. coli* MW. The cultures, in either BHIB, THB, or CDM were grown under anaerobic conditions (Macintosh & Fields jar) or under aerobic (either static or shaken culture) conditions at 37°C. Growth was measured by withdrawal of samples every 2 h and absorbance measured at 600nm. Repeated experiments (three times) with any of streptococcal isolates shown in Table 15 and Fig. 32 & 33 failed to detect any inhibition of bacterial growth under conditions of iron-restriction. From results presented in Figs. 32 & 33 and Table 15, it was concluded that 17 strains of *S. pyogenes* GrA and 3 strains of *S. milleri* were able to grow unrestrained under iron-limited conditions, with a 20-fold molar-excess of EDDA over Fe as well as in iron-replete media. This finding agrees with the results of Marcelis *et al.*, (1978) who reported that EDDA had no effect on growth of *S. faecalis*. Under similiar conditions of Fe-restriction, both *S. aureus* strain C336 and *E. coli* MW failed to grow.

#### 11.2. Effect of $\alpha, \alpha$ -dipyridyl on the growth of *S. pyogenes* Gr A

The growth kinetics of of *S. pyogenes*, *E. coli* MW and *Staph. aureus* (see Table 16) in the presence or absence of the synthetic iron chelator,  $\alpha, \alpha$ -dipyridyl, up to a 20-fold molar excess over Fe was monitored. *S. pyogenes* showed no growth inhibition (Fig. 34) whereas growth of *E. coli* MW (Fig. 35) and *Staph. aureus* was inhibited at a 10 fold molar excess of the chelator (data not shown).

#### 11.3. Effect of desferal (desferrioxamine) on growth *S. pyogenes* Gr

##### A

All isolates of group A streptococci (see Table 15) grew in BHIB, THB, or CDM in the presence of desferrioxamine up to a 20-fold molar excess. It was observed that desferal had no effect on

Fig 32 and 33

Growth kinetics of *S. pyogenes* strain 55903M and strain 52948 in the  
B. H. I. Broth in presence of EDDA

Bacteria were incubated at 37°C in shaken aerobic condition .  
EDDA was added to the medium 12 hr before inoculation.

Fig. 32. Strain 55903M

Key:

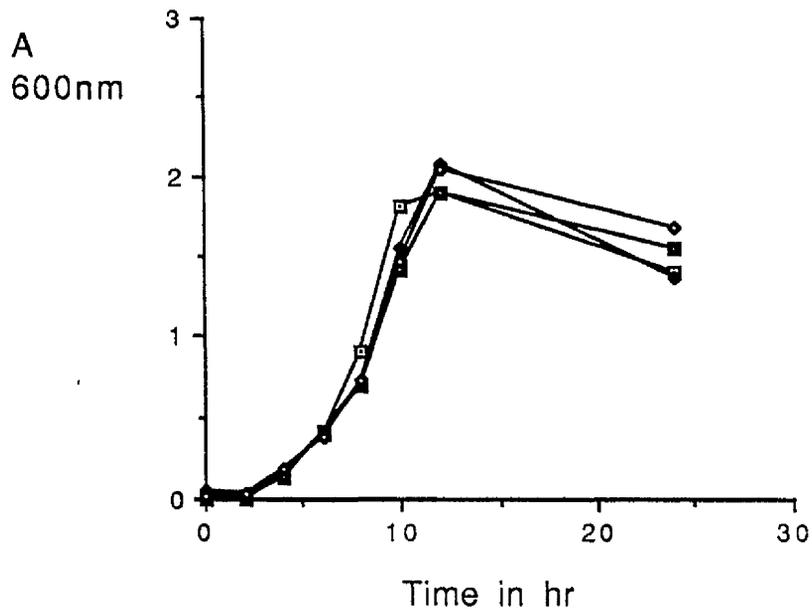
- control, no addition
- ◆—◆ addition of 1:1 EDDA to Fe
- addition of 2:1 molar excess of EDDA over Fe
- ◇—◇ addition of 10:1 molar excess of EDDA over Fe

Fig. 33. Strain 52942

Key:

- control, no addition
- ◆—◆ addition of 10:1 molar excess of EDDA over Fe
- addition of 20:1 molar excess of EDDA over Fe

**Growth pattern of strain 55903M  
in presence of EDDA**



**Growth pattern of strain 52942  
in presence of EDDA**

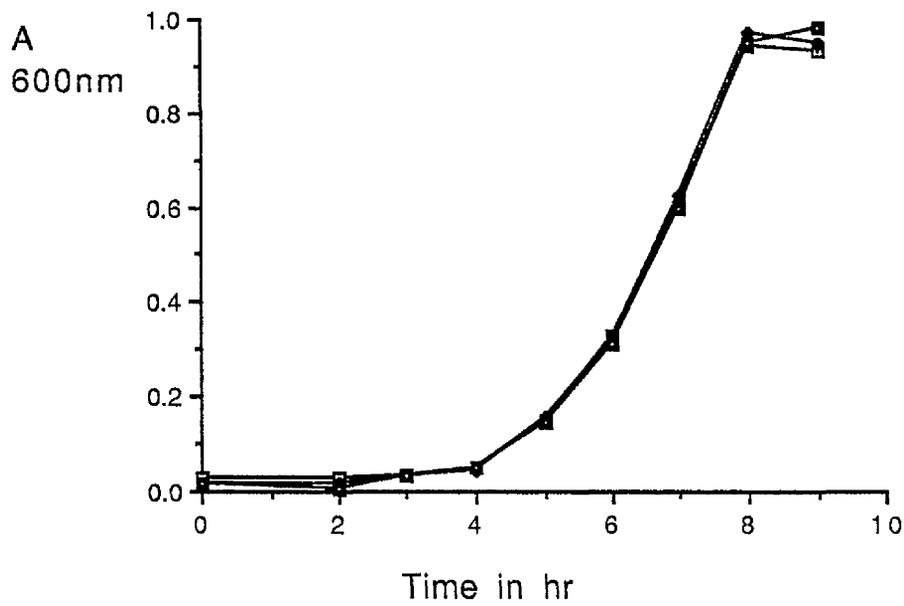


Fig 34 and 35

Growth kinetics of *S. pyogenes* strain 02750 and *E. coli* MW in  
B. H. I. Broth in presence of  $\alpha, \alpha$ -dipyridyl

Bacteria were incubated at 37°C in shaken aerobic condition.  $\alpha, \alpha$ -dipyridyl was added to the medium 12 hr before inoculation.

Fig. 34. Strain 02750

Key:

□—□ , control, no addition

◆—◆ , addition of 10:1 molar excess of  $\alpha, \alpha$ -dipyridyl over Fe

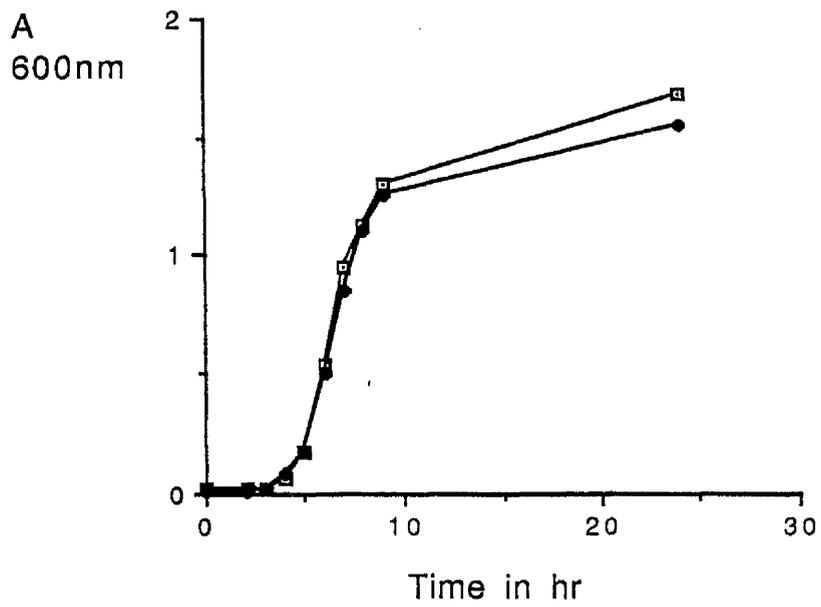
Fig. 35. *E. coli* MW

Key:

□—□ , control, no addition

◆—◆ , addition of 10:1 molar excess of  $\alpha, \alpha$ -dipyridyl over Fe

Growth pattern of strain  
02750 in presence of  $\alpha$ - $\alpha'$  dipyridyl



Growth pattern of *E. coli* MW in presence  
of  $\alpha$ - $\alpha'$  dipyridyl

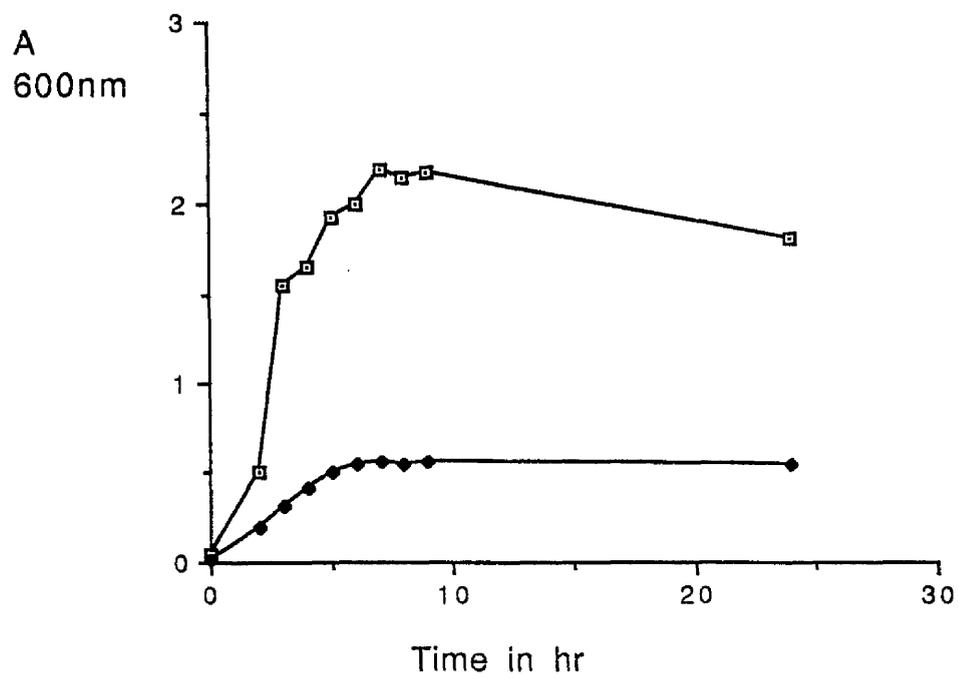


Fig. 36

Growth kinetics of *S. pyogenes* strain 55903M in presence of Desferal

Bacteria were incubated at 37°C in shaken aerobic condition.  
Desferal was added to the medium 12 hr before inoculation.

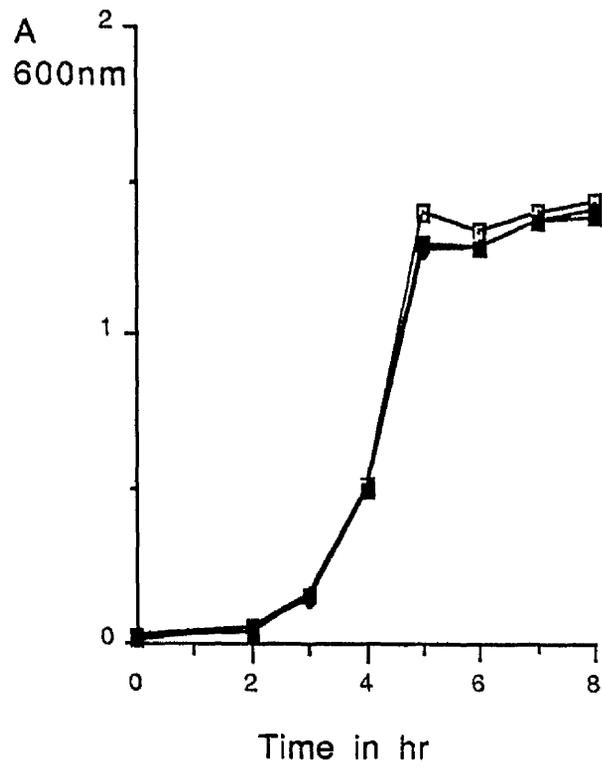
Key:

□—□ , control, no addition

◆—◆ , addition of 10:1 molar excess of Desferal over Fe

■—■ , addition of 20:1 molar excess of Desferal over Fe

Growth pattern of strain 55903M  
in presence of Desferal



multiplication of group A streptococcus strains and suggested iron-independent growth, as shown in representative results for a group A streptococcus strain shown Fig. 36. Desferrioxamine has previously been shown to enhance the growth of *Staph. aureus*, *Yersinia enterocolitica*, *Klebsiella aerogenes*, *Salmonella typhimurium* but it had no effect on the multiplication of *S. faecalis* (Brock *et al.*, 1983, 1988) .

#### 11.4. Inhibition of growth by EDDA in the agar plate assay

Fig. 37. a showed the zone of growth inhibition of *E. coli* MW after 18 h at 37°C in the presence of various concentrations of EDDA. Similar results have been obtained with a number of strains of *Staph. aureus* (A. Bensoltane , pers. comm. ). All strains of *Staph. aureus* were inhibited by 14 mM EDDA. Growth of all strains of *S. pyogenes* was unaffected by concentrations of EDDA up to 44 mM. An example of the pattern of the growth on the plate assay for strain 55903M is shown in Fig. 37. B. These observations corresponded with the findings of Marcellis *et al.*, (1978) where *S. faecalis* was shown to be insensitive to the presence of EDDA up to 44 mM.

#### 11.5. Search for siderophores under different growth conditions

To investigate whether or not the insensitivity to the presence of Fe chelators was the result of the production of siderophores by *S. pyogenes* strains, variations in growth conditions were introduced in an attempt to enhance any siderophore production (see Table 16). All strains were grown in either BHIB, THB or CDM under iron-restricted conditions. Inocula obtained from different isolates grown under iron-restricted conditions were washed with normal saline and used as starter for 50 ml cultures either grown

Fig. 37. Plate assay for inhibition of growth by EDDA

Key:

(A): Effect of EDDA on the growth of *E. coli* M4

a = 5  $\mu$ l of 1.5 mM EDDA .

b = 5  $\mu$ l 4.4 mM EDDA .

c = 5  $\mu$ l 14 mM EDDA .

d = 10  $\mu$ l 14 mM EDDA .

(B): Effect of EDDA on the growth of *Strep. pyogenes* GrA

a = 5  $\mu$ l of 1.5 mM EDDA .

b = 5  $\mu$ l 4.4 mM EDDA .

c = 5  $\mu$ l 14 mM EDDA .

d = 10  $\mu$ l 44 mM EDDA .

A



B

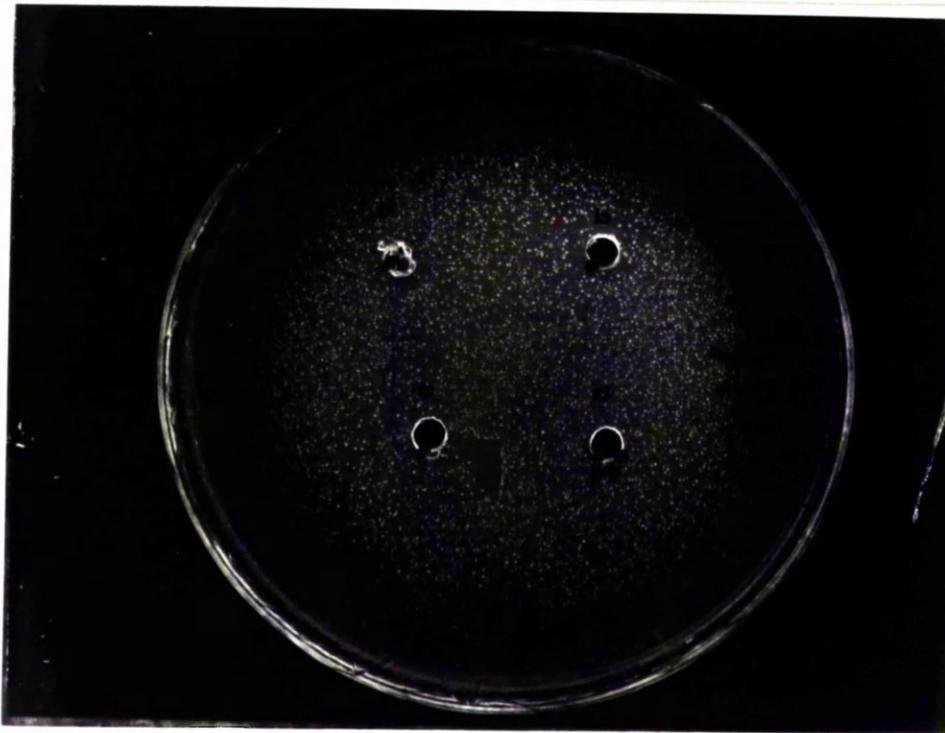


Table 16

Assay for <sup>\*</sup>siderophore production in the presence of iron  
chelators in B.H.I.B , T.H.B , and C.D. Broth

Bacterial strain	Transferrin	EDDA	$\alpha$ - $\alpha$ -dipyridyl	Desferal
<u>Gr A streptococci</u>				
54359V	-	-	-	-
55903M	-	-	-	-
60343X	-	-	-	-
00657	-	-	-	-
00657	-	-	-	-
52114	-	-	-	-
52011	-	-	-	-
47061	-	-	-	-
48137	-	-	-	-
05790	-	-	-	-
12757	-	-	-	-
12997	-	-	-	-
10132	-	-	-	-
05790	-	-	-	-
52986	-	-	-	-
52942	-	-	-	-
01594	-	-	-	-
02750	-	-	-	-
<u>S. milleri</u>				
505	-	-	-	-
586	-	-	-	-
591	-	-	-	-
<u>E. coli</u> MW	+	+	+	NT
<u>Staph. aureus</u>	+	+	+	NT

NT = Not tested

\* Possible siderophore production by isolates of *S. pyogenes* was investigated. The bacteria were grown under iron-restricted conditions and culture supernates were tested by Arnow and Csaky test (or ferric perchlorate test).

under high oxygen tension or low oxygen tension (shaken or static cultures). Samples (2 ml) were removed from a 50 ml flask culture after 4, 8, 12, and 24h of growth at 37°C and were assayed for phenolate or hydroxamate type siderophores by the Arnow assay and Csaky test (or ferric perchlorate test) respectively. When negative results were obtained after 24h growth in iron-restricted media, supernate samples were lyophilised, concentrated 10-fold and retested. Under these conditions phenolate-type siderophores were detected only from *E. coli* MW and *Staph. aureus* C336. There was no evidence of phenolate siderophores in concentrated lyophilized culture supernates of 20 streptococcal isolates. Hydroxamate type siderophores were not detected in supernates of any of the strains tested except *E. coli* MW (see Table 17).

16.6. Assay for Phenolate-and Hydroxamate-type siderophores in cultures of Streptococcal isolates grown in three different iron-limiting media

Three different iron-limiting media were used in attempts to induce siderophore production by *S. pyogenes* GrA.

(i) BHI broth with either EDDA, transferrin,  $\alpha$ - $\alpha$ -dipyridyl or desferal in a 20 fold molar excess were used as culture media. When lyophilised stationary phase culture supernates were tested for the presence of siderophores, all samples from streptococcal isolates gave negative results, whereas that from the culture of *E. coli* MW gave a positive phenolate and hydroxamate type siderophore reaction and *Staph. aureus* C336 gave a positive phenolate type siderophore reaction.

(ii) THB with either EDDA, transferrin,  $\alpha$ , $\alpha$ -dipyridyl and desferal in a 20 fold-molar excess was used as culture medium. None of the

Table 17      Detection of Siderophores by different assays

No of strains used	Arnow assay	Perchlorate assay	Csaky assay	TLC	Blue plate assay
<i>S. pyogenes</i>					
27	-	-	-	-	-
<i>S. milleri</i>					
3	-	-	-	-	-
<i>E. coli MW</i>	+	+	-	+	+
<i>Staph. aureus C336</i>	+	-	-	+	+

streptococcal isolates gave positive reactions for either phenolate or hydroxamate type siderophores when lyophilised stationary phase culture supernates were tested by the Czaky or Arnow assay,

(iii) CDM (see Appendix 13.1.b) with EDDA, transferrin,  $\alpha, \alpha$ -dipyridyl or desferal was used as the culture medium. CDM was used either before or after chelex treatment to measure  $Fe^{3+}$ . All isolates of streptococci gave negative reactions for both phenolate and hydroxamate type siderophore. *E. coli* MW gave positive reactions for both hydroxamate and phenolate siderophores.

#### 11.7. Assay for phenolate-type siderophores by Thin Layer

##### Chromatography

Lyophilised culture supernates for detection of phenolates were extracted with ethyl acetate, as outlined in Materials and Methods. *E. coli* MW and *Staph. aureus* C336 produced one iron-binding compound (see Fig. 39) whereas no evidence was found for phenolate production by any of the streptococcal isolates tested. Iron-binding phenolic compounds form a purple/blue complex with iron and migrate with an  $R_f$  value of approximately 0.5. The control (3,4-dihydroxybenzoic acid) migrated with an  $R_f$  value = 0.5 and extracted material from culture samples from *Staph. aureus* migrated with an  $R_f$  value of approximate 0.55.

#### 11.8. Detection of hydroxamate-type siderophores by Thin Layer Chromatography

For the detection of hydroxamates from *E. coli* MW, *Staph. aureus* and possibly from *S. pyogenes* grown under iron-limiting conditions (see Materials & Methods 7.2.h), culture supernates were concentrated by lyophilisation and separated by thin layer

chromatography using a solvent system of n-butanol-water-acetic acid (60:15:25;v/v). *E. coli* MW produced an iron-binding compound which formed a red /brown complex with iron similar to desferal, which migrated with an  $R_f$  value of approx 0.5. No hydroxamate type siderophores (see Fig.39) were detected in culture supernates from streptococcal isolates or *Staph. aureus* (Table 17).

11.9. Assay of siderophores by a universal assay (Blue agar plate assay)

The blue agar plate assay (Schwyn and Neilands, 1987) was used as a screening technique for possible production of siderophores from the isolates of *S. pyogenes*, *S. milleri*, *E. coli* MW and *Staph. aureus*. To remove the iron from the dye, the bacterium must produce the high affinity iron uptake system but only to a level that satisfies its requirements for the metal. This may result in relatively small orange halos around colonies. None of the isolates of group A streptococci nor the three strains of *S. milleri* tested produced any siderophore-like activity (see Fig .40). Streptococcal isolates under investigation were able to grow unimpaired under iron-restricted conditions suggesting an iron-independent metabolism.

12. Effect of Potassium cyanide (KCN) and Sodium azide ( $\text{NaN}_3$ ) on the growth of *S. pyogenes* Gr A

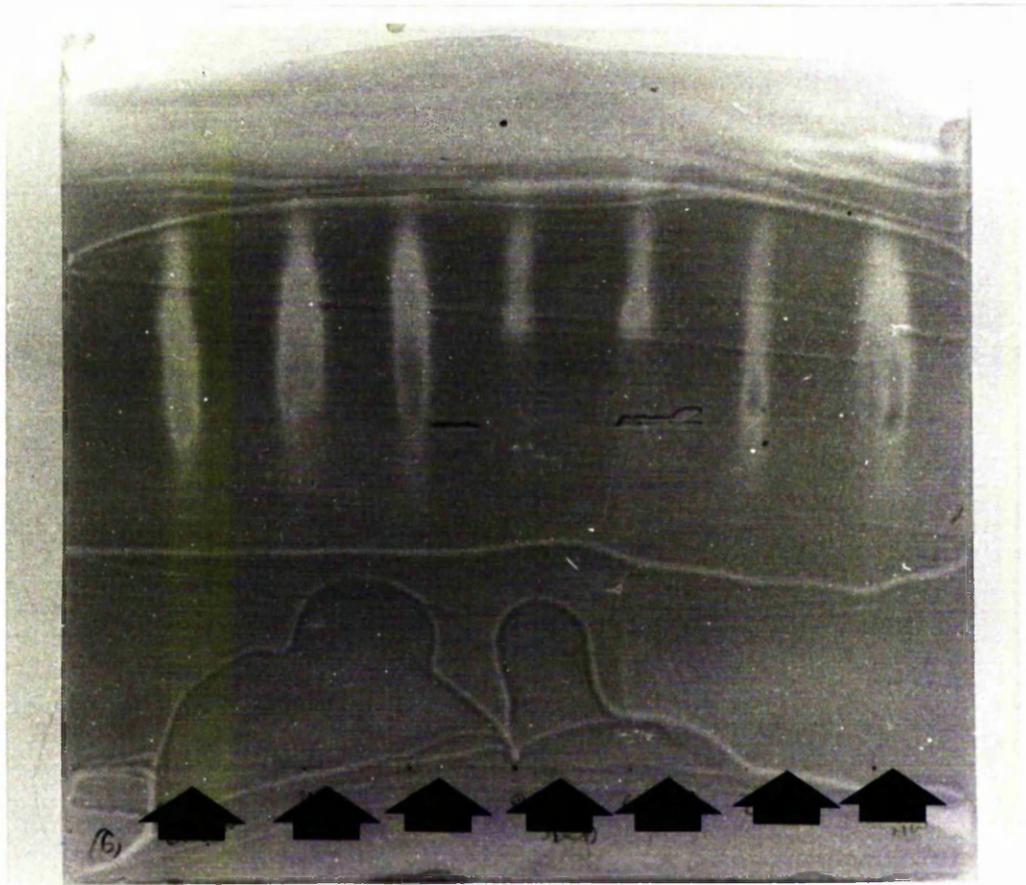
The effect of KCN and  $\text{NaN}_3$  was tested on a number of isolates of group A streptococci, *S. milleri*, *E. coli* MW, and *Staph. aureus* (Table 18). Generally, KCN, a respiratory inhibitor, binds to and inhibits both the oxidized and reduced forms of cytochrome oxidase of the  $\text{aa}_3$ -type (Smith, 1954, Ritchy and Seeley, 1976). Azide ( $\text{N}_3^-$ ) acts as a respiratory inhibitor by combining with, and preventing the reduction of oxidized cytochrome oxidases of the

Fig. 38

Assay for phenolate-type siderophores by Thin Layer Chromatography

Key:

- a= Culture supernate of *Staph. aureus*
- b= Culture supernate of *Staph. aureus* grown in 10:1 molar excess of EDDA
- c= Culture supernate of *Staph. aureus* in grown 20:1 molar excess of EDDA
- d= Culture supernate of *S. pyogenes* in 20:1 molar excess of EDDA
- e= Culture supernate of *S. pyogenes*
- f= Control (3,4 dihydroxybenzoic acid)
- g= Culture supernate of *E. coli* MW grown in 20:1 molar excess of EDDA



a

b

c

d

e

f

g

Fig. 39

Assay for hydroxamate-type siderophores by Thin Layer Chromatography

Key:

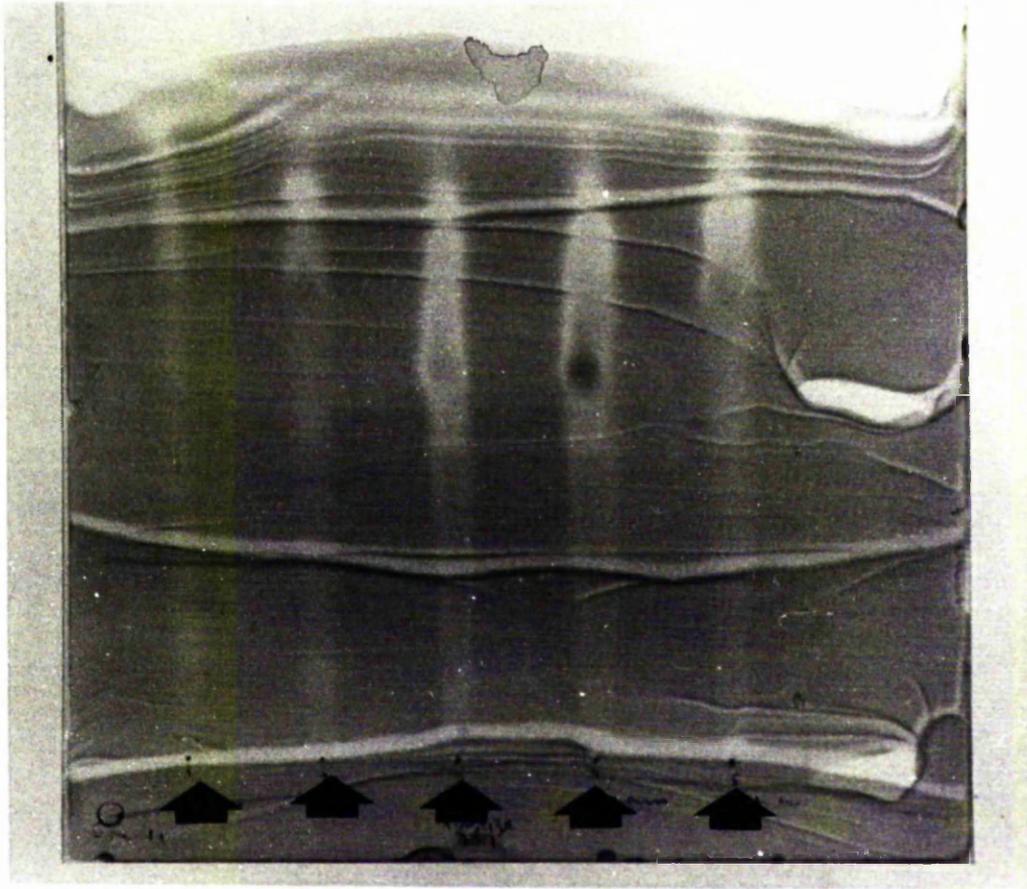
a= Culture supernate of *S. pyogenes*

b= Culture supernate of *S. pyogenes* grown in 20:1 molar excess of  
EDDA

c= Control (Desferal)

d= Culture supernate of *E. coli* MW grown in 20:1 molar excess of  
EDDA

e= Culture supernate of *E. coli* MW



a b c d e

Fig 40Detection of siderophores by universal assay (Blue agar plate assay)

Strains were point inoculated on the plate and incubated for 24 h at 37° before the plate was inspected for orange halos around the colonies which would indicate siderophore production.

## Key:

a=	<i>S. pyogenes</i>	strain	55903M
b=	..	..	00657
c=	..	..	52114
d=	..	..	54359V
e=	..	..	05790
f=	..	..	52942
g=	<i>S. milleri</i>	strain	505
h=	..	..	586
i=	..	..	591



Table 18

Growth inhibitory concentration of sodium azide, and potassium cyanide for streptococci and other organisms.

---

<u>Species</u>	<u>No. of strains tested</u>	<u>Inhibitory concentration ( mM)</u>	
		Sodium azide	Potassium cyanide
<i>S. pyogenes</i>	9	100 mM	100 mM
<i>S. milleri</i>	3	100 mM	100 mM
<i>E. coli MW</i>	1	10 mM	10 mM
<i>Staph. aureus</i>	1	10 mM	10 mM

---

aa<sub>3</sub>-type. *S. pyogenes* was resistant to the effect of both these respiratory inhibitors (KCN and NaN<sub>3</sub>) when added 2h after initiation of growth. The final concentrations of these respiratory inhibitors tested in B.H.I.B were 1 mM, 10 mM, 50 mM, and 100 mM. BHI broth cultures containing either KCN or NaN<sub>3</sub> were incubated aerobically at 37°C in shaken and unshaken conditions or anaerobically. Strains of *S. pyogenes* showed no inhibition of growth by 50 mM KCN (Fig. 41); however growth inhibition did occur at 100 mM final concentration of KCN and NaN<sub>3</sub> in both aerobic (shaken or unshaken) and anaerobic conditions. In contrast, growth of *E. coli* MW (see Fig. 42) and *Staph. aureus* (data not shown) was markedly inhibited at concentrations of 1 mM and 10 mM of KCN and NaN<sub>3</sub> and completely inhibited at a concentration of 50 mM. These results correspond with those reported by Whittenbury, (1978), Britton *et al.*, (1978), and Archibald and Fridovich, (1981).

12.1. Effect of KCN plus iron chelator EDDA on the growth of *S. pyogenes*

*S. pyogenes* was grown under iron-restricted conditions in the presence of 1:1, 10:1 and 20:1 EDDA over Fe in B.H.I broth either aerobically (shaken or unshaken) or anaerobically at 37°C. KCN was added after 2h of growth at final concentrations of 1 mM, 10 mM, and 50 mM. EDDA and KCN or NaN<sub>3</sub> had no effect on the growth of *S. pyogenes* (Fig. 43), even at 50 mM final concentration of either KCN or NaN<sub>3</sub> whereas iron-restriction plus the presence of either respiratory inhibitor had a profound effect on the growth of both *E. coli* MW and *Staph. aureus* (data not shown).

12.2. Membrane protein (MP) profile under iron-restricted conditions

The membrane protein profile of one clinical isolate (5903M)

Fig. 41.

Effect of KCN in B. H. I. Broth on the growth of 55903M

Bacteria were grown aerobically shaken at 37°C. KCN was added (arrow) after two hr of growth.

Key:

- control, no addition
- ◆—◆ addition of 1 mM KCN after 2 hr growth.
- addition of 10 mM KCN after 2 hr growth..
- ◊—◊ addition of 100 mM KCN after 2 hr growth.

# Growth pattern of strain 55903M in presence of KCN

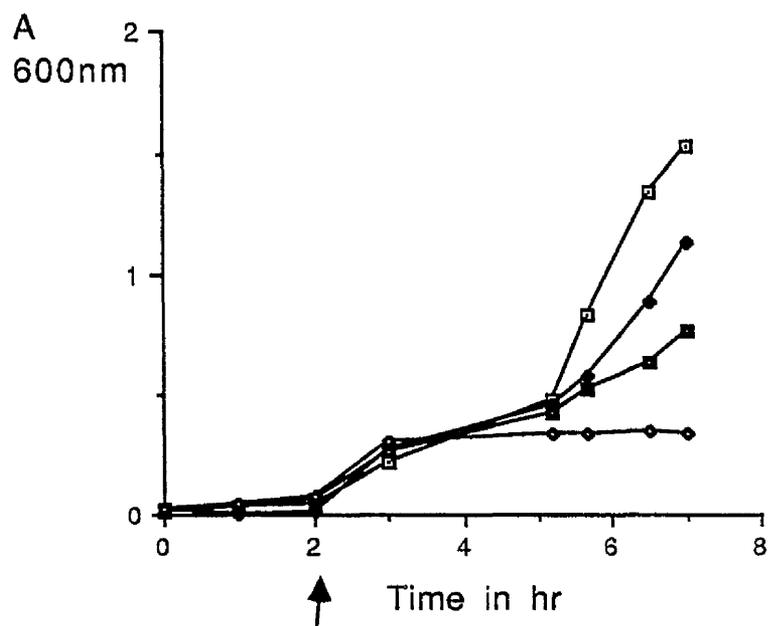


Fig. 42Effect of KCN in B. H. I. Broth on the growth of *E. coli* MW

Bacteria were grown aerobically shaken at 37°C. KCN was added (arrow) after two hr of growth.

Key:

□—□ , control, no addition

◆—◆ , addition of 1 mM KCN after 2 hr growth.

■—■ , addition of 10 mM KCN after 2 hr growth.

Growth pattern of *E. coli* MW in presence of KCN

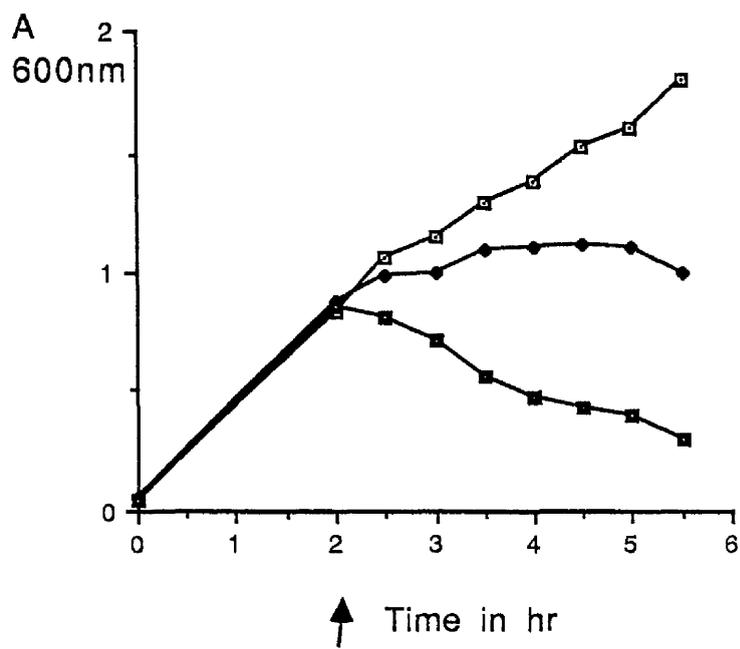


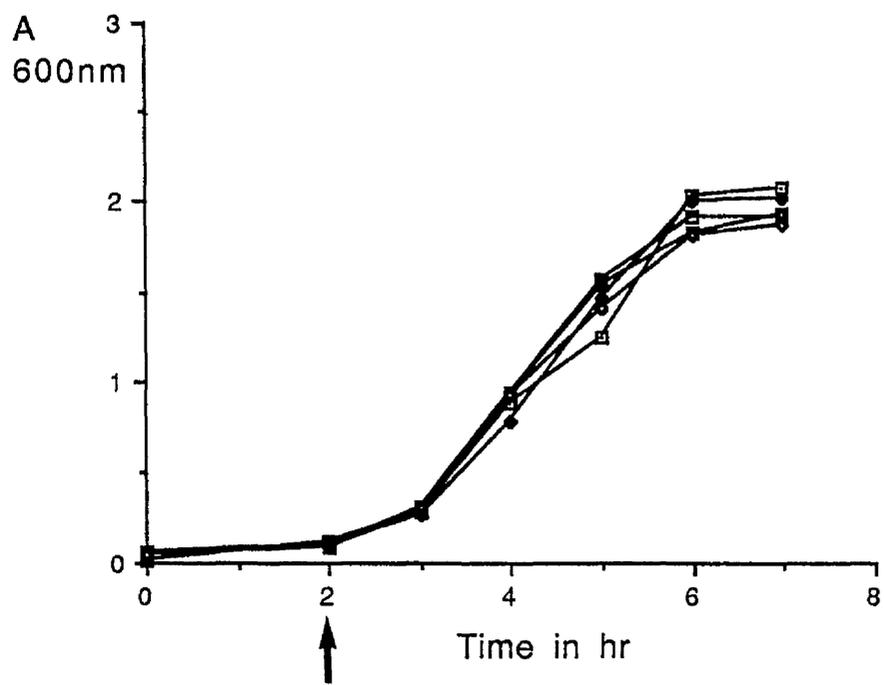
Fig. 43Effect of KCN + EDDA in B. H. I. Broth on the growth of 55903M

Bacteria were grown under iron-restricted conditions in presence of 10:1 molar excess of EDDA over Fe in aerobic shaken conditions at 37°C. KCN was added after two hr growth.

Key:

- control, no addition
- ◆—◆ 10:1 molar excess of EDDA over Fe
- 10:1 EDDA plus addition of 1 mM KCN after 2 hr growth .
- ◇—◇ 10:1 EDDA plus addition of 10 mM KCN after 2 hr growth .
- 10:1 EDDA plus addition of 50 mM KCN after 2 hr growth.

Growth of strain 55903M in presence of  
EDDA and KCN.



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of *S. pyogenes* (group A) grown aerobically in BHI broth under iron-restricted conditions imposed by EDDA is shown in Fig 44. Comparison of the band patterns in lanes B and C indicated that there was no differences in the protein profile between cells grown under iron-replete (lane B) and iron-restricted conditions (lane C) as visualized by silver staining.

Fig. 44Membrane protein (MP) profile under iron-restricted conditions

A= SDS-7 mol. wt. standard

B= Strain 55903M grown under iron-replete conditions

C= Strain 55903M grown under iron-restricted conditions



## Discussion

13. Production (induction and release) , purification and characterisation of SLS

Two strains of *S. pyogenes* group A, strain C203S and strain 55093M were examined for streptolysin S (SLS) production. Strain C203S was originally selected because of its ability to produce large quantities of SLS from resting cells (Bernheimer, 1949 ; Arbuthnott and Symington, 1973 ; Ginsburg, 1970 ; Hryniewicz and Pryjma 1977 ; Alouf and Loridan, 1986). The procedure for induction and release of SLS is not only simple, but economical and reproducible, yielding appreciable quantities of SLS at 1000 HU/ml.

A single batch of washed streptococci was shown to produce, upon 14 induction cycles, the same total haemolytic activity of SLS after each induction. In the past , Weld (1934), Bernheimer (1949), Ginsburg *et al.*, (1963, 1965), Alouf and Loridan (1986) all have shown that a single batch of cells could be induced 4 times (Alouf and Loridan 1986) for the production of high amounts of SLS, provided that optimal amounts of RNA-core (albumin or Tween), an energy source and  $Mg^{++}$  ions were present. On the other hand, Lai *et al.*, (1978), and Akao *et al.*, (1983) induced the release of SLS from washed cells with RNA-core for 90 min and were able to obtain haemolytic material after one induction but none on further induction.

In the present study, the interaction of washed streptococci, prepared under specified conditions with RNA-core and a fermentable carbohydrate (maltose) and  $PO_4^{-2}$  in the presence of  $Mg^{++}$  resulted in an appearance of haemolytic activity identified as SLS. This finding is in accordance with the earlier reports of Weld (1934), Bernheimer (1949), Ginsburg *et al.*, (1963, 1965) and Alouf and

Lorridan (1986). It is interesting to note that both Bernheimer (1949) and Okamoto (1962) showed that in addition to RNA-core active fraction (AF), an energy source (maltose, glucosamine),  $K^+$  and  $PO_4^{-2}$  were essential for maximal haemolysin formation by resting streptococci. Okamoto (1964) also demonstrated that the inhibitory effect of glucose on SLS formation by growing streptococci might be due to the glucose effect in enzyme synthesis, and not the killing effect of low pH. According to Bernheimer (1949) there was a direct relationship between SLS yield and the quantity of glucosamine fermented. His findings indicated that the cocci were actively metabolizing when SLS appeared in the medium and that this process was energy-dependent.

The induction of the same cell suspension for 14 cycles showed that, under appropriate conditions, washed cocci were capable of forming appreciable quantities of SLS and that the system studied can be utilized for preparation of crude toxin of high potency and large yield. A possible explanation of the cyclic release of toxin may be that each induction cycle causes autolysis of a fraction of the bacterial cells, and that SLS is released from an intracellular pool by this process. That the presence of SLS in the induction buffer does not depend upon autolysis is indicated by several facts. First, there is no decrease in optical density of the bacterial suspension while SLS is formed. Second, SLS develops as a specific response to very low concentrations of RNA-core. Third, when induction buffer was used without RNA-core, there was no release of haemolytic material (SLS).

Although it is clear that SLS does not have its origin in autolysis, the actual mechanism by which toxin arises is not clear (Bernheimer, 1949). The necessity for an energy source, the effect of

temperature on the formation of SLS, and the inhibition of toxin formation by a variety of enzymes demonstrates that SLS is formed only during active metabolism.

It has already been pointed out by Bernheimer (1949) that appearance of SLS depends upon the rate of synthesis. In agreement with this is the observation that only traces of SLS can be found in sonically-disrupted cocci, and this fact has already disproved conclusively the extraction hypothesis. It seems likely that SLS is synthesized in the resting cells, and that the rate of appearance of SLS is limited by an energy-yielding or other metabolic processes in such resting cells. The most highly purified fraction of SLS (fraction 19) had a specific haemolytic activity  $3.5 \times 10^5$  HU (mg protein)<sup>-1</sup>, a value similar to that reported by Lai *et al.*, (1978) and Alouf and Loidan (1986).

The data presented here suggest that SLS is synthesised *de-novo* during each induction cycle and that inhibitors of protein synthesis such as chloramphenicol block its formation. An alternative, but more complex interpretation might involve synthesis of a protein (possibly catalytic) which promotes release of presynthesised SLS. The two possible interpretations were not differentiated by this experimental approach. According to Akao *et al.*, (1983) the production of SLS by streptococci was inhibited by treatment with the protease inhibitor, tosylphenylalanine chloromethyl ketone (TPCK), even in the presence of the inducer oligonucleotides. Other protease inhibitors, antipain, leupeptin, or pepstatin had little or no effect. Trypsin reversed the effect of TPCK or TLCK. The reversal was dependent upon the amount of added trypsin and the incubation time at 37°C suggesting

that a protease activity was involved in haemolysin formation. The effect of trypsin was not observed if chloramphenicol was also added, suggesting that a precursor of SLS was processed as it was synthesized and released into medium as the active haemolysin, by the concerted action of a protease and inducer oligonucleotides.

Chloramphenicol blocked protein synthesis of "cells A" while "cells B" (used as control) not exposed to antibiotic were not affected and yielded the same haemolytic titre after each induction (1000 HU/ml). As the activity of such haemolytic material was inhibited by trypan blue but not by cholesterol, it was considered to be due to SLS.

Estimated molecular weights of less than 4,000 have been reported for SLS (Hryniewicz and Pryjma<sup>1964</sup>1978, Koyama, and Egami and Lai *et al.*, 1978) but clear and direct supporting evidence has not been published. However, Alouf and Loidan (1986) for the first time presented data concerning the analysis of SLS on SDS and native-PAGE. According to their findings, a band which they identified as SLS migrated with the dye front in SDS and native PAGE, although the gel profile of the RNA-core carrier molecule was not reported. From these results the molecular weight of SLS was estimated to be below 4000.

The data presented in this thesis suggest that the purified hydroxylapatite column product contained the low molecular weight peptide which always ran at the dye front of the gel as visualized through silver staining whereas the appearance of 5-8 higher molecular weight bands were indicative of the presence of the carrier molecule yeast RNA-Core. There was no discernible difference in the low molecular weight region of the gel between profiles of carrier alone

and carrier with SLS bound. Almost the same pattern of bands was observed and it was difficult to determine the possible molecular weight of SLS from such gels. When crude and purified SLS was analyzed by native-PAGE with RNA-core as a control, it was observed that the RNA-core profile lacked one band as compared to the SLS gel profile. This band was suspected of being SLS but one cannot estimate the molecular weight by this method.

Since the molecular weight and identity of the SLS peptide was not clear from the SDS and native PAGE analysis, a zymogram technique was used to locate SLS by its haemolytic activity on native-PAGE. Comparison of the pattern of haemolysis with that of the stained bands after silver staining showed that the haemolytic activity resided in a component not at the dye front but at a position expected of a small peptide. Three fractions were assayed by this method and fraction 19 produced a relatively larger haemolytic zone than fractions 18 and 20 as expected from the haemolytic assay of these fractions.

Hryniewicz and Prýjma (1978) and Alouf and Loridan (1986) claimed to have observed the haemolytic activity of SLS on gels by overlays containing sheep RBCs, but no data were published in their reports. The zymogram technique demonstrated clearly that SLS is haemolytically active in the absence of carrier, a finding which contradicts earlier claim that SLS was inactive in a carrier free state (Lai *et al.*, 1978).

Proteins are usually fragile molecules that often require great care during purification to ensure that they remain intact and fully active. Removal of proteins from the cellular environment

subjects them to a variety of conditions and processes that can lead to loss of activity or alteration of structure. These include dilution, change in solution conditions, exposure to degradative enzymes, oxygen, heavy metals, and surfaces and changes in physical conditions (e.g. freezing and thawing). SLS is normally assayed by its haemolytic activity and this property is remarkably labile.

Poor stability of SLS activity has been the subject of comment by previous investigators. According to Ginsburg (1970), haemolysin complexed with serum albumin, detergent or RNA-core are very unstable in aqueous solutions and they lose most of their activity on standing at 25°C for a few hours. According to Koyama and Egami (1964), in the purified state, the haemolytic activity rapidly decreased even at ice-cold temperature and at neutral pH and only 10 per cent of original activity remained after overnight standing. However, in partially purified preparations, the haemolytic activity could be conserved by lyophilization without any appreciable inactivation. Lai *et al.*, (1978), Bernheimer, (1983), and Alouf and Loridan, (1986) reported that SLS was unusually labile and its instability through manipulative steps was probably due to its hydrophobicity.

The result presented here showed that SLS lost almost all haemolytic activity if kept in 37°C, 4°C, or -20°C, with or without glycerol for 12 h. However SLS retained 100% haemolytic activity for more than six months if stored in the presence of 0.1% extraneous protein (bovine serum albumin) plus 20% glycerol at -20°C. If one is primarily interested in studying enzyme activity, the presence of serum albumin may not matter. In contrast the presence of extraneous

protein is undesirable in structural studies. The presence of 20% glycerol may also be undesirable. Although it can be removed by dialysis, it is likely that biological activity would be lost during this process. The role of serum albumin in stabilising the activity of SLS is not known.

Attempts to electro-transfer SLS from PAGE gels onto nitrocellulose or Hybond C membranes for further characterization by amino acid sequencing and animal inoculation for immunogenicity studies were unsuccessful. Likewise, attempts to remove SLS after excision of gel bands followed by electroelution resulted in no recovery of activity. Purified SLS, in its native state was run on non-denaturing PAGE. SLS proved difficult to visualize by Ponceau S and Coomassie-blue staining, possibly because of the relatively low amounts of protein and its high specific activity. This observation suggested that Ponceau S staining was not sensitive enough to detect SLS or that SLS failed to bind to the membranes during transfer. Further efforts directed towards conclusively identifying the SLS peptide on gels and effecting its extraction or transfer to nitrocellulose would be very worthwhile, since this could facilitate the sequencing of the peptide, which would provide the key to further studies on this elusive toxin. In similar studies with a small hydrophobic peptide,  $\delta$ -lysin, produced by *S. aureus*, the sequence of this poorly antigenic peptide allowed its chemical synthesis, and a series of  $\delta$  lysin analogues were studied extensively in relation to their haemolytic activity and possible modes of interaction with membrane lipids (Alouf, 1986).

### 13.1. The growth of *S. pyogenes* in the presence of chelators

The growth response of *S. pyogenes* and *S. milleri* to Fe-deprivation was compared to that of *E. coli* MW and *S. aureus*, both of which contain several well-documented high-affinity Fe acquisition systems (Neillands, 1981). All the streptococcal strains examined in this study were able to grow and multiply in media (BHIB, CDM and THB) containing powerful ferric iron chelators such as EDDA, transferrin,  $\alpha$ ,  $\alpha$ -dipyridyl and desferal. A large molar excess of any of these chelators (over Fe) in the growth medium had no significant effect on the streptococcal growth rate or final cell density, and the absence of siderophore-mediated iron acquisition systems indicate either extraordinarily efficient Fe acquisition or a very low or zero Fe requirement. Our findings coincide with the findings of Neillands (1974) who indicated that iron is not essential for lactic acid bacteria. Marcelis *et al.*, (1978) could not detect significant differences in the growth of *S. faecalis* in the presence or absence of available iron. Archibald (1983) explicitly stated that *Lactobacillus-plantarum* is an organism not requiring iron and his experiments regarding Graphite furnace atomic absorption analysis revealed that washed *Lactobacillus plantarum* cells contained less than 0.1  $\mu\text{M}$  intracellular Fe while *E. coli* B cells contained 900 $\mu\text{M}$ . Verstraete *et al.*, (1989) also reported that lactic acid bacteria (using species of different genera such as *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc*) were able to compete and grow in the absence of available iron and copper.

Brock and Janet (1983) and Brock *et al.*, (1988) reported that an excess of iron markedly increased the rate of multiplication

of both *Staph. aureus*, and *Y. enterocolitica*, but had no effect on the multiplication of *S. faecalis*. These observations were similar to earlier studies showing that growth of many microorganisms, but not streptococci, is inhibited by serum or transferrin and that this inhibition is reversible by iron (Schade, 1963, Weinberg, 1978, and Marcelis *et al.*, 1978). The fact that desferrioxamine (desferal) had no effect on multiplication of *Strep. faecalis* supports our findings for Fe-independent growth of *S. pyogenes* and *S. milleri*, since if desferrioxamine were acting in some other way not related to iron chelation, it might have been expected to enhance the growth of all streptococcal strains.

In contrast to these reports, Francis *et al.*, (1985) reported "in the case of *S. pyogenes*, it seems unclear whether or not it even uses hemoproteins for aerobic respiration. Although heme has been observed to be necessary for the aerobic existence of streptococci such as *S. faecalis* and *S. sanguis* (Whittenbury, 1978), *S. pyogenes* was reported in one study to lack heme induced NADH oxidase, thereby suggesting a lack of a cytochrome system (Ritchey and Seelley, 1976). However, that same study indicated *S. sanguis* to be devoid of NADH oxidation. If the studies of *S. sanguis* are accurate, it appears that although this bacterial species does not require a cytochrome system for aerobic respiration, it still requires heme for growth (probably to synthesize a catalase-like protein for protection against H<sub>2</sub>O<sub>2</sub> formation). Although Ritchey and Seeley (1976) reported *S. pyogenes* to either facilitate NADH oxidation using flavin-like proteins or to lack the oxidation capacity altogether (depending on the strain), they did not indicate whether or not *S. pyogenes* required heme for peroxidase

activity. Since it was observed that *S. pyogenes* (strain unknown) required Hb for growth, it is likely that this species needs intact hemin for at least the formation of a catalase [since all streptococci are believed to be incapable of synthesizing protoporphyrin IX].

Francis *et al.*, (1985) proposed that if *S. pyogenes* required haemin for growth (as with other streptococci) (Ritchey and Seelley, 1976), it might secrete a high haem. affinity protein such as haemopexin which would be bound by a membrane receptor when carrying haem. The haem. could then be transferred into the cell interior.

The experiments reported in this thesis on growth in the presence of chelators and the search for siderophores support the conclusion of Marcelis *et al.*,<sup>(1978)</sup> Archibald (1983) and Verstraete *et al.*, (1989) that *S. pyogenes* and *S. milleri* can grow in the absence of Fe. No evidence was obtained to support the conclusion of Francis *et al.*, (1985) that *S. pyogenes* required haemin, nor that of Griffiths and McClain (1989) that Fe stimulated growth of *S. pyogenes* and that iron limitation induced a higher yield of SLS.

Nutrient limitation is known to have a profound effect on the composition and surface structure of bacteria (Williams and Brown, 1985) as well as on gene regulation. Present investigations suggested that there was no minor or major differences in the SDS-PAGE profiles of membranes from cells grown under iron-replete and iron-restricted conditions. This study confirms that iron does not alter the membrane protein profile of *S. pyogenes* group A and had no impact on bacterial growth.

### 13.2. Absence of siderophore productions

The data presented indicate that *S. pyogenes* and *S. milleri*

do not excrete a readily detectable, classic phenolate or hydroxamate siderophore. On other hand *E. coli* MW produced both phenolate and hydroxamate-type of siderophore whereas *Staph. aureus* produced only a phenolate-type of siderophore as detected by TLC. Our findings coincide with the observation of Evans *et al.*, (1986) and Francis *et al.*, (1985), that *S. mutants* and *S. pyogenes* did not produce ferric chelating and transporting siderophores respectively. These data confirmed that a high affinity siderophore-mediated iron acquisition system was not involved. Evans *et al.*, (1986) proposed a model for reductive assimilation of iron by *S. mutants* and demonstrated the effect of an environmental reductant (sodium ascorbate) on iron uptake, as well as possible chelation and withholding of iron by certain ferric or ferrous chelators.

13.3. Effect of potassium cyanide (KCN) and sodium azide ( $\text{NaN}_3$ ) on the growth of *S. pyogenes* Gr A

The effect of KCN and  $\text{NaN}_3$  was tested on a number of isolates of group A streptococci, *S. milleri*, *E. coli* MW, and *Staph. aureus*. Generally, KCN, a respiratory inhibitor, binds to and inhibits both the oxidized and reduced forms of cytochrome oxidase of the  $\text{aa}_3$ -type (Smith, 1954, Ritchy *et al.*, 1976). Azide ( $\text{N}_3^-$ ) acts as a respiratory inhibitor by combining with, and preventing the reduction of oxidized cytochrome oxidases of the  $\text{aa}_3$ -type. The data presented show that *S. pyogenes* was resistant to the effect of both these respiratory inhibitors (KCN and  $\text{NaN}_3$ ) when added 2 h after initiation of growth. Strains of *S. pyogenes* showed no inhibition of growth by 50 mM KCN ; however growth inhibition did occur at 100 mM final concentration of KCN and  $\text{NaN}_3$  in both aerobic (shaken or unshaken) and anaerobic conditions. This probably reflects a non-specific effect

unrelated to cyt A/A<sub>3</sub>. Hence it is not surprising that the streptococci, which are generally catalase negative and lacking in cytochromes, are amongst the most resistant organisms. Streptococci and other lactic bacteria are conventionally considered to be facultative anaerobes with a preference for anaerobic conditions but it was observed that both *S. pyogenes* and *S. milleri* grew very well in both aerobic and anaerobic culture. They are unique amongst bacteria able to grow aerobically in that they are not able to synthesize porphyrins - and therefore cytochromes and catalase - and are not, apparently, capable of forming ATP via the electron transport chain. Aerobically they are presumed to carry out substrate level ATP synthesis via the fermentation mechanisms they use when growing anaerobically (Whittenbury, 1978). The univalent reduction of oxygen to the superoxide radical is a commonplace event in biological systems, and the superoxide dismutases (SODs), which catalytically scavenge this radical, appear to function as a primary defence against its potential cytotoxicity (Britton *et al.*, 1978). There appear to be manganese enzymes and iron enzymes in both gram-positive and gram-negative bacteria. According to Britton *et al.*, (1978) *S. pyogenes* gr A, and *S. faecalis* lack an FeSOD but contain only MnSOD. This observation supports our finding that *S. pyogenes* gr A might not require iron and lacks a cytochrome system. In contrast, growth of *E. coli* MW and *Staph. aureus* was markedly inhibited at a concentration of 1 mM and 10 mM of KCN and NaN<sub>3</sub> and completely inhibited at a concentration of 50 mM. These results correspond with those reported by

Whittenbury, (1978), Britton *et al.*, (1978), and Archibald and Fridovich, (1981).

13.4. Effect of KCN or  $\text{NaN}_3$  plus iron chelator EDDA on the growth of *S. pyogenes*

*S. pyogenes* grew profusely in the presence of high concentration of Fe chelators such as EDDA, Desferal,  $\alpha, \alpha$ -dipyridyl in an aerobic environment. In order to show that such growth was independent of both Fe and  $\text{O}_2$  as a terminal electron acceptor, KCN and  $\text{NaN}_3$  were added to the growth medium at levels which completely inhibited growth of aerobic bacteria. These agents have no effect on the growth of *S. pyogenes* at concentration below 100 mM, proving the the growth was independent of cyt  $\text{aa}_3$  oxidoreductase and  $\text{O}_2$ .

13.5. Iron and SLS production in *S. pyogenes* Gr A

Griffths and McClain (1988) reported that Fe-limitation stimulated SLS production in *S. pyogenes*. Reports of toxin synthesis being derepressed by Fe have been made for diphtheria toxin in *Corynebacterium diphtheriae*; also Fe is known to affect the levels of Shiga toxin production in *Shigella dysenteriae*. All the data on growth and SLS production in C203S suggest that SLS is not regulated by iron and nor there any evidence for a dependence of SLS production on iron. Addition of EDDA at a 20 fold molar excess over Fe in BHI-BM did not restrict the growth of C203S and the level of haemolysin (SLS) production was not affected. This finding is in contrast with those of Griffths and McClain (1988), who claimed that growth of *S. pyogenes* was stimulated by iron and that low iron-concentration (1.2  $\mu\text{g/ml}$ ) in medium was conducive to high haemolysin production, while an increase of iron upto 5.0  $\mu\text{g/ml}$  offered no correlation.

13.6. The influence of purified SLS on the chemiluminescence response of rabbit peritoneal neutrophils to FMLP

Various strategies have evolved in microorganisms and their products to interfere with phagocytic functions (Mims, 1982). The most straightforward antiphagocytic action is to kill the phagocytic cells. Another mechanism is the inhibition of chemotaxis or the mobilization of phagocytic cells. A number of cytolytic toxins elicit, at lytic doses, the disruption of cytoplasmic membrane and that surrounding the intracellular organelles of phagocytic cells, thereby provoking the explosion of vesicles containing autolytic enzymes (Arbuthnott, 1982).

Earlier investigators have demonstrated that most group A streptococci possess cell-bound haemolysin (CBH), in the form of SLS which is capable of haemolysing red blood cells and causing cytopathic changes in mouse peritoneal leucocytes (Elias *et al.*, 1966, Hryniewicz and Pryjma 1977). According to Ofek<sup>*et al.*</sup> (1970), the haemolytic and leukotoxic activity of streptococci is abolished by the neutralization of CBH activity with trypan blue. Leukocytes that engulfed streptococci (40% phagocytosis) lacking CBH activity (heat killed or trypan blue treated) did not undergo cytopathic changes. On the other hand, under similar conditions 100% of the leukocytes died following exposure to CBH-containing streptococci.

Chemiluminescence is an indirect measure of the activity of bacterial toxins on phagocytes. McCall *et al.*, (1979) reported that the responses induced by FMLP in neutrophils are similar to those induced during an acute bacterial infection. SLS inhibited the chemiluminescence response of peritoneal neutrophils in response to the pro-inflammatory mediator FMLP. This is the first report of the effect

of purified SLS on neutrophil response to pro-inflammatory mediators. The results suggest that SLS inhibited the CL response in a dose-dependent manner. However, it proved difficult to count the dead / live neutrophils in response to FMLP because of a pronounced cell aggregation response and shape change, although one could count the dead and live cells without FMLP.

Little is known about the exact mechanism by which this toxin kills the neutrophils. The lytic effects of SLS are thought to be due to its direct disruptive actions on cell membranes, and the crucial role of membrane phospholipid as the binding site and probably the target of SLS has been suggested in a variety of experiments (Elias *et al*, 1966, Bernheimer, 1972).

Although cell death by lysis can be induced by sufficiently high doses of toxin, there may be significant changes in cell function with sublytic concentrations of toxin. Alouf (1986) stated that "At sublytic doses, certain cytolytic toxins impair the chemotactic response of phagocytes which may be of significance in the pathogenesis of bacterial infections". This impairment was reported to be due to "moderate modification" of the phagocyte membrane. In most cases, the membrane modification of the oxidative metabolism of neutrophils is activated and results in an intense burst of chemiluminescence as shown, for example, in the case of treatment of PMNs with SLO (Anderson and Duncan, 1980) or with *E. coli* haemolysin (Cavaliere, *et al.*, 1984). The chemotactic response of macrophages was not investigated here but would be of interest in future studies.

### 13.7. Effect of SLS on opsonophagocytosis

The effects of SLS on the complement system and opsonization

have been largely unexplored. Complement proteins interact in a precise sequence of reactions leading to the production of biologically active cleavage fragments capable of interacting with microorganisms promoting opsonization on the one hand, and cell damage on the other. An important consideration is the relevance of the *in vitro* effects to what happens *in vivo*.

This is the first report where the effect of SLS on opsonization of zymosan has been examined. The result, blockage of opsonization and decreased uptake of opsonized zymosan, was unpredicted. It was interesting to find both washed and unwashed opsonized zymosan particles behaving in a similar fashion suggesting that washing had little effect in removing SLS from the SLS+serum+zymosan complex. Furthermore, no haemolytic activity was observed when supernates of these washings were examined. Blockage of opsonization of zymosan could be explained in the following ways :

(i) Phagocytosis occurred more slowly in heat-inactivated (no complement activity) than in MgEGTA-chelated samples (alternate pathway only remaining active) suggesting that, although MgEGTA blocked the classical pathway, it did not completely block the opsonisation of zymosan. Thus opsonization was due partly to the classical and partly to the alternate pathway. Although it was not directly proved that SLS could inactivate both classical and alternate pathways, it is assumed that SLS may have an inhibitory effect on a step common to both pathways. More extensive work in this area will be worthwhile.

(ii) More evidence of inhibition of opsonization and resistance to phagocytosis came from the effects of SLS on bound opsonin (serum),

where zymosan particles were preopsonized with serum and then exposed to SLS. After washing the complex, zymosan particles behaved in a similar fashion to serum + SLS + zymosan complex (i.e., decreased uptake of zymosan by phagocytes). This suggested that SLS may cause C3b to detach from zymosan particles, or that SLS inactivated the C3b deposited on the zymosan surface, for example by binding to C3b to block the receptor site recognized by PMNs. Alternatively, SLS may bind to C3b or other complement proteins which may act as carriers subsequently releasing SLS to kill PMNs. It is well established that haemolytic activity can be transferred from one chemically unrelated carrier (inducer) to another (Duncan and Masson, 1976). These inducers are serum albumin, RNA, ribonuclease-resistant core RNA, trypan blue,  $\alpha$ -lipoprotein and some non-ionic detergents such as Tween 40, 60, 80, and triton X-205 (Wannakmer, 1983).

The finding regarding interaction with serum and opsonophagocytosis clearly suggests that SLS needs serum in order to bind to zymosan. Once bound to zymosan (serum-treated), SLS does not elute from the zymosan on washing. PMNs see opsonized zymosan treated with SLS like unopsonized zymosan. PMNs appear to be either unresponsive because they are dead or because they are paralysed in terms of their CL response to opsonized zymosan.

### 13.8. Possible synergism between M-protein and SLS

Group A streptococci can persist in tissues for weeks, primarily because of the M-protein on their outer surface (Fischetti, 1991). Studies by Phillips *et al.*, (1981) indicated that purified M-protein released from the streptococcal cell wall with phage lysin was similar to the size of the native cell-wall-bound M-molecule. M-

proteins extracted with pepsin are almost half the size of the lysin-extracted molecule and are likely to be the enzymically cleaved products derived from the native M-molecule. The M-protein used in this study was purified pepsin-extracted fragment of type 24 streptococci. The data presented in this thesis did not show synergism between SLS and M-protein and it proved difficult to assess the influence of the mixture of M-protein and SLS on serum components and on opsonization.

### 13.9. Brief evaluation of the role of SLS in the pathogenicity of group A streptococci

This thesis presents details of study on the possible roles of iron and SLS in pathogenicity of group A streptococci which may help in the understanding of the disease process in infections caused by these organisms. The multiplicity of factors and their contributions to the pathogenicity of the organisms are summarized in Fig. 4. Such a comprehensive armoury endows the organism with great powers of adaptability.

The present study clearly shows that this potential pathogen can proliferate *in vitro* under iron-depleted conditions and has no need for iron or production of siderophores. It seems that *S. pyogenes* group A lacks Fe-containing enzymes and probably the Fe is replaced by Mn in case of SOD as reported by Britton *et al.*, (1978). Other metals may act as prosthetic groups in essential metalloenzymes but this aspect of streptococcal metabolism warrants further study. Studies of Francis *et al.*, (1985) found no requirement or transport system for Fe in *S. pyogenes* group A. Hence, these pathogenic bacteria may establish infections in mammalian hosts irrespective of levels of free iron,

which is known to be a limiting factor in the virulence of the pathogenic bacteria. Also, the ability to thrive either in oxygen rich or oxygen free conditions adds further to the versatility of this organism.

Although considerable work has been done on iron and its acquisition by invading bacteria, little is known about the requirement and acquisition of other metals by bacteria. It is well accepted that iron plays an essential role in some microbial enzymes, yet insufficient data is available on the role of other metals. The findings of this thesis regarding *S. pyogenes* draw attention to the need to study the role of other metals in microbes where iron is either replaced by other metals or is not an absolute requirement.

SLS is apparently not immunogenic. This property may result from its action on lymphocytes, from the small size of the active polypeptide moiety or its affinity for phospholipids of cell membranes. It may bind rapidly to cells and not be available to stimulate immune response. So, the lack of immunogenicity may enable its free circulation attached to carrier serum proteins and thus cause disturbance of cells even in remote organs because of the apparent ease with which non-specific carriers can exchange SLS. Since no neutralizing antibody is found after immunisation or in convalescent sera, the effect of toxin may be unimpaired even after repeated streptococcal infections ; such a sequence may occur in the pathogenesis of rheumatic fever.

In this thesis a detailed analysis of SLS on both SDS and native-PAGE was done and SLS was localized on a zymogram. This will facilitate excision of the relevant protein band, which can then be

used for immunogenic studies by injecting the emulsified gel band with a suitable carrier either adsorbed or the toxin covalently linked as a hapten. It is interesting to note that staphylococcal delta lysin, a small lytic peptide, is more-immunogenic when toxoided than when in the native form. Further studies on immunogenicity and the structure of SLS would aid the understanding of the relationship between SLS and various unrelated carriers. Also, further studies on the interaction of SLS with serum and in opsonophagocytosis will further help in understanding of the complexity and role of SLS in streptococcal pathogenicity.

Only *in vitro* studies were carried out in this thesis and the role of SLS in infections is difficult to assess without good animal models. Two major trends on experimental streptococcal infections are evident in the literature.

- 1) studies of the dynamics of infection in laboratory animals
- 2) studies devoted to the elucidation of the pathogenic mechanisms that lead to the development of tissue damage. This is principally in relation to the pathogenesis of post-streptococcal sequelae in humans. The significance of SLS production by groups A, C, and G streptococci in the pathogenesis of streptococcal disease is not understood. Streptococcal strains, the virulence of which has been increased by passage in animals, did not produce greater amounts of haemolysins. In fact, decreased production of SLS has been reported (Leedom and Barkulis, 1959). The toxic manifestations in mice injected with serum haemolysin have been studied by Weld (1934). Animals surviving for 90 min after injection developed haematuria, and their livers and spleens became enlarged. Rabbits injected i. v. with sublethal doses of RNA-

haemolysin experienced mild proliferative glomerulonephritis. Rabbits injected i. v. with lethal doses of RNA-haemolysin (approximately 6,000 haemolytic units/kg body weight) died within 45-60 min with severe intravascular haemolysis. SLS injected into the knee joint of rabbits regularly elicited chronic arthritis (Cook and Fincham, 1966). Thus modern studies of the *in vivo* effects of streptococci are lacking. However, it has been reported (Duncan, 1983) that SLS is produced during *in vivo* (mice) growth of the streptococci, and it will be worthwhile to study the role of SLS in pathogenesis by using SLS<sup>-</sup> and SLS<sup>+</sup> strains.

Naturally occurring SLS<sup>-</sup> strains of *S. pyogenes* are seldom seen, therefore, SLS is generally considered a conserved trait. The incidence of SLS<sup>-</sup> strains in clinical specimens is unknown because SLS is primarily responsible for beta-haemolysis on blood agar plates, and non-haemolytic colonies are ignored by clinical laboratory workers (James and McFarland, 1971). The role of SLS in clinical infections or in the pathogenesis of group A streptococci has been questioned because SLS<sup>-</sup> and SLS<sup>+</sup> strains (assayed *in vivo*, however) have been isolated from incidents of epidemic pharyngitis, rheumatic fever and other infections. However, toxicity of SLS to erythrocytes, leucocytes and smooth muscle has qualified this extracellular product as an accessory virulence factor (Bernheimer, 1954). The use of transposon Tn916 from *S. faecalis* to inactivate the genes required for the production of SLS has been reported by Nida and Cleary (1983). The same study reported that upon excision of the transposon, SLS was once again expressed. Such mutants will be invaluable in investigating of

the role of SLS in infection and in molecular analysis of this toxin, which is apparently simple, yet very difficult to study .

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## APPENDICES

15.1. Media and DiluentsMediaA. Brain Heart Infusion broth (BHI, BM) g/l

	<u>OXOID</u>	<u>DIFCO</u>
Calf Brain Infusion Salts	12.5g	20. g
Beef Heart infusion Solids	5.0g	25. g
Protease peptone	10.0g	10g
Sodium Chloride	5.0g	5g
Dextrose	2.0g	2g
Disodium phosphate	2.5g	2.5. g

Either of the medium preparations was supplemented with 1% (w/v) maltose and 2% (w/v) sodium bicarbonate .

B. Chemical Defined Medium (C. D. M. )

A modification of the original C. D. M (1980) composition was used .

<u>Chemical</u>	<u>Concentration (mg/L)</u>
1. FeSO <sub>4</sub> . 7H <sub>2</sub> O	5
K <sub>2</sub> HPO <sub>4</sub>	200
KH <sub>2</sub> PO <sub>4</sub>	1,000
MgSO <sub>4</sub> . 7H <sub>2</sub> O	700
2. Casamino acids ( see below)	
3. p. Aminobenzoic acid	0.2
Biotin	0.2
Folic acid	0.8
Niacinamide	1.0





(ii) Lower Buffer (4X concentration) pH 8.9

Tris	18.1g
SDS	0.4g
Distilled water	70 ml

The pH was adjusted to 8.9 with concentrated HCl and the final volume made up to 100ml with distilled water .

(iii) Upper buffer (4X concentration ) pH 6.8

Tris	6.06g
SDS	0.4g
Distilled water	70 ml

The pH was adjusted to 6.8 with concentrated HCl and the final volume made up to 100ml with distilled water .

(iv) Temed (undiluted stock)(v) Ammonium Persulphate Solution

A 10% solution was made up freshly (50mg in 0.5 ml distilled water ).

(vi) Solubilising Buffer for proteins

Glycerol	10 ml
2-mercaptoethanol	5 ml
SDS	3 g
Bromophenol Blue	0.01g
Upper Buffer ( 1 in 8 dilution of (iii) to 100 ml	

(vi) Running Buffer pH 8.3

Tris	3.03
Glycine	14.4
SDS	1.0g
Distilled water	1000 ml

The pH was adjusted to 8.3 with concentrated HCl .

(vii) Staining Solution

Coomasie Blue R250	1.25g
50% (v/v) methanol	454 ml
Glacial acetic acid	46 ml

(ix) Destaining Solution

Methanol	50 ml
Glacial Acetic Acid	75 ml
Distilled water	875 ml

B. Slab-Gel Preparations(i) Lower Gel-Separating Gel

	12.5% (1.5mm gel)	15% (0.8mm gel)
Lower Buffer (4X)	10 ml	5 ml
Distilled water	13.4 ml	5 ml
Acrylamide/Bis	16.6 ml	10 ml

After degassing for 20 minutes the following were added:

Ammonium Persulphate solution	200 $\mu$ l	200 $\mu$ l
Temed	20 $\mu$ l	20 $\mu$ l

(i) Upper Gel-Stacking Gel (4.5%)

Upper Gel Buffer (4X)	2.5 ml
Distilled water	6ml
Acrylamide/Bis	1.5 ml

After degassing for 10 minutes the following were added:

Ammonium persulphate solution	30 $\mu$ l
Temed	20 $\mu$ l

15.3. Buffers and Diluents(i) Induction Buffer (IB) pH. 7.0

$\text{KH}_2\text{PO}_4$	100 mM
$\text{MgSO}_4$	2 mM

The final volume of 40ml was adjusted to pH 7.0 with NaOH and then was supplemented with 30 mM maltose.

(ii) Blood Washing Buffer pH 6.8

$\text{NaH}_2\text{PO}_4$	150 mM
$\text{Na}_2\text{HPO}_4$	150 mM
NaCl	150 mM

Mix equal volumes of sodium phosphate buffer + NaCl .

pH was adjusted to 6.8 and was kept at 4°C .

(iii) Hepes Buffered Saline (HBS)

<u>Chemical</u>	<u>Concentration(g/l)</u>
NaCl	8.00g
KCl	0.40g
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.20g
$\text{CaCl}_2$	0.14g
Glucose	1.00g

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HEPES	2.388g
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Ingredients were dissolved in 900 ml of distilled, deionised water and the pH adjusted to 7.4 with 1 NaOH. The volume was then made up to 1 litre with distilled, deionised water and the preparation autoclaved at 121°C for 15 minutes at 15 p.s.i. The divalent cation-free HBS-EDTA solution was prepared by the omission of the Ca- and Mg-containing salts and the addition of 0.292 g of monosodium EDTA.

(iv) Phosphate-buffered saline (Dulbecco A)

<u>Chemical</u>	<u>Concentration (g L<sup>-1</sup>)</u>
NaCl	8.00g
KCl	0.20g
NaH <sub>2</sub> PO <sub>4</sub>	0.20g
Na <sub>2</sub> HPO <sub>4</sub>	1.15g

Phosphate-buffered saline tablets (Oxoid) were used. Each tablet was added to 100 ml of distilled water and the solution autoclaved at 121°C for 15 min at p.s.i. The pH of the subsequent solution was 7.3.

(v) Buffer for protein immunoblotting

Transfer buffer

Tris	3.03g
glycine	14.4g
20% (v/v) methanol to	1000ml

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(v) MgEGTA The preparation of 100 mM stock saline solution of ethylene glycol tetraacetic (EGTA) was supplemented with with an equimolar concentration of  $MgCl_2$  (MgEGTA) . In order to dissolve EGTA in saline , it is necessary to heat the mixture to  $60^{\circ}C$  . add 5M NaOH dropwise until the EGTA goes into solution , titrate back to pH 7.45 with 1 N HCl , and then bring to final volume with normal saline . The stock solution was stored at  $4^{\circ}C$  ; 0.1 ml of 100 mM solution was added to 1ml of serum to yield a final concentration of approximately 10 mM. (Fine *et al.*, 1972) .

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