STUDIES ON THE DELTA HAELOLYSIN
OF STAPHYLOCOCCUS AUREUS

BY

GILLIAN MARGARET SMITH B.Sc.

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at the University of Leicester.

University of Leicester,
Department of Biochemistry,
Adrian Building,
University Road,
Leicester LE1 7RH.

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<tr>
<td>A</td>
<td>Absorbance, A$_{280}$ = absorbance at 280 nm.</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Carboxy-terminus</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNAase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>ε</td>
<td>Extinction coefficient</td>
</tr>
<tr>
<td>HU</td>
<td>Haemolytic units</td>
</tr>
<tr>
<td>ITC glass</td>
<td>Isothiocyanate glass</td>
</tr>
<tr>
<td>MCD peptide</td>
<td>Mast-cell degranulating peptide</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>M.W.</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N-terminus</td>
<td>Amino-terminus</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>PTH amino acids</td>
<td>Phenylthiohydantoin amino acids</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylene diamine</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TSB medium</td>
<td>Trypticase soy broth medium</td>
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ABSTRACT

The production of haemolysins by several strains of *Staphylococcus aureus* was investigated and strain RN25 chosen for further study.

Three methods for the purification of delta haemolysin were compared for efficiency and purity of the products. The products were characterised using polyacrylamide gel electrophoresis, amino acid analysis and N-terminal sequence analysis. The method of Heatley (1971 and 1976) was found to be the most efficient and yielded the product of highest purity and specific activity. From polyacrylamide gel electrophoresis in the presence of SDS the molecular weight of delta haemolysin was estimated to be less than 5,000. N-terminal sequence analysis of the products of the purification procedures led to the observation that the preparations of delta haemolysin contained a proportion of N-terminally blocked protein.

Thin layer chromatography of the products revealed the three preparations of delta haemolysin to be heterogeneous. The toxin was further purified using hydrophobic affinity chromatography.

The molecular weight of native delta haemolysin was estimated from gel filtration to be 150,000.

The effect of chemical modification of delta haemolysin on its haemolytic activity indicated that the N-terminus of the toxin is involved in its mechanism of action and that the amino groups of the molecule are very important for its activity. The results were closely comparable to those observed for melittin of bee venom.

Inhibitors of extracellular protein production, procaine and cerulenin, were found to inhibit the production of delta haemolysin and total extracellular protein. Using cerulenin and harvesting cultures of strain RN25 in the mid-exponential phase of growth, the possibility of the existence of a precursor of delta haemolysin was investigated using techniques of immunoprecipitation.
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References
INTRODUCTION

1.1. The study of bacterial toxins

Many bacteria produce a multitude of extracellular products, including toxins and enzymes, which have a wide range of biological activities. Studies on the modes of action of these products and their possible interactions may lead to a better understanding of their roles in microbial pathogenicity.

The study of bacterial toxins has also been greatly stimulated by their utility as tools in the investigation of other biochemical systems, for example the use of cholera toxin as a probe for cellular functions regulated by cyclic AMP and the use of bacterial phospholipases and neuraminidases in the study of membrane structure and function. The different specificities of bacterial phospholipases, e.g. *Staphylococcus aureus* beta toxin (sphingomyelinase C) and *Clostridium perfringens* alpha toxin (phospholipase C) have been exploited in studies on the lipid composition of various membranes. Such studies revealed the asymmetric arrangement of phospholipids in the erythrocyte membrane.

1.2. Staphylococcal toxins

Staphylococci were first identified as the causative agents of acute abscesses and septicaemia in 1880 by Sir Alexander Ogston (cited by McCartney and Arbuthnott, 1978). De Christmas and von Leber independently showed eight years later that the culture filtrates of staphylococci contained a toxic principle which caused inflammatory reactions when injected into experimental animals (cited by McCartney and Arbuthnott, 1978). *Staphylococcus aureus* produces a variety of extracellular products (Table 1.1.), a single strain producing as many as 25 - 30 extracellular proteins (Bernheimer and Schwartz, 1961).
Table 1.1. Extracellular toxins and enzymes of *Staphylococcus aureus*

<table>
<thead>
<tr>
<th>Enzyme or toxin</th>
<th>Molecular weight</th>
</tr>
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<tbody>
<tr>
<td>Amidase</td>
<td>30 000</td>
</tr>
<tr>
<td>Coagulase</td>
<td>18 000</td>
</tr>
<tr>
<td>Deoxyribonuclease</td>
<td>17 000</td>
</tr>
<tr>
<td>Endo-β-N-acetylglucosaminidase</td>
<td>70 000</td>
</tr>
<tr>
<td>Enterotoxin B</td>
<td>30 000</td>
</tr>
<tr>
<td>Enterotoxin C₁</td>
<td>34 000</td>
</tr>
<tr>
<td>Epidermolytic toxin</td>
<td>22 000 - 33 000</td>
</tr>
<tr>
<td>Esterase</td>
<td>-</td>
</tr>
<tr>
<td>Alpha haemolysin</td>
<td>36 000</td>
</tr>
<tr>
<td>Beta haemolysin</td>
<td>33 000</td>
</tr>
<tr>
<td>Gamma haemolysin</td>
<td>26 000 - 29 000</td>
</tr>
<tr>
<td>Delta haemolysin</td>
<td>35 000</td>
</tr>
<tr>
<td>Hyaluronate lyase</td>
<td>82 000</td>
</tr>
<tr>
<td>Leucocidin</td>
<td>32 000</td>
</tr>
<tr>
<td>Lipase</td>
<td>100 000</td>
</tr>
<tr>
<td>Lymphocyte mitogen</td>
<td>21 000</td>
</tr>
<tr>
<td>Lymphocyte mitogen</td>
<td>14 000</td>
</tr>
<tr>
<td>Peptidase</td>
<td>35 000</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>58 000</td>
</tr>
<tr>
<td>Protease I</td>
<td>21 000</td>
</tr>
<tr>
<td>Protease II</td>
<td>12 000</td>
</tr>
<tr>
<td>Protease III</td>
<td>28 000</td>
</tr>
<tr>
<td>Staphylokinase</td>
<td>13 000 - 15 000</td>
</tr>
</tbody>
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*After Wadström, T. in "Bacterial toxins and cell membranes" (J. Jeljaszewicz and T. Wadström, eds.) (1978).*
The study of staphylococcal toxins is of considerable medical importance since, in spite of the availability of a number of useful anti-staphylococcal antibiotics, *S. aureus* is still an important cause of infection in man and animals and can cause disease in nearly every organ of the body.

Several of the staphylococcal toxins have been shown to exert their toxic action through interaction with the membranes of susceptible cells. As discussed in Section 1.1, the toxins are therefore of interest to workers in membrane research for use as tools in the study of membrane structure and function.

Staphylococcal toxins include 4 haemolysins; so named because they were found to be haemolytic for erythrocytes of many species and haemolytic potency has been used extensively as a measure of their biological activity. In fact, since the haemolysins can cause lysis of many cell types, Bernheimer (1968) coined the term "cytolytic" to describe such bacterial haemolysins. However Thelestam and Köllby (1975 a and b) have shown that at sub-lytic concentrations these toxins induce permeability changes in the membranes of susceptible cells. For this reason McCartney and Arbuthnott (1978) suggested the term "membrane-damaging" to describe these toxins.

1.2.1. Alpha haemolysin

Although alpha haemolysin has been the most studied of the staphylococcal membrane-damaging toxins, its mechanism of action at the molecular level is still unknown. Freer et al. (1968) showed that the monomeric (3S) form of alpha haemolysin polymerised on erythrocyte membranes and liposomes to form a hexameric (12S) polymer. Bernheimer et al. (1972) showed that the ring structures were formed on mammalian membranes but not on membranes of various bacterial species. Various lipids in aqueous dispersion have been shown to induce formation of the polymer (Bernheimer et al., 1972 and
Arbuthnott et al., 1973) and investigation of the surface activity of the toxin revealed that it penetrates lipid monolayers to differing degrees (Buckelew and Colacicco, 1971). Evidence from freeze-etching studies also suggests that the toxin penetrates the hydrophobic region of the membrane (Bernheimer et al., 1972). This evidence suggests that alpha haemolysin is surface-active and that its mode of action may involve penetration and disruption of the hydrophobic regions of the cell membrane by polymers of the toxin.

However, Cassidy et al. (1974), showed that sensitivity to lysis by alpha haemolysin of liposomes prepared from rabbit erythrocytes was similar to that of liposomes prepared from human erythrocytes. As rabbit erythrocytes are 100 times more sensitive to lysis by the toxin than human erythrocytes it seems unlikely that penetration of the membrane by the toxin is the sole basis for the mechanism of action of alpha haemolysin and that some protein receptor might be involved.

Work by Wiseman and Caird (1970 and 1972) and Wiseman et al. (1975) led them to postulate that alpha haemolysin is a proteolytic enzyme activated by a membrane-bound protease. These observations have not been confirmed by other workers, and Freer et al. (1973) failed to demonstrate protease activity for alpha haemolysin. This anomaly might be explained by the difficulties encountered in purification of bacterial toxins as discussed in Section 1.3.1.

Alpha haemolysin has been detected on the surface of rabbit erythrocytes during haemolysis using immunofluorescence techniques (Klainer et al., 1964). A decrease in fluorescence after maximal haemolysis was observed and this was thought to be due to release of alpha haemolysin. This would agree with earlier work presenting evidence for the recovery of alpha haemolysin after haemolysis (Lominski and Arbuthnott, 1962). Also Cassidy and Harshman (1973) have shown that $^{125}$I-labelled alpha haemolysin binds very rapidly
during the pre-lytic period with some release of the toxin occurring just before the onset of visible lysis. Wiseman and Caird (1972) found that the capacity of erythrocytes or erythrocyte ghosts of different species to bind alpha haemolysin correlated with their sensitivity to lysis. Such evidence suggests that specific receptors exist in the rabbit erythrocyte membrane which are present either in lower amounts or with different activities in the erythrocyte membranes of other species.

1.2.2. Beta haemolysin

The mechanism of action of beta haemolysin (sphingomyelinase C) is the best understood of all the staphylococcal haemolysins. It is an enzyme which attacks sphingomyelin in the target membrane resulting in hydrolysis of the molecule to yield N-acetylsphingosine and phosphorylcholine. The sensitivity of the erythrocytes of different species correlates with their sphingomyelin content.

Beta haemolysin is unique in its exhibition of the phenomenon of "hot-cold" lysis. Incubation of the toxin with sheep erythrocytes (the most sensitive to lysis by the toxin) at 37°C results in little or no lysis. However if the mixture is subsequently chilled below 10°C rapid lysis occurs. Bernheimer (1974) suggested that hydrolysis of sphingomyelin of the target membrane resulted in areas of the membrane existing as lipid monolayers which collapsed when the membrane was chilled. Smyth et al. (1975) suggested that the phenomenon of "hot-cold" lysis is a consequence of the temperature-dependence of divalent ion stabilisation of the membrane and showed that an effect resembling that caused by cooling to 4°C could be achieved by treatment with chelating agents.
1.2.3. Gamma haemolysin

Gamma haemolysin has been shown by Guyonnet et al. (1966) and Guyonnet and Plommet (1970) to consist of 2 protein components, which could be separated by hydroxylapatite chromatography and act synergistically, both components being necessary for haemolysis. Other workers using different procedures for purification of the haemolysin maintained that gamma haemolysin is a single protein. However the work of the French group was supported by Taylor and Bernheimer (1974) who also showed that gamma haemolysin consisted of 2 distinct cationic proteins with pI values of 9.8 and 9.9, which act synergistically and could be separated by hydroxylapatite chromatography but not by isoelectric focusing.

The mode of action of this toxin however is still not understood.

1.2.4. Delta haemolysin

Delta haemolysin was first discovered by Williams and Harper (1947) who noticed that various strains of *S. aureus* grown on sheep blood agar produced a haemolytic agent that was not neutralised by antitoxins to either alpha or beta haemolysins.

Delta haemolysin has several unique properties that distinguish it from the other staphylococcal haemolysins.

i) Delta haemolysin is relatively thermostable; it can withstand heating to 80°C for 1 h (Kreger et al., 1971), whereas alpha and beta haemolysins are heat labile (Rogolsky, 1979 and Wiseman, 1965).

ii) Hydrophobicity; the toxin contains a high proportion of hydrophobic amino acids (38%) and is soluble in a mixture of chloroform and methanol (2:1 by volume).

iii) The toxin has a low specific haemolytic activity compared with those of alpha and beta haemolysins; it has a low degree of species specificity and lyses many different cell types as well as protoplasts, spheroplasts and sub-cellular organelles, lysosomes.
and mitochondria.

iv) Delta haemolysin is inhibited by serum (Jackson and Little, 1958), the alpha and beta globulin fractions of serum (Gladstone and Yoshida, 1967), some fatty acids and phospholipids.

v) The toxin has been shown to be highly surface-active and its mode of action detergent-like.

vi) Delta haemolysin lacks the amino acids proline, arginine, tyrosine, cysteine and histidine.

The properties of delta haemolysin have been compared with those of surfactin of Bacillus subtilis and melittin, the major lytic component of bee venom.

Thelestam et al. (1973) have shown that, unlike alpha and beta haemolysins, delta haemolysin exhibits no lag period prior to causing changes in membrane permeability. It has an immediate effect on the cell membranes of human diploid lung fibroblasts demonstrated by the release of radioactively labelled nucleotides at concentrations of toxin that resulted in no microscopically visible cell damage. The leakage of radioactively labelled compounds induced by delta haemolysin was shown to be similar to that induced by the non-ionic detergent Triton X-100. However, in a more detailed study using intracellular radioactive labels of different molecular sizes, Thelestam and Müllby (1975b) went on to show that the lytic effects of Triton X-100 do differ from those of delta haemolysin and melittin in the apparent size of the lesions produced. Delta haemolysin and melittin resulted in small lesions initially, demonstrated by the preferential leakage of labelled nucleotides (M.W. less than 1000), and after prolonged incubation or incubation with higher concentrations of the toxin the release of labelled RNA (M.W. greater than 200 000) was observed. In contrast, treatment with Triton X-100 resulted in rapid release of both labelled substances indicating the formation of large lesions immediately.
1.3. The purification of delta haemolysin

1.3.1. The problems of purification of bacterial toxins

After it was realised that many bacteria produce several extracellular toxins and enzymes, workers in the 1930's and 1940's tried to purify these products in order to study them. They used highly toxic strains of bacteria and studied the optimisation of their growth and production of extracellular proteins. However the task of purifying a single product from the culture supernatants of these bacteria has proved difficult even with modern methods of protein separation.

The problems encountered are twofold. Firstly, in most cases the protein to be purified has to be separated from the other extracellular products of the bacteria most of which have the potential to interfere with biological studies on the "purified" protein even if the contaminating proteins are present only in small amounts. For example, preparations of staphylococcal alpha haemolysin have often been found to be contaminated with delta haemolysin (Bernheimer et al., 1968). Such contamination can lead to many misconceptions about the properties of a toxin or enzyme.

Secondly the problem of purification is further complicated by the complex heterogeneity of many bacterial products for example staphylococcal alpha haemolysin (Table 1.2.). This heterogeneity may be due to postsynthetic modification of the proteins, for example by proteases or glycosidases in the culture supernatant.

For these reasons earlier work on bacterial extracellular products must be interpreted with great caution as many of these investigations were performed with preparations which are now assumed to have been impure. However since the early 1970's the development of improved methods of purification and assessment of purity have made homogenous preparations feasible.
Table 1.2. The heterogeneity of staphylococcal alpha haemolysin

<table>
<thead>
<tr>
<th>Method of separation</th>
<th>Number of forms</th>
<th>Main component</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose gradient</td>
<td>4</td>
<td></td>
<td>Bernheimer and Schwartz (1963)</td>
</tr>
<tr>
<td>centrifugation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ion exchange</td>
<td>2</td>
<td>7.2</td>
<td>28 000</td>
</tr>
<tr>
<td>chromatography</td>
<td></td>
<td>8.4</td>
<td>28 000</td>
</tr>
<tr>
<td>Isoelectric focusing</td>
<td>4</td>
<td>8.6</td>
<td>41 000 Wadström (1968)</td>
</tr>
<tr>
<td>Isoelectric focusing</td>
<td>4</td>
<td>8.55</td>
<td>36 000 McNiven et al. (1972)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.98</td>
<td>51 000 Watanabe and Kato (1974)</td>
</tr>
<tr>
<td>Isoelectric focusing</td>
<td>4</td>
<td>8.6</td>
<td>Dalen (1975)</td>
</tr>
<tr>
<td>Isoelectric focusing</td>
<td>4</td>
<td></td>
<td>Dalen (1975)</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>4</td>
<td></td>
<td>Dalen (1975)</td>
</tr>
</tbody>
</table>

After Wadström, T. in "Bacterial toxins and cell membranes" (J. Jeljaszewicz and T. Wadström, eds.) (1978).
1.3.2. Comparison of methods of purification of delta haemolysin

Delta haemolysin was first purified by Yoshida (1963) and subsequently by Kreger et al. (1971), Heatley (1971) and Kantor et al. (1972). Table 1.3. lists some of the properties of delta haemolysin investigated by these workers and compares their findings.

Comparison of the haemolytic activity of the culture supernatants used and the products obtained after purification is difficult as assays for haemolytic activity varied in the different studies with regard to composition of diluent, pH, time of incubation, origin and concentration of erythrocytes and estimation of end-point. This is demonstrated by the different values for the haemolytic activity of the culture supernatant of the same strain (from the same source) reported by Kreger et al. (1971) and Kantor et al. (1972).

It can be seen from Table 1.3. that although the findings of these workers were in general agreement there were some discrepancies concerning the physical properties of purified delta haemolysin. For example the behaviour of delta haemolysin in ultracentrifugation, polyacrylamide gel electrophoresis and gel filtration. However Figure 1.1. illustrates that the amino acid analyses performed in the different laboratories were in fairly good agreement. The virtual lack of 5 amino acids, proline, cysteine, tyrosine, histidine and arginine reported by all of the authors is good evidence that the protein under study was the same in each case.

Part of this study was undertaken to compare these methods of purification, using culture supernatant from one strain of *S. aureus*, in order to determine whether the differences in the properties of delta haemolysin reported were due to differences in the strains of *S. aureus* used or due to the different methods of purification employed.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain used in the study</td>
<td>Poggie</td>
<td>W46M a mutant of Wood 46</td>
<td>186X derived from strain Newman</td>
</tr>
<tr>
<td>Haemolytic activity of the culture supernatant</td>
<td>30 - 60 HU.ml⁻¹</td>
<td>300 - 400 HU.ml⁻¹</td>
<td>40 - 50 HU.ml⁻¹</td>
</tr>
<tr>
<td>% recovery and specific activity of the product</td>
<td>15 - 16% 120 and 400 HU.mg⁻¹</td>
<td>20 - 30% 400 and 200 HU.mg⁻¹</td>
<td>250 - 300 HU.mg⁻¹</td>
</tr>
<tr>
<td>Sedimentation coefficient (M.W. 74 000)</td>
<td>Product was a mixture of 11.9S and 4.9S forms with a value of 1.95 in alkali and urea</td>
<td>4.9S</td>
<td></td>
</tr>
<tr>
<td>Polyacrylamide gel electrophoresis</td>
<td>2 bands at pH 4.3 and 2 bands at pH 9.5</td>
<td>At pH 4.6 + 6M urea and at pH 8.4 + 6M urea a diffuse main band with a faint 2nd band</td>
<td>A single broad band seen to enter the gel</td>
</tr>
<tr>
<td>Sucrose gradient centrifugation</td>
<td>1 broad peak</td>
<td></td>
<td>1 peak (M.W. 103 000)</td>
</tr>
</tbody>
</table>
Table 1.3 Continued

Gel filtration on Biogel A5M

Isoelectric focusing

Estimation of $<0.05\%$ phosphorus content

Estimation of carbohydrate content

Estimation of lipid content

Absorbance at 280 nm

UV absorption spectrum

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel filtration on Biogel A5M</td>
<td>2 peaks</td>
</tr>
<tr>
<td>Isoelectric focusing</td>
<td>2 peaks pI 9.5, 5.0</td>
</tr>
<tr>
<td>Estimation of phosphorus</td>
<td>1 - 2%, 0.25%</td>
</tr>
<tr>
<td>content</td>
<td></td>
</tr>
<tr>
<td>Estimation of carbohydrate</td>
<td>Less than 1%, 1.7%</td>
</tr>
<tr>
<td>content</td>
<td></td>
</tr>
<tr>
<td>Estimation of lipid content</td>
<td>0.5 - 1%, less than 1%</td>
</tr>
<tr>
<td>Absorbance at 280 nm</td>
<td>1 mg. ml$^{-1}$</td>
</tr>
<tr>
<td>gave OD$_{280}$ of 0.8</td>
<td>1 mg. ml$^{-1}$</td>
</tr>
<tr>
<td>UV absorption spectrum</td>
<td>distinct shoulder at 291 nm</td>
</tr>
<tr>
<td></td>
<td>3 peaks pI 4.65, 6.7, 9.0</td>
</tr>
<tr>
<td></td>
<td>1 peak in 0.1% Tween 80 pI 5.2</td>
</tr>
<tr>
<td></td>
<td>1 mg. ml$^{-1}$</td>
</tr>
<tr>
<td>gave OD$_{280}$ of 1.1</td>
<td>1 mg. ml$^{-1}$</td>
</tr>
<tr>
<td></td>
<td>gave OD$_{280}$ of 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1.1. Amino acid analyses of delta haemolysin

The levels of each amino acid are expressed as a percentage of the total amino acid content of each preparation.

- Delta haemolysin purified by Kreger et al. (1971).
- Delta haemolysin purified by Heatley (1971).
- Delta haemolysin purified by Kantor et al. (1972).
1.4. **Melittin from bee venom**

The mode of action of delta haemolysin has been observed to be comparable with that of melittin from bee venom (Thelestam and Mullby, 1975b) as described in Section 1.2.4.

Bee venom has 5 major constituents; the main component, melittin (making up roughly half the dry weight of bee venom), 2 enzymes, phospholipase A\textsubscript{2} and hyaluronidase, and 2 other peptides, apamine and mast-cell degranulating (MCD) peptide. Melittin is important in the action of bee venom in that, as well as MCD peptide, it causes histamine release. It also acts on cell membranes affecting their permeability and resulting in lysis or rendering the membranes vulnerable to the action of phospholipase A\textsubscript{2}, which also leads to lysis.

The amino acid sequence of melittin was elucidated over 10 years ago by Habermann and Jentsch (1967). The peptide was found to consist of 26 amino acids; a hydrophobic segment (residues 1 - 20) and a group of basic amino acids at the C-terminal end of the molecule (residues 21 - 26) as shown in Figure 1.2.

Much work has been done on the relation of the structure of melittin to its activity. Succinylation of the amino groups of melittin has been found to abolish its haemolytic activity with very little affect on the surface activity of the molecule (Habermann and Kowellek, 1970). It seems that the surface activity of the molecule is not sufficient alone for haemolysis. Acetylation of the amino groups of the molecule would also alter the asymmetric charge distribution supposed to confer the detergent-like properties of melittin with less steric interference than succinylation. This was also found to reduce the activity of melittin (Maulet et al., 1980).

Thus it appears that the basic C-terminus of the protein is essential for activity.

Peptides constituting different parts of the melittin molecule
Figure 1.2. The amino acid sequence of bee venom melittin

GLY-NH$_2$
ILE
GLY
ALA
VAL
LEU
LYS
VAL
LEU
THR
THR
GLY
LEU
PRO
ALA
LEU
ILE
SER
TRP
ILE
LYS
ARG
LYS
ARG
GLN
GLN-CONH$_2$

After Habermann and Jentsch (1967).
Figure 1.3. A model proposed for the orientation of melittin in the lipid bilayer.

After Dawson et al. (1978).
have been synthesised and assayed for activity. Peptides containing amino acids 1-8, 1-17, 7-17 and 14-17 were all found to be devoid of activity (Habermann, 1972) as were peptides containing residues 20-26 and 8-26 (Dawson et al., 1978). It seems, therefore, that both ends of the molecule are necessary for the full biological activity of the molecule.

The single tryptophan residue does not appear to be essential for the activity of the molecule as it can be destroyed photochemically without gross alteration of the surface activity or haemolytic potency the molecule (Habermann, 1972).

Secondary structure analysis of melittin by a modification of the method of Chou and Fasman (1974), (Dufton and Hider, 1977) led Dawson et al. (1978) to propose a model for the orientation of the melittin monomer when it interacts with the membrane surface (Figure 1.3.). They propose that the initial interaction between melittin and lipid membranes is probably electrostatic, the highly basic C-terminal end forming salt links with phosphate anions. In membranes residues 2-11 and 15-21 are predicted to be alpha-helical by secondary structure analysis resulting in the "hinged" molecule depicted in Figure 1.3. This is thought to produce a wedge effect resulting in disruption of the acyl chain region of the membrane and leading to increased ion permeability.

It was hoped that this study would include similar work on purified delta haemolysin in order to ascertain whether the modes of action of these 2 lytic peptides are also similar at the molecular level.

1.5. Tumour promoters, melittin and delta haemolysin

Tumour promoters constitute a class of compounds which, by themselves, are not carcinogenic, but greatly increase the incidence
of tumours when applied to animals that have previously been exposed to a low dose of a carcinogen. These compounds also enhance transformation in cell cultures previously treated with a chemical carcinogen. Elucidation of the molecular mechanism of action of tumour promoters might facilitate an understanding of the multistage process of carcinogenesis and aid in the design of screening systems for such agents.

The compound 12-O-tetradecanoyl-phorbol-13-acetate (TPA), which was isolated from croton oil (Hecker, 1967), has emerged as the most potent tumour promoter. In cell cultures TPA alone induces several phenotypic changes which resemble those seen in cells transformed by viruses or chemical carcinogens. These include altered cell morphology, increased saturation density, altered lipid metabolism, altered cell surface glycoproteins, enhanced membrane transport of 2-deoxyglucose and possibly other nutrients, induction of the enzymes plasminogen activator and ornithine decarboxylase, and induction of prostaglandin synthesis (Lee and Weinstein, 1978). TPA also inhibits terminal differentiation in cell cultures of diverse species.

The stimulation of prostaglandin biosynthesis by TPA in dog kidney cells was observed at nanomolar concentrations of the compound. The stimulation was found to result from increased deacylation of cellular phospholipids releasing arachidonic acid, a precursor of prostaglandin biosynthesis (Levine and Hassid, 1977a,b). Interestingly, melittin of bee venom has also been shown to result in increased susceptibility of phospholipid to hydrolysis by phospholipase (Mollay et al., 1976). The toxin has been found to stimulate phospholipase A₂ activity in a wide variety of cells in culture at nanomolar concentrations, resulting in the synthesis and release of prostaglandins (Shier, 1979). For this reason, melittin was tested to ascertain whether or not it could produce some of the other phenotypic
effects in cell culture that can be produced by phorbol ester tumour promoters such as TPA (Yufson et al., 1979). It was found that both melittin and TPA, at similar concentrations, inhibited differentiation in mouse melanoma cells and induced arachidonic acid release and prostaglandin synthesis in mouse fibroblasts.

The cell membrane is generally considered to be the target site for TPA as it causes alterations in several membrane-associated processes. Lee and Weinstein (1978) postulated that TPA might act by reacting with cell receptors in the membrane whose normal function is to mediate the action of a growth regulator or hormone. They suggested epidermal growth factor (EGF) as a possible candidate, as it shares a number of biological effects with TPA (Lee and Weinstein, 1978), including the stimulation of phospholipase activity and prostaglandin synthesis in dog kidney cells, at nanomolar concentrations of the hormone (Levine and Hassid, 1977b). TPA was found to be a potent inhibitor of the binding of EGF to its cell surface receptors in HeLa cells (Lee and Weinstein, 1979). The effect was found to be temperature sensitive, much greater at 37°C than at 4°C. It was suggested that TPA might induce changes in the lipid microenvironment of the EGF receptors, perhaps leading to clustering or altered conformation of the receptors. However, the inhibitory effect of TPA on the binding of EGF to its cell surface receptor sites was found to be preferential for the EGF receptor sites, for example the binding of insulin to its receptors in HeLa cells was not inhibited (Lee and Weinstein, 1979).

Delta haemolysin was tested in this system (Umezawa et al., 1980) and was also found to inhibit the binding of EGF to cell surface receptors in rat embryo and HeLa cells, at nanomolar concentrations of the toxin. At these concentrations the delta haemolysin did not result in cell lysis. The toxin was also found to induce arachidonic acid release from membrane phospholipids and increase prostaglandin
synthesis.

The effects of TPA and melittin have been studied in another system. Dicker and Rozengurt (1978) showed that the normally serum-dependent stimulation of DNA synthesis and cell proliferation by TPA could be demonstrated in the absence of serum with the addition of polypeptide hormones such as EGF, insulin or fibroblast-derived growth factor (PDGF). TPA was found to act synergistically with these factors, stimulating DNA synthesis and cell proliferation. The neurohypophyseal polypeptide vasopressin was shown to have similar mitogenic properties to those of TPA (Rozengurt et al., 1979). Also like TPA, vasopressin has been shown to inhibit the binding of EGF to its receptors in a temperature dependent fashion (Rozengurt et al., 1981). Dicker and Rozengurt (1979) also report the inhibition of binding of EGF to its receptors by TPA, but suggest that it binds to a site other than the EGF receptor. They also suggest that it is unlikely that TPA exerts its effects via a pathway involving the EGF receptor because of the synergistic stimulation of DNA synthesis observed with EGF and TPA. Also the involvement of the EGF receptors could not be the only mechanism by which TPA acts on cells, as it also affects cells which do not possess EGF receptors (Lee and Weinstein, 1978).

Dicker and Rozengurt (1979) also report that vasopressin and TPA do not act synergistically or additively to stimulate DNA synthesis and suggest that they may act via a common pathway (Dicker and Rozengurt, 1980). Both TPA and vasopressin have been shown to stimulate ion-flux (Na-K pump activity) in quiescent cells, a phenomenon thought to be an early event in the initiation of cell proliferation (Dicker and Rozengurt, 1979). Melittin has also been shown to stimulate monovalent ion-fluxes (Na-K pump activity) at subtoxic concentrations. The toxin also stimulates DNA synthesis in quiescent cells, acting synergistically with insulin, EGF and PDGF (Rozengurt et al., 1981).
It has also been demonstrated that melittin does not act synergistically with TPA or vasopressin and suggested that these three chemically diverse compounds may modulate cell function through a closely related pathway.

It can be seen from this work that the tumour promoter TPA, vasopressin, melittin and delta haemolysin share several effects on cells in culture suggesting they may share a similar mechanism of action. Further studies on the modes of action of these compounds may reveal the molecular mechanisms involved.

1.6. The biosynthesis and secretion of delta haemolysin

In the course of this study, delta haemolysin was found to be a small protein with a molecular weight of approximately 4,000. Attempts to sequence the N-terminal end of delta haemolysin prepared by the method of Kreger et al. (1971) were initially unsuccessful. This was thought to be due to the existence of a blocked N-terminus in the delta haemolysin. Subsequently Pitton et al. (1980) reported that delta haemolysin purified by the method of Heatley (1971 and 1976) contained two forms of delta haemolysin, detected by mass spectrometry, one containing an N-formyl methionine block at the N-terminus and the other unblocked. They reported that the blocked form represented 80% of the purified delta haemolysin.

The small size of delta haemolysin and the existence of the N-formyl methionine-containing species suggested that it might not be a total gene product but the N-terminal region of a larger gene product. The problem of secretion of this small, hydrophobic molecule supports the existence of a larger precursor. It was suggested that delta haemolysin might act as a signal sequence for a larger protein, itself being secreted after cleavage.

Much work has been done on the mechanism of secretion of proteins
across membranes. The signal hypothesis was first put forward by Blobel and Dobberstein (1975). Many secreted proteins, both eukaryotic and prokaryotic have been found to have N-terminal extensions of their sequences, present only transiently during their synthesis. These have been found either from in vitro translation studies with mRNA, or from DNA sequences, and are called signal sequences. Over 30 eukaryotic signal sequences and 7 bacterial sequences have been elucidated. They are generally 15 - 29 residues in length, have no sequence homology, but have similar secondary structure. Figure 1.4.a illustrates a model of the characteristics of these sequences. There is usually a positively charged N-terminal region followed by a region of 9 - 25 consecutively uncharged residues, many of them hydrophobic, with a region of high probability of beta turns before the cleavage site. The residue before the cleavage site always has a short side chain, GLY, ALA, SER, CYS or THR (Inouye and Halegoua, 1980).

The signal hypothesis of Blobel and Dobberstein (1975) proposed the binding of the signal sequence to a receptor protein in the membrane, formation of a proteinaceous tunnel through the membrane, elongation-mediated translocation of the nascent polypeptide through the tunnel and proteolytic removal of the signal sequence leaving the protein on the other side of the membrane. For membrane proteins it was proposed that a second hydrophobic sequence acted as a stop translocation signal leaving the protein sitting across the membrane (Blobel, 1980). However, there is little evidence for the existence of protein receptors in the membrane.

DiRienzo et al. (1978) proposed that the signal sequence inserts directly into the membrane. They divided the signal sequence into 4 regions as shown in Figure 1.4.b. They proposed that the positively charged N-terminus (S1) could attach to the negatively charged phospholipid head groups of the lipid bilayer bringing the protein
Figure 1.4.a General characteristics of signal sequences

The last residue before the cleavage site always has a short side chain (Inouye and Halexoua, 1980).

Figure 1.4.b A model of a signal peptide

After DiRienzo et al. (1978).
and its ribosome to the membrane. The hydrophobic core (I1) could then
draw the growing polypeptide chain into the lipid bilayer and the
second hydrophilic region (S2) would anchor the signal peptide across
the membrane, by interacting with the outer phospholipid head groups.
As in the signal hypothesis, they propose elongation-mediated
translocation of the polypeptide chain through the membrane and
cleavage of the signal peptide by a proteolytic enzyme.

There have followed several models for cotranslational
translocation of proteins based on the principles described above,
for example the loop model of Inouye and Halegoua (1980).

Von Heijne and Blomberg (1979) and von Heijne (1980) have put
forward the direct transfer model, which discusses the transfer of a
nascent chain through the lipophilic core of a membrane from a
physicochemical point of view. They considered the free energies
involved in the transfer of amino acid residues from an aqueous phase
into a non-polar phase. The direct transfer mechanism, illustrated
in Figure 1.5.a, depends on two assumptions; that the hydrophobic
signal sequence will bind in an alpha helical conformation to the
lipophilic core of any membrane, and that the subsequent binding of
the ribosome to a binding site in the membrane, possibly a ribophorin
(Kreibich et al., 1978 a and b), will force even strongly hydrophilic
residues into the lipophilic phase. The energy of folding of the
nascent chain on the other side of the membrane should serve to "pull"
residues through the membrane. For the insertion of membrane proteins,
the hydrophobic, membrane-spanning sequence must be followed by a
strongly hydrophilic, preferably charged region, to detach the
ribosome from its binding site in the membrane.

The sequence of delta haemolysin purified by the method of Heatley
(1971 and 1976) from the culture supernatant of S. aureus strain 186X,
was elucidated during the course of this study by Pitton et al. (1980),
Figure 1.5.a The direct transfer model for cotranslational translocation of proteins

After von Heijne and Blomberg (1979).

Figure 1.5.b Models for the folding of delta haemolysin which would satisfy the direct transfer model

Possible orientations of delta haemolysin in the lipid bilayer which would satisfy the requirements of the direct transfer model (von Heijne, personal communication to W.V. Shaw).
and is illustrated in Figure 1.6. It can be seen that although delta haemolysin is the same size as the "model signal peptide" and has a high content of hydrophobic amino acids, it lacks many of the general features of the signal peptides. It has no positive charge at the N-terminus, a hydrophobic segment which is interrupted the positive charge of a lysine residue and none of the characteristic amino acids at the C-terminus. However, if the molecule were folded in the membrane so that these charges were neutralised (Figure 1.5.b), the molecule would satisfy the free energy requirements of the direct transfer model (von Heijne, personal communication to W.V. Shaw).

These considerations prompted the search for a precursor of delta haemolysin as part of this study. It was hoped that the use of inhibitors of extracellular protein production might result in the build up of any precursor of delta haemolysin and facilitate its identification using techniques of immunoprecipitation. Cerulenin, an antibiotic discovered by Hata et al. (1960), has been found to cause inhibition of extracellular protein production. Altenbern (1977) reported that the production of staphylococcal enterotoxins B and C was completely inhibited by concentrations of cerulenin of less than 5 \( \mu g.ml^{-1} \), which did not affect either the growth rate of the bacteria, or the final cell density of the culture. Studies of the mode of action of cerulenin have revealed that it specifically inhibits the biosynthesis of fatty acids (Omura, 1976). This would be expected to cause alterations in the membranes of target cells which would affect the process of exporting proteins.

Alterations in the physical state of bacterial membranes can also be achieved by treatment with a variety of alcohols and anaesthetics (Eliasz et al., 1976 and Papahadjopoulos, 1972). Such agents have also been shown to selectively suppress the appearance of exoenzymes produced by a variety of bacteria under conditions
<table>
<thead>
<tr>
<th>NHCHO</th>
<th>MET</th>
<th>ALA</th>
<th>GLN</th>
<th>ASP</th>
<th>ILE</th>
<th>ILE</th>
<th>SER</th>
<th>THR</th>
<th>ILE</th>
<th>GLY</th>
<th>ASP</th>
<th>LEU</th>
<th>VAL</th>
<th>LYS</th>
<th>TRP</th>
<th>ILE</th>
<th>ILE</th>
<th>ASP</th>
<th>THR</th>
<th>VAL</th>
<th>ASN</th>
<th>LYS</th>
<th>PHE</th>
<th>THR</th>
<th>LYS</th>
<th>LYS</th>
<th>COOH</th>
</tr>
</thead>
</table>

After Pitton et al. (1980).
which allow normal growth and hence the synthesis of essential cell proteins (Fishman et al., 1980). One such agent is the anaesthetic procaine hydrochloride.

In this study the effects of procaine hydrochloride and cerulenin on the production of delta haemolysin by *S. aureus* were investigated. It was hoped that if these compounds resulted in the inhibition of delta haemolysin secretion, they might result in a build up of any precursor of delta haemolysin, which might then be identified using techniques of immunoprecipitation.
2.1. Materials

2.1.1. Chemicals

Unless listed below all chemicals were of analytical grade and were obtained from Fisons Scientific Apparatus (Loughborough, Leicestershire, U.K.).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide, specially purified for electrophoresis</td>
<td>BDH Chemicals Ltd. (Poole, England).</td>
</tr>
<tr>
<td>Alumina C ß gel</td>
<td>Sigma Chemical Company (St. Louis, U.S.A.).</td>
</tr>
<tr>
<td>Biogel HTP</td>
<td>Bio-Rad Laboratories (Richmond, California, U.S.A.).</td>
</tr>
<tr>
<td>Citric acid.H₂O</td>
<td>Riedel-De Haen AG (Seelze, Hannover).</td>
</tr>
<tr>
<td>Cyanogen bromide</td>
<td>Sigma Chemical Company.</td>
</tr>
<tr>
<td>4-Dimethylaminobenzaldehyde</td>
<td>BDH Chemicals Ltd. (Analar).</td>
</tr>
<tr>
<td>1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide</td>
<td>Sigma Chemical Company.</td>
</tr>
<tr>
<td>Ethylene glycol monomethyl ether</td>
<td>Pierce Chemical Company (Rockford, Illinois, U.S.A.).</td>
</tr>
<tr>
<td>Folin and Ciocalteau's reagent</td>
<td>BDH Chemicals Ltd.</td>
</tr>
<tr>
<td>Glycine methyl ester</td>
<td>Sigma Chemical Company.</td>
</tr>
<tr>
<td>Guanidine hydrochloride</td>
<td>Sigma Chemical Company.</td>
</tr>
<tr>
<td>Hydrochloric acid for protein chemistry</td>
<td>BDH Chemicals Ltd. (Aristar).</td>
</tr>
<tr>
<td>Maleic anhydride</td>
<td>Sigma Chemical Company.</td>
</tr>
<tr>
<td>Ninhydrin</td>
<td>Pharmacia Fine Chemicals AB</td>
</tr>
</tbody>
</table>
Octyl sepharose  & Pharmacia Fine Chemicals AB.  
Page Blue 83  & BDH Chemicals Ltd.  
Phenylmethylsulphonylfluoride  & Sigma Chemical Company.  
Phosphoric acid  & BDH Chemicals Ltd. (Analar).  
Polyethylene glycol 20 000  & BDH Chemicals Ltd.  
Protein A sepharose  & Pharmacia Fine Chemicals AB.  
Sephadex G-10, G-25, G-50, G-150  & Pharmacia Fine Chemicals AB.  
Sodium β-glycerophosphate  & Sigma Chemical Company.  
Stannous chloride.2H₂O  & BDH Chemicals Ltd. (Analar).  
TEVRED  & BDH Chemicals Ltd.  

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boric acid</td>
<td>May and Baker Ltd. (Dagenham, England).</td>
</tr>
<tr>
<td>Brij 35</td>
<td>BDH Chemicals Ltd.</td>
</tr>
<tr>
<td>Citric acid.H₂O</td>
<td>Raedel-De Haen AG.</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>BDH Chemicals Ltd. (Aristar).</td>
</tr>
<tr>
<td>Methanol</td>
<td>Rathburn Chemicals (Walkerburn) Ltd.</td>
</tr>
<tr>
<td></td>
<td>(Walkerburn, Peebleshire, Scotland).</td>
</tr>
<tr>
<td>Ninhydrin</td>
<td>Pierce Chemical Company.</td>
</tr>
<tr>
<td>Pentachlorophenol</td>
<td>Pierce Chemical Company.</td>
</tr>
<tr>
<td>Sodium acetate buffer solution</td>
<td>BDH Chemicals Ltd. (Sepramar).</td>
</tr>
<tr>
<td>pH 5.5 (4M Na)</td>
<td>BDH Chemicals Ltd. (Aristar).</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>BDH Chemicals Ltd. (Analar).</td>
</tr>
<tr>
<td>Stannous chloride.2H₂O</td>
<td>Pierce Chemical Company.</td>
</tr>
<tr>
<td>Thiodiglycol</td>
<td>Pierce Chemical Company.</td>
</tr>
<tr>
<td>Trisodium citrate.2H₂O</td>
<td>BDH Chemicals Ltd. (Sepramar).</td>
</tr>
</tbody>
</table>
2.1.3. Chemicals for N-terminal sequence analysis

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminopropyl glass (75 Å porosity)</td>
<td>Pierce and Warriner (U.K.) Ltd. (Chester, Cheshire, England).</td>
</tr>
<tr>
<td>Dimethylallylamine buffer</td>
<td>Pierce Chemical Company.</td>
</tr>
<tr>
<td>Dimethyl formamide</td>
<td>Pierce Chemical Company.</td>
</tr>
<tr>
<td>Methanol</td>
<td>Rathburn Chemicals (Walkerburn) Ltd.</td>
</tr>
<tr>
<td>Phenylenediisothiocyanate</td>
<td>Eastman Kodak Company (Rochester, N.Y., U.S.A.).</td>
</tr>
<tr>
<td>Trifluoroacetic acid</td>
<td>Pierce Chemical Company.</td>
</tr>
</tbody>
</table>

2.1.4. Radiochemicals

Radiochemicals used are listed below with their specific activities and were all purchased from Amersham International (Amersham, England).

<table>
<thead>
<tr>
<th>Radiochemical</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-(U-14C) lysine monohydrochloride</td>
<td>340 mCi.mmol⁻¹</td>
</tr>
<tr>
<td>L-(4,5-3H) lysine monohydrochloride</td>
<td>108 Ci.mmol⁻¹</td>
</tr>
<tr>
<td>(1,4-14C) Maleic anhydride</td>
<td>32.6 mCi.mmol⁻¹</td>
</tr>
</tbody>
</table>

2.1.5. Biological materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-rabbit antiserum</td>
<td>Wellcome Research Laboratories (Beckenham, Kent, U.K.).</td>
</tr>
<tr>
<td>(Donkey) RD 17</td>
<td></td>
</tr>
<tr>
<td>Bovine serum albumin fraction V</td>
<td>Sigma Chemical Company.</td>
</tr>
<tr>
<td>Casein acid hydrolysate</td>
<td>Oxoid Ltd. (England).</td>
</tr>
<tr>
<td>Cerulenin</td>
<td>Makor Chemicals Ltd. (Jerusalem, Israel).</td>
</tr>
<tr>
<td>Chymotrypsinogen</td>
<td>The Boehringer Corporation</td>
</tr>
</tbody>
</table>
Cytochrome C  The Boehringer Corporation (London) Ltd. (Bell Lane, Lewes, East Sussex, England).
DNAase  Sigma Chemical Company.
Lysine decarboxylase  Sigma Chemical Company.
Lysostaphin  Sigma Chemical Company.
Melittin  Sigma Chemical Company.
Mitomycin C  BDH Chemicals Ltd.
Molecular weight standards for gel filtration  Pharmacia Fine Chemicals AB.
Myoglobin molecular weight markers  BDH Chemicals Ltd.
Ovalbumin grade V  Sigma Chemical Company.
Pen-Assay base agar  Difco Laboratories (Detroit, Michigan, U.S.A.).
Procaine hydrochloride  BDH Chemicals Ltd.
Trypticase soy broth  Difco Laboratories.
Yeast extract  Oxoid Ltd.

2.1.6. Water

Glass distilled water was used in all experiments except protein chemistry for which glass distilled water was treated with ion exchange resins and charcoal and sterilised using a Millipore Super Q2 filter unit.
2.2. Methods

Generally used methods not described in the following chapters are described below.

2.2.1. Centrifuges

The centrifuges used are listed below;

- microfuge (Quickfit Instrumentation, England.)
- bench centrifuge (M.S.E. Scientific Instruments, Manor Royal, Crawley, Sussex, England.)
- preparative centrifuge, M.S.E. 1800 (M.S.E. Scientific Instruments).

2.2.2. Scintillation counting

Radioactivity in aqueous samples (not more than 100 μl) was estimated by counting in a Packard Tricarb Liquid Scintillation Counter, model 3385 (Packard Instrument Ltd., Caversham, Berks., U.K.) using as scintillant:

- naphthalene: 60 g
- 2,4-diphenyloxazole: 4 g
- methanol: 100 ml
- ethanediol: 20 ml

made up to 1 l with 1,4-dioxan.

2.2.3. Lyophilisation

Samples were frozen in a mixture of acetone and solid carbon dioxide at -86°C and then dried under vacuum.
Table 2.1. Strains of *Staphylococcus aureus* used in this study

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Genotype</th>
<th>Prophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN1</td>
<td>8325</td>
<td>Ø 11, Ø 12, Ø 13</td>
</tr>
<tr>
<td>RN25</td>
<td>8325-3</td>
<td>Ø 13</td>
</tr>
<tr>
<td>RN27</td>
<td>8325-3 (80X)</td>
<td>Ø 13</td>
</tr>
<tr>
<td>RN450</td>
<td>8325-4</td>
<td>cured</td>
</tr>
<tr>
<td>A003</td>
<td>8325-3 (80X pC221)</td>
<td>Ø 13</td>
</tr>
<tr>
<td>RN451</td>
<td>8325-4 (Ø 11)</td>
<td>Ø 11</td>
</tr>
<tr>
<td>RN740</td>
<td>8325-4 (Ø 12)</td>
<td>Ø 12</td>
</tr>
<tr>
<td>186X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P392</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P391</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P484</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P363</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Strain 186X was a gift from Dr. N.G. Heatley, the strains with the prefix P were donated by Dr. P.A. Pattee, all of the remaining strains were donated by Dr. R.P. Novick.
2.2.4. Microbiology

2.2.4.1. Sterilisation

Sterilisation was achieved by autoclaving at a pressure of 15 lb.in$^{-2}$ for 15 min. Some glassware was sterilised by heating overnight in an oven at 180°C.

2.2.4.2. Bacterial strains

The organisms used were all strains of Staphylococcus aureus, their strain numbers and genotypes are outlined in Table 2.1.

Strain 186X, derived from S. aureus strain Newman, was kindly provided by Dr. N.G. Heatley. The strains with the prefix P were kindly donated by Dr. P.A. Pattee, and all the remaining strains were generously donated by Dr. R.P. Novick from his collection.

2.2.4.3. Maintenance of bacterial strains

Bacteria were maintained on plates of Pen-Assay base agar (25 g.l$^{-1}$). The bacteria were streaked onto the plates, incubated 24 - 36 h at 37°C and then stored at 4°C. The strains were sub-cultured monthly.

Samples of the strains were also stored in glycerol. Bacteria from overnight cultures in 3 ml of medium were harvested by centrifuging at 1 600 g for 5 min at room temperature (bench centrifuge). The supernatants were removed and the pellets resuspended in 2 ml of 0.05M phosphate buffer pH 7.0 containing 50% (w/v) glycerol. Sterile plastic vials were used to store 1 ml aliquots of the strains at -20°C. When required the strains were streaked onto agar plates and incubated overnight at 37°C.
2.2.4.4. Growth of bacteria

The organisms were grown in the yeast diffusate medium of Gladstone and van Heyningen (1957). This contained per litre:

- casein acid hydrolysate 20 g
- yeast diffusate 200 ml
- sodium β-glycerophosphate 20 g
- MgSO$_4$·7H$_2$O 20 mg
- MnSO$_4$·4H$_2$O 10 mg
- KH$_2$PO$_4$ 0.4 g
- Na$_2$HPO$_4$·12H$_2$O 6.25 g

Solution containing:
- 0.32% (w/v) FeSO$_4$·7H$_2$O
- 0.32% (w/v) citric acid 2 ml

Sodium lactate included in the original medium was omitted as its omission was found to result in increased production of delta haemolysin.

Yeast diffusate was prepared by dialysis of 280 g of yeast extract in 200 ml of water against 1.8 l of water in a 2 l measuring cylinder overnight at 4°C.

The medium and yeast diffusate were stored at 4°C after sterilisation.

Cultures were grown overnight at 37°C shaking at 150 - 200 rpm on a gyrotary shaker. Small scale cultures in 5 - 25 ml of medium were grown up in Erlenmeyer flasks with capacities equal to 10 times the volume of the culture to allow adequate aeration. Sterile medium was inoculated with a small volume of a starter culture, 10 ml of medium inoculated with bacteria directly from a plate and grown overnight. Bacteria were harvested by centrifuging at 1 600 g for 15 min at room temperature (bench centrifuge).

Large scale cultures were grown in 500 ml batches, the medium
in 2 l Erlenmeyer flasks was inoculated with 3 ml of a starter culture. The bacteria were harvested by centrifuging at 13 000 g for 15 min at 20°C (preparative centrifuge). Culture supernatant was treated with FMSP (20 mg l\(^{-1}\)) to inhibit the action of proteases and stored for 24 - 48 h at 4°C or at -20°C for longer periods.

Growth was monitored spectrophotometrically, either by measuring the absorbance at 540 nm of diluted samples of the cultures, or by using sidearm flasks as culture vessels and estimating growth in a Klett-Summerson photoelectric colorimeter (Klett MFG. Co., Inc., N.Y., U.S.A.).

The use of absorbance as a measure of cell density, and therefore growth of bacterial cultures is most reliable for organisms that do not clump. Staphylococci do clump rendering absorbance measurements subject to some error. Absorbance values estimated after brief sonication of the cultures may have been more reliable, however this was not carried out in order to avoid producing aerosols of potentially pathogenic strains of \(S\).\textit{aureus}.

2.2.5. Assay for haemolytic activity

The assay used for the estimation of haemolytic activity was based on that described by Heatley (1971).

2.2.5.1. Preparation of blood for use in the assay

The preparation of blood was carried out under sterile conditions. Fresh human blood, type 0, Rh positive, from a single donor was added to 2 ml of 3.8% (w/v) sodium citrate in a capped centrifuge tube and mixed well. The erythrocytes were separated from the plasma by centrifuging at 1 600 g for 3 min at room temperature (bench centrifuge). The supernatant was removed using a Pasteur pipette. The erythrocytes were washed by resuspending in
6 ml of 0.9% (w/v) NaCl and centrifuging as before but for 2 min. The supernatant was removed as above and the washing procedure repeated 5 times. Finally the erythrocytes were resuspended in phosphate buffered saline (PBS1) to a total volume of 10 ml and stored at 4°C. PBS1 contained per litre;

- NaCl 5.86 g
- Na₂HPO₄·12H₂O 8.96 g
- KH₂PO₄ 3.40 g

2.2.5.2. Construction of a standard curve

For each fresh preparation of blood a standard curve was constructed. Stock erythrocyte suspension was diluted 25-fold in PBS1 for use in the assay (assay erythrocyte suspension). Delta haemolysin purified by Dr. N.G. Heatley by his method (Heatley, 1971) from the culture supernatant of S. aureus strain 186X was used in the construction of standard curves. As different preparations differed slightly in specific activity the same preparation was used throughout the study. A stock solution of 0.25 mg.ml⁻¹ (by weight) in PBS1, for use in the assay, was made from more concentrated solutions of delta haemolysin which were stored at -20°C.

Different volumes of the stock solution of delta haemolysin were made up to 0.5 ml with PBS1 in duplicate as outlined in Table 2.2. in 1.5 ml microfuge tubes. Assay erythrocyte suspension, 0.5 ml, was added to the tubes which were then incubated for 15 min at 37°C with mixing. Stock erythrocyte suspension diluted 25-fold in water was taken to represent 100% haemolysis. After incubation the tubes were centrifuged at 12 000 g for 1.5 min at room temperature (microfuge) to remove unlysed cells and cell debris. Samples of the supernatants, 0.5 ml, were removed and added to 2.5 ml of 0.1% (w/v) Na₂CO₃ with mixing. The sample representing 100% haemolysis was used
Table 2.2. Construction of a standard curve for the assay of haemolytic activity

<table>
<thead>
<tr>
<th>Reagent</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS1</td>
<td>500</td>
<td>495</td>
<td>490</td>
<td>480</td>
<td>460</td>
<td>420</td>
<td>380</td>
<td>350</td>
<td>-</td>
</tr>
<tr>
<td>2 x PBS1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>500</td>
</tr>
<tr>
<td>Delta haemolysin (0.25 mg.ml⁻¹)</td>
<td>-</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>40</td>
<td>80</td>
<td>120</td>
<td>150</td>
<td>-</td>
</tr>
<tr>
<td>Assay erythrocyte suspension</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>-</td>
</tr>
<tr>
<td>25-fold dilution of stock erythrocyte suspension in water</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>500</td>
</tr>
</tbody>
</table>

All volumes are expressed in µl. The assays were done in duplicate.
to measure the absorption spectrum of the haemoglobin released (Figure 2.1.). The blank used was 0.5 ml of PBS1 in 2.5 ml of 0.1% (w/v) Na₂CO₃. The absorbance maximum was found to be at 543 nm. At this wavelength samples representing 100% haemolysis gave an absorbance value of 0.45 - 0.5. The extent of haemoglobin release in the other samples was measured spectrophotometrically at 543 nm. Figure 2.2. shows a standard curve constructed from the mean values from 21 standard curves. One haemolytic unit (HU) was taken to be that amount of delta haemolysin which resulted in 50% haemolysis.

Erythrocyte suspensions more than 7 days old were found to give values in a standard curve which fell outside 1 standard deviation (Figure 2.2.). The erythrocytes were less sensitive to the haemolysin. For this reason erythrocyte suspensions used for the assay of haemolytic activity were never more than 7 days old.

2.2.5.3. Assay of unknown samples

Samples to be assayed for haemolytic activity (up to 0.2 ml) were made up to 0.5 ml with PBS1 and treated as described above. The number of haemolytic units in the samples were calculated from the standard curve. Samples to be assayed for only delta haemolysin, which might contain other haemolysins were first heat-treated by heating to 60°C for 1 h. Any precipitate was then removed by centrifuging at 12 000 g for 5 min at room temperature (microfuge) and the supernatants assayed as described above.

2.2.6. Estimation of protein

The adaptation of the method of Polin and Ciocalteau by Lowry (1951) was used.

Solutions

A - 2% (w/v) Na₂CO₃ in 0.1M NaOH
Figure 2.1. The absorption spectrum of haemoglobin released in the assay for haemolytic activity.

The sample representing 100% haemolysis was used to measure the absorption spectrum of the haemoglobin released in the assay for haemolytic activity. The absorbance maximum was found to be at 543 nm.
Figure 2.2. Standard curve for the assay of haemolytic activity

The standard curve was constructed from the mean values from 21 standard curves, the bars indicate 1 standard deviation.
B - 1% (w/v) CuSO\(_4\)·5H\(_2\)O
C - 2% (w/v) sodium tartrate
D - was prepared immediately before use by mixing A, B and C, 100:1:1 by volume.
E - Folin and Ciocalteau's reagent diluted 1:1 by volume with water before use.

Solution D, 2.0 ml, was added to 0.4 ml of the protein sample, diluted to contain 10 - 100 µg of protein, with mixing. After 10 min at room temperature 0.2 ml of solution E was added with mixing. After a further 30 min at room temperature the colour which had developed was estimated spectrophotometrically at 500 nm using a blank in which water had replaced the protein sample. The concentration of the protein samples was calculated from a standard curve constructed using bovine serum albumen (BSA). Unless otherwise stated all protein estimations were performed using this method.

2.2.7. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out in gel slabs, 15.0 x 14.0 x 0.2 cm in a vertical gel apparatus

2.2.7.1. Polyacrylamide gel electrophoresis in the presence of SDS

SDS PAGE was carried out using a method based on that of Laemmli et al. (1970) which varies the ratio of methylene bisacrylamide to acrylamide for different percentage gels so that;

\[
\text{methylen bisacrylamide (mg.100 ml}^{-1}) = 1.3 \times \text{acrylamide (g.100 ml}^{-1})
\]

Table 2.3 details the compositions of the various percentage gels used in this study.

To construct a gel the first 4 components listed were mixed and degassed under vacuum. After the addition of the SDS the ammonium persulphate and the TEMED were added, the gel poured and allowed to polymerise. The surface of the gel was overlaid with water-saturated
Table 2.3. The composition of polyacrylamide gels used in this study

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Percentage acrylamide gel (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>30% (w/v) acrylamide</td>
<td>10.0</td>
</tr>
<tr>
<td>60% (w/v) acrylamide</td>
<td>-</td>
</tr>
<tr>
<td>1% (w/v) methylene bisacrylamide</td>
<td>3.9</td>
</tr>
<tr>
<td>1.5M Tris-HCl buffer pH 8.7</td>
<td>7.5</td>
</tr>
<tr>
<td>water</td>
<td>8.0</td>
</tr>
<tr>
<td>20% (w/v) SDS</td>
<td>0.15</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulphate</td>
<td>0.1</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01</td>
</tr>
</tbody>
</table>

**Stacking gel (5% (w/v) acrylamide)**

<table>
<thead>
<tr>
<th>Reagent</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>30% (w/v) acrylamide</td>
<td>1.67</td>
</tr>
<tr>
<td>1% (w/v) methylene bisacrylamide</td>
<td>1.30</td>
</tr>
<tr>
<td>1M Tris-HCl buffer pH 6.8</td>
<td>1.25</td>
</tr>
<tr>
<td>water</td>
<td>4.40</td>
</tr>
<tr>
<td>20% (w/v) SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulphate</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005</td>
</tr>
</tbody>
</table>

All volumes are expressed in ml; ammonium persulphate was made up immediately before use.
n-butanol to prevent inhibition of polymerisation by oxygen and to ensure a flat surface on the top of the gel. This was removed when the gel was set and the stacking gel poured in a similar manner.

Sample buffer contained per 100 ml:

- glycerol 10 ml
- dithiothreitol 1.54 g
- 20% (w/v) SDS 10 ml
- 1M Tris-HCl buffer pH 6.8 1.1 ml.

Samples of 25 μl or less were mixed with 2 or more volumes of sample buffer (final volume not more than 75 μl), larger samples were lyophilised and taken up in 30 - 60 μl of sample buffer. Samples were heated to 80°C for 5 min, cooled and 5 μl of 0.1% (w/v) bromophenol blue tracking dye added to each.

Running buffer contained per litre:

- glycine 144 g
- Tris 30 g
- SDS 5 g

Electrophoresis was towards the anode and was performed at constant voltage at room temperature. The samples were stacked at 60 V for 30 - 60 min and then electrophoresed in the running gel at 120 V for 3 - 4 h.

2.2.7.2. Electrophoresis in polyacrylamide gradient gels in the presence of SDS

The procedure was the same as outlined above except that the running gels were constructed from a linear gradient of 10 - 30% (w/v) acrylamide. Samples were electrophoresed in the running gel for 4 - 5 h.
2.2.7.3. Native polyacrylamide gel electrophoresis

The system described for SDS PAGE was used omitting SDS from all buffers. The sample buffer was replaced by 50mM Tris-HCl buffer pH 7.8 containing 0.1M 2-mercaptoethanol. A drop of glycerol was added to each sample with tracking dye as above. The gels were electrophoresed as described for SDS PAGE.

2.2.7.4. Native PAGE in the acidic system of Reisfeld et al. (1962)

Solutions

A - 1M KOH 48.0 ml
   glacial acetic acid 17.2 ml
   TEMED 4.0 ml
made up to 100 ml with water.

B - 1M KOH 48.0 ml
   glacial acetic acid 2.87 ml
   TEMED 0.46 ml
made up to 100 ml with water.

C - acrylamide 60.0 g
   methylene bisacrylamide 0.4 g
made up to 100 ml with water.

D - acrylamide 10.0 g
   methylene bisacrylamide 2.5 g
made up to 100 ml with water.

E - freshly prepared ammonium persulphate solution (5 mg.ml⁻¹).

P - reservoir buffer;
   glacial acetic acid 8.0 ml
   β-alanine 31.2 g
made up to 1 l with water.

The running gel was prepared by adding 3 ml of solution A to 6 ml of solution C and 8.28 ml of water. After degassing the solution
under vacuum 6.72 ml of degassed solution E was added with gentle mixing and the gel poured and overlaid with water-saturated n-butanol as described before. This constituted a 15% (w/v) acrylamide gel. The stacking gel was prepared by mixing 1 ml of solution B with 2 ml of solution D and 3 ml of water. This was degassed and then 2 ml of degassed solution E was added with gentle mixing and the gel poured.

Samples were mixed with a drop of glycerol, 10 μl of 0.5% (w/v) methyl green tracking dye and solution B to make the total volume not more than 75 μl. Electrophoresis was carried out as described before but towards the cathode.

2.2.7.5. Procedures used for staining polyacrylamide gels

**Method A** Gels were fixed in 10% (v/v) acetic acid, 15% (v/v) methanol for 30 min at 37°C. The gels were then stained overnight at 37°C in 0.4% (w/v) Page Blue 83, 3.5% (w/v) perchloric acid. The gels were destained in 7% (v/v) acetic acid.

**Method B** "Quick stain" Gels were stained for 30 min in 0.1% (w/v) Page Blue 83, 50% (v/v) methanol, 10% (v/v) acetic acid and then destained in 5% (v/v) methanol, 7% (v/v) acetic acid for 1 h or overnight.

**Method C** Gels were stained overnight in 0.2% (w/v) Page Blue 83, 10% (w/v) trichloroacetic acid, 40% (v/v) methanol and destained in 7% (v/v) acetic acid.

**Method D** This stain was developed for the detection of low molecular weight peptides or basic proteins using formaldehyde fixation (Steck et al., 1980). Gels were stained for 1 h in:

- ethanol 180 ml
- water 420 ml
- 35% (w/v) formaldehyde 100 ml
- Page Blue 83 0.8 g.
Gels containing SDS were then stained for a further 3 h in:

ethanol 250 ml
water 750 ml
35% (w/v) formaldehyde 10 ml
Page Blue 83 1.2 g.

Gels were destained in:

ethanol 250 ml
water 750 ml
35% (w/v) formaldehyde 10 ml.

2.2.7.6. Fluorography of polyacrylamide gels

Gels were prepared for fluorography as described by Bonner and Laskey (1974), either directly after electrophoresis or after staining and destaining. Gels were soaked in 20 volumes of dimethyl sulphoxide (DMSO) for 30 min and then in a second wash of 20 volumes of DMSO for a further 30 min. The gels were then soaked in 4 volumes of DMSO containing 22.2% (w/v) diphenyl oxazole for 3 h. Finally the gels were soaked in several changes of water for 1 h and then dried under vacuum at 70°C for 1.5 h. X-omat RP film (Kodak Ltd., England) was applied to the gels and exposed at -70°C. After exposure the films were developed for 5 min in D19 developer (Kodak Ltd.), rinsed in water and then fixed in Amfix fixer (Kodak Ltd.) for 5 min. The films were then rinsed well in water and dried.

2.2.8. Amino acid analysis

Samples for amino acid analysis, normally about 10 nmol of protein (30 μg of delta haemolysin), were lyophilised in hydrolysis tubes and then 0.2 ml of 6M HCl containing 10μM phenol was added to each sample. The tubes were centrifuged at 180 g for 30 s at room temperature (bench centrifuge) to ensure that all the liquid was at
the bottom of the tubes. The hydrolysis tubes were then drawn out in an oxygen-gas flame to form a narrow neck. The samples were flushed with nitrogen and degassed under vacuum twice, and finally sealed under vacuum in a bunsen flame. Samples were hydrolysed for 24, 48 and 72 h or just 48 h at 105°C. After hydrolysis the samples could be stored at -20°C. For analysis the tubes were centrifuged as before, broken open and the acid removed under vacuum. The hydrolysates were resuspended in 0.02M HCl and analysed using a single column Locarte amino acid analyser operating with sodium citrate buffers (Spackman et al., 1958). Chromatograms were integrated automatically or manually, and data for 24, 48 and 72 h hydrolysates normalised by the method of Ambler (1975).

2.2.9. N-terminal sequence analysis

Automated sequencing of protein samples attached to an inert support was performed using an Anachem APS 2 400 solid-phase sequencer based on the principles described by Laursen (1971). The column support used was isothiocyanate-glass (ITC-glass). This was made by the addition of 1 g of aminopropyl-glass (0.2 meq.g⁻¹) in 100 mg aliquots at 5 min intervals to a solution of 1.5 g of phenylene diisothiocyanate in 5 ml of dimethyl formamide (DMF) under nitrogen at room temperature. After the final addition the suspension was stirred slowly under nitrogen for 1 h and the glass then collected by centrifuging at 180 g for 1 min at room temperature (bench centrifuge). The derivatised glass was washed extensively by resuspension in DMF and methanol alternatively and finally with methanol. The glass was then dried under vacuum.

Protein samples (5 mg) were dialysed exhaustively against water and then lyophilised. The samples were then dissolved in 0.5 ml of dimethylallylamine buffer pH 9.5 and 200 mg of ITC-glass was added
in 50 mg aliquots at 10 min intervals under nitrogen with stirring at 45°C. After the final addition stirring was continued under nitrogen at 45°C for a further 45 min. Excess isothiocyanate groups were blocked by the addition of 0.1 ml of ethanolamine with subsequent stirring at 45°C for 30 min. The resulting protein-derivatised glass was washed with 5 x 5 ml of methanol and dried under vacuum. Perham (1975) and Machleidt et al. (1973).

Identification of phenylthiohydantoin-amino acids (PTH amino acids) was achieved as follows. Fractions containing the sequentially released phenylthiocarbamoyl derivatives of the amino acids in trifluoroacetic acid and methanol were collected from the sequencer and the solvent removed by evaporation at 50°C under a stream of nitrogen. Conversion to the PTH amino acids was achieved by the addition of 0.2 ml of 1.0M HCl and heating at 80°C for 15 min. After cooling, the PTH amino acids were extracted from the acid by partition into 1.5 ml of redistilled ethylacetate. The organic phase was removed and evaporated at 50°C under a stream of nitrogen. The residues were each taken up in 20 μl of ethylacetate and spotted on the origin of a thin-layer silica gel plate (DC-Fertigplatten Kieselgel 60F254, Merck). The PTH amino acids were identified by comparison of the fluorescence quenching with standards using chromatography in 2 solvents;

solvent 1 - chloroform, ethanol 98:2 (v/v)
solvent 2 - chloroform, ethanol, methanol 88.2:1.8:10 (v/v/v)

Bridgen et al. (1975).

2.2.10 Silica gel thin layer chromatography (TLC)

The plates used for TLC were 20 x 20 cm and layered with 0.25 mm of silica gel (Polygram Sil G, Camlab, Cambridge, U.K.). Samples for TLC, 30 - 500 μg of protein (10 - 170 nmol of delta haemolysin) were
lyophilised and taken up in water or a mixture of chloroform and methanol (2:1 v/v) and spotted on the origin.

Chromatograms were developed in n-butanol, water, acetic acid (45:30:25 v/v/v) for 4 h and then dried in a fume cupboard. The chromatograms were stained first by dipping in cadmium-ninhydrin reagent (Glazer et al., 1975). The plates were dried as before and developed in an oven at 45°C. The stained areas were traced and chromatograms then stained for tryptophan-containing peptides using Erlich's reagent (Glazer et al., 1975). The plates were dried and developed as above and the stained areas traced.

2.2.11. **Further purification of delta haemolysin by chromatography on octyl sepharose**

Samples of delta haemolysin (not more than 40 mg) were loaded in 1 - 2 ml of water onto a column of octyl sepharose of bed-volume 18 - 20 ml which had been equilibrated in water. Samples were washed onto the column with 15 ml of water. The column was then eluted with 15 ml of 35% (v/v) ethanol, a linear gradient made up from 30 ml of 35% (v/v) ethanol and 30 ml of 70% (v/v) ethanol, 30 ml of 70% (v/v) ethanol and finally with 30 ml or more of ethanol. The solvents were pumped through the column at a flow rate of 1.5 ml.min⁻¹ and 1.5 ml fractions were collected. The elution profiles were recorded using a continuous flow spectrophotometer at 280 nm. The fractions were assayed for haemolytic activity.

2.2.12. **Quantitative estimation of amino groups using ninhydrin**

Ninhydrin reagent, 100 ml, was made up from 80 mg of SnCl₂·2H₂O in 50 ml of sodium citrate buffer which contained:

- citric acid·H₂O: 2.1 g
- NaOH: 0.8 g.
To this was added \( 2 \text{ g} \) of ninhydrin in \( 50 \text{ ml} \) of methyl cellosol (ethylene glycol monomethylether). The solution was saturated with nitrogen and stored under nitrogen in the dark at \( 4^\circ \text{C} \).

Ninhydrin reagent, \( 1 \text{ ml} \), was added to the samples, \( 0.1 \text{ ml} \), containing \( 0.01 - 1 \text{ mg} \) of protein in water or buffer. The samples were then heated in a vigorously boiling water bath for \( 20 \text{ min} \). After cooling for \( 5 \text{ min} \) at room temperature the samples were rapidly mixed with \( 5 \text{ ml} \) of \( 50\% \text{(v/v)} \) aqueous isopropanol. The colour that developed after a further \( 5 \text{ min} \) was estimated spectrophotometrically using a blank in which water or the appropriate buffer replaced the sample. The absorbance maximum of the colour that developed was found at \( 572 \text{ nm} \); the colour that developed in the samples was estimated at this wavelength.

2.2.13 Double diffusion tests (Ouchterlony, 1958)

Test plates were made in \( 10 \times 10 \text{ cm} \) square Petri dishes from \( 16 \text{ ml} \) of \( 0.75\% \text{(w/v)} \) agarose in PBS2, pH 7.3, which contained per litre; \( \text{NaCl} \) \( 8.0 \text{ g} \), \( \text{KCl} \) \( 0.2 \text{ g} \), \( \text{Na}_2\text{HPO}_4 \) \( 1.15 \text{ g} \), \( \text{KH}_2\text{PO}_4 \) \( 0.2 \text{ g} \).

Wells of diameter \( 3 \text{ mm} \) were cut into the agarose, \( 7 \text{ mm} \) apart. Samples (up to \( 10 \mu\text{l} \)) were placed in the wells and the plates incubated for \( 72 \text{ h} \) at \( 4^\circ \text{C} \). Precipitin lines that developed were visible, but to facilitate photography the test plates were stained in the following way.

The plates were flooded with PBS2 for \( 24 \text{ h} \) at room temperature to remove unprecipitated protein. The plates were then dried by covering with blotting paper and several layers of paper towel and applying a weight of \( 1 \text{ kg} \). The plates were then rinsed with water and stained for
30 - 60 min in 0.123% (w/v) amido black in 5% (v/v) acetic acid. The plates were destained by rinsing several times in 7% (v/v) acetic acid. The plates were then dried as before.

2.2.14. Preparation of the immunoglobulin fraction from the serum of rabbits (Turner and Pickard, 1980)

Immunoglobulin (Ig) fractions were prepared from both immune and preimmune sera. Serum, 5 ml, was mixed with 5 ml of ammonium sulphate at 60% saturation, pH 7.0, at 4°C. The mixture was then centrifuged at 40 000 g for 20 min at 4°C (preparative centrifuge). The supernatant was removed and the pellet dissolved in 5 ml of PBS3, pH 7.0, which contained per litre;

\[
\begin{align*}
\text{NaCl} & \quad 5.86 \text{ g} \\
\text{NaH}_2\text{PO}_4 & \quad 5.42 \text{ g} \\
\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} & \quad 21.90 \text{ g}.
\end{align*}
\]

The whole procedure was repeated twice. The Ig fraction in PBS3 was stored at -20°C.
CHAPTER THREE
3.1. Investigation of the production of haemolysins by different strains of *Staphylococcus aureus*

Table 3.1. lists the different strains of *S. aureus* investigated, showing their genotypes and the different prophages carried by each. A small scale culture (10 ml) of each strain was grown up and the cells removed as described in Section 2.2.4.4. The culture supernatants of the different strains were assayed for haemolytic activity as described in Section 2.2.5, in three different ways, in order to give some indication of the amounts of three different haemolysins, alpha, beta and delta haemolysins.

i) Assay of untreated supernatants.

ii) Assay of heat-treated supernatants, samples of the supernatants were heated to $60^\circ C$ for 1 h, the precipitated protein was removed by centrifuging at 12 000 g for 5 min at room temperature.

iii) Assay of heat-treated supernatants including a second incubation of the assay mixture at $4^\circ C$ for 15 min.

As alpha haemolysin is destroyed by heating to $60^\circ C$ for 1 h (Rogolsky, 1979) and beta haemolysin only causes lysis after additional incubation of the assay mixture at $4^\circ C$ ("hot-cold" haemolysis) the haemolytic units detected in assay ii) were taken to be due to delta haemolysin. Additional haemolytic units detected in assay i) were taken to represent the activity of alpha haemolysin. Additional haemolytic units detected in assay iii) were taken to represent the activity of beta haemolysin. Beta haemolysin has been reported to be heat-labile (Wiseman, 1965), however in this study using fresh supernatants the greatest increase in activity detected with additional incubation of the assay mixtures at $4^\circ C$ was observed with heat-treated supernatants.
Table 3.1. Strains of *Staphylococcus aureus* investigated for haemolytic activity and total extracellular protein production

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Genotype</th>
<th>Prophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN1</td>
<td>8325</td>
<td>Ø 11 Ø 12 Ø 13</td>
</tr>
<tr>
<td>RN25</td>
<td>8325-3</td>
<td>Ø 13</td>
</tr>
<tr>
<td>RN27</td>
<td>8325-3 (80K)</td>
<td>Ø 13</td>
</tr>
<tr>
<td>RN450</td>
<td>8325-4</td>
<td>cured</td>
</tr>
<tr>
<td>A003</td>
<td>8325-3 (80K, pC221)</td>
<td>Ø 13</td>
</tr>
<tr>
<td>RN451</td>
<td>8325-4 (Ø 11)</td>
<td>Ø 11</td>
</tr>
<tr>
<td>RN740</td>
<td>8325-4 (Ø 12)</td>
<td>Ø 12</td>
</tr>
</tbody>
</table>
Figure 3.1 illustrates the activities of the different haemolysins detected in the culture supernatants of the different strains. It must be noted that the levels of the different haemolysins can only be compared for the same haemolysin in different strains. The levels do not represent the actual amounts of the haemolysins present in the supernatants. Alpha and beta haemolysins could be more accurately assayed using rabbit and sheep erythrocytes respectively, which are the most sensitive to lysis by them.

It can be seen however that strains RN1, RN25 and RN27 exhibit fairly high levels of delta haemolysin with low levels of alpha and beta haemolysins. Strains A003 and RN740 exhibit higher levels of delta haemolysin, but with high levels also of alpha and beta haemolysins. Strain RN451 exhibits lower levels of all the haemolysins. There was no detectable haemolytic activity in the supernatant of strain RN450. This strain is derived from strain RN25, and has been "cured" of prophages by several cycles of growth in the presence of mitomycin C to induce resident prophages. Survivors were judged to be free of prophages (R.P. Novick, personal communication to W.V. Shaw). This would suggest that the production of normal levels of haemolysins may in some way be linked to the presence of prophages. This is supported by the fact that haemolysins are produced by strains RN451 and RN740 which are derived from RN450 and have been reinfected with prophages Ø 11 and Ø 12 respectively.

3.2. Investigation of the patterns of extracellular protein production of different strains of Staphylococcus aureus

Total extracellular protein production was investigated using SDS PAGE. Samples of the culture supernatants (750 μl) of the different strains (Table 3.1.) were concentrated to a volume of 375 μl using Minicon concentrators (Amicon Corporation, Lexington, Mass., U.S.A.)
Figure 3.1. Production of different haemolysins by different strains of Staphylococcus aureus

Culture supernatants were assayed for haemolytic activity as outlined in the text.

- Delta haemolysin
- Alpha haemolysin
- Beta haemolysin
The volumes of the samples, which were taken for analysis by SDS PAGE, were corrected to allow for the different final cell densities of the strains, estimated spectrophotometrically at 540 nm. The samples (containing approximately 500 μg of protein) were treated with sample buffer and electrophoresed in a 10% (w/v) acrylamide gel in the presence of SDS as described in Section 2.2.7.1. The gel was stained and destained using method A described in Section 2.2.7.5.

Plate 3.1. illustrates the results. A sample was also prepared from the culture supernatant of *S. aureus* strain Wood 46, kindly provided by Dr. G. Coleman, which contains alpha haemolysin as one of the major proteins. This sample was electrophoresed in Track 1 and the main protein band observed is believed to be alpha haemolysin. The sample prepared from the culture supernatant of strain RN450, which has been "cured" of prophages, can be seen in Track 5 to produce less extracellular protein than most of the other strains.

3.3. Search for haemolytic activity in cell lysates of strains of *Staphylococcus aureus*

The cell lysates of strains RN25, RN450 and 186X were assayed for haemolytic activity in order to determine whether the inability of strain RN450 to produce detectable haemolytic activity in the supernatant was due to an inability to produce the proteins or an inability to export the proteins.

Small scale cultures (10 ml) of the strains were grown up and the cells harvested as described in Section 2.2.4.4. The cells were washed by resuspension in 10 ml of 0.01M Tris-HCl buffer pH 7.5 containing 0.15M NaCl and harvested as before. The supernatants were removed and the washing procedure repeated. The cells were finally resuspended in 1.25 ml of the buffer and 15 μl of a 1 mg.ml⁻¹ solution of lysostaphin added to each sample. The mixtures were then incubated
Samples of the culture supernatants of the different strains were concentrated 2-fold. Volumes of these samples taken for electrophoresis were calculated to correct for differences in the final cell densities of the cultures and contained approximately 500 μg of protein. The samples were electrophoresed in a 10% (w/v) acrylamide gel in the presence of SDS and stained and destained by method A (Section 2.2.7.5.).

Track 1 extracellular proteins of *S. aureus* strain Wood 46.
Track 2 extracellular proteins of *S. aureus* strain RN1.
Track 3 extracellular proteins of *S. aureus* strain RN25.
Track 4 extracellular proteins of *S. aureus* strain RN27.
Track 5 extracellular proteins of *S. aureus* strain RN450.
Track 6 extracellular proteins of *S. aureus* strain A003.
Track 7 extracellular proteins of *S. aureus* strain RN451.
Track 8 extracellular proteins of *S. aureus* strain RN740.
Plate 3.1. Patterns of total extracellular protein production of different strains of Staphylococcus aureus

Samples were then treated with 5 μl of a 1 mg ml⁻¹ solution of Elamine and incubated for a further 10 min at 37°C. The viscosity of the samples decreased.

Samples of each lysate (0.5 μl) were treated and heat-treated as described in Materials and Methods. The ELA activity was assayed as described in section 5.2.4.4. No activity was detected on the surface of the Delta-2,2,2,6,6-lysates. All lysates used in the assay were treated with 50 μl of a 0.2 M Tris-HCl buffer, pH 7.4 and then heated to 90°C and then cooled to 0°C. No activity was detected on the surface of the lysates.

The experiment was repeated using lysates prepared from the Delta-2,2,2,6,6-lysates. The activity of the Delta-2,2,2,6,6-lysates was significantly increased compared to the Delta-2,2,2,6,6-lysates. The activity of the lysates was determined using the ELA assay as described in section 5.2.4.4. No activity was detected on the surface of the lysates.
for 30 min at 37°C, after which time lysis was apparent by the greatly increased viscosity of the samples due to the release of DNA. The samples were then treated with 5 μl of a 1 mg.ml⁻¹ solution of DNase and incubated for a further 30 min at 37°C. The viscosity of the samples decreased.

Samples of each lysate (0.2 ml), untreated and heat-treated as described in Section 3.1. were then assayed for haemolytic activity as described in Section 2.2.5. The results are shown in Table 3.2.a. No haemolytic activity was detected.

The effects of the cell lysates, lysostaphin and DNase on the activity of delta haemolysin was then investigated. Stock delta haemolysin solution (0.25 mg.ml⁻¹) described in Section 2.2.5.2. was used. Aliquots of 0.25 ml were added to 0.25 ml of each of the cell lysates, untreated and heat-treated, to 0.25 ml of 0.01M Tris-HCl buffer pH 7.5 containing 0.15M NaCl as a control and to buffer containing lysostaphin and DNase in the concentrations used in the lysates. The mixtures were then incubated for 5 min at 37°C and then 80 μl assayed for haemolytic activity as described in Section 2.2.5. The results are shown in Table 3.2.b. It can be seen that neither lysostaphin or DNase had any inhibitory effect on the delta haemolysin, but that all the lysates inhibited the activity of the delta haemolysin completely.

It was possible that any delta haemolysin inside the cells might have been destroyed by proteases released in the cell lysates. This might also explain the inhibition of delta haemolysin by the cell lysates. To investigate this a cell lysate was prepared from *S. aureus* strain RN25 as described before except that when the lysostaphin was added 50 μl of a 0.5 mg.ml⁻¹ solution of PMSF was also added. When 0.2 ml of the lysate was assayed for haemolytic activity, none was detectable. A sample of the lysate (0.1 ml) was
Table 3.2.a  Search for haemolytic activity in cell lysates of different strains of *Staphylococcus aureus*

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Untreated cell lysate</th>
<th>Heat-treated cell lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN25</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>RN450</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>186X</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Table 3.2.b  Investigation of the effects of cell lysates, lysostaphin and DNAase on the activity of delta haemolysin

<table>
<thead>
<tr>
<th>Sample</th>
<th>Haemolytic activity (HU.ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated RN25 cell lysate</td>
<td>0.0</td>
</tr>
<tr>
<td>Heat-treated RN25 cell lysate</td>
<td>0.0</td>
</tr>
<tr>
<td>Untreated RN450 cell lysate</td>
<td>0.0</td>
</tr>
<tr>
<td>Heat-treated RN450 cell lysate</td>
<td>0.0</td>
</tr>
<tr>
<td>Untreated 186X cell lysate</td>
<td>0.0</td>
</tr>
<tr>
<td>Heat-treated 186X cell lysate</td>
<td>0.0</td>
</tr>
<tr>
<td>Lysostaphin</td>
<td>12.25</td>
</tr>
<tr>
<td>DNAase</td>
<td>12.75</td>
</tr>
<tr>
<td>Control</td>
<td>11.75</td>
</tr>
</tbody>
</table>
incubated with 0.1 ml of the stock delta haemolysin solution as before and then 80 µl assayed for haemolytic activity. Again the delta haemolysin was completely inhibited.

In conclusion, although no haemolytic activity was detected in the cell lysates, the inhibitory effects of one or more components of the cell lysates may have masked the activity of any intracellular delta haemolysin. Such inhibition may be due to the presence of membrane material in the lysates which would be expected to bind delta haemolysin.

3.4. Investigation of the growth of the strain of Staphylococcus aureus chosen for further study and its production of delta haemolysin

_S. aureus_ strain RN25 was chosen for further study as it exhibited fairly high production of delta haemolysin with lower levels of alpha and beta haemolysins (refer to Section 3.1.) and total extracellular protein (refer to Plate 3.1.) when compared with the other strains investigated. It was hoped that this would minimise the problems of contamination in the purification of delta haemolysin.

The growth of the strain in yeast diffusate medium was investigated. Sterile yeast diffusate medium (500 ml) in a 2 l Erlenmeyer flask was inoculated with 5 ml of a starter culture of _S. aureus_ strain RN25 and the culture grown as described in Section 2.2.4.4. At recorded time intervals 1 ml samples were removed, 0.5 ml was heat-treated and then assayed for haemolytic activity (Section 2.2.5.) and the remainder used to estimate growth spectrophotometrically at 540 nm (Section 2.2.4.4.). Measurement of the total extracellular protein produced was not possible due to the presence of high amounts of "Lowry-positive" material in the rich yeast diffusate medium.

The results are illustrated in Figure 3.2. It can be seen that delta haemolysin first appears in the supernatant in the late
Figure 3.2. Investigation of the growth of *Staphylococcus aureus* strain RN25 and its production of delta haemolysin

Samples of the culture were taken at the times indicated and growth estimated spectrophotometrically at 540 nm (o). Heat-treated samples of the culture supernatant were assayed for haemolytic activity (e). Growth was at 37°C in yeast diffusate medium.
exponential phase of growth and that the amount of delta haemolysin in
the supernatant levels off in the stationary phase. As delta haemolysin
is known to be a fairly stable molecule, this would indicate that
production of delta haemolysin has ceased.

3.5. The effect of induction of prophage Ø 13 in Staphylococcus aureus
strain RN25 using mitomycin C

If the ability to produce delta haemolysin was directly linked
to the presence of a prophage, for example if the haemolysin was a
phage protein or its production was controlled by phage protein, then
induction of the prophage might be expected to result in a surge in
production of delta haemolysin.

Induction of the prophage was first attempted in the rich yeast
diffusate medium. A range of concentrations of mitomycin C was used;
1 μg.ml⁻¹, 2.5 μg.ml⁻¹ and 5 μg.ml⁻¹ in 50 ml cultures. A control
culture contained no mitomycin C. Aliquots of yeast diffusate medium
(50 ml) were inoculated with 0.1 ml of a starter culture of S.aureus
strain RN25 and the cultures allowed to grow to the early exponential
phase of growth (OD₅₄₀ of 0.4). The appropriate amount of mitomycin C
(400 μg.ml⁻¹ in sterile water) was added and the cultures allowed to
continue growing for 30 min at 37°C. The cells were then harvested
by centrifuging at 10 000 g for 15 min at 20°C (preparative centrifuge)
and washed by resuspension in 50 ml of sterile medium at 37°C. The
cells were harvested as before and again resuspended in 50 ml of
prewarmed sterile medium. The cultures were then allowed to continue
growing. At timed intervals samples were removed for estimation of
growth spectrophotometrically at 540 nm.

Figure 3.3. illustrates the results. The higher concentrations
of mitomycin C were judged to have led to cell death as no growth
was observed. The lowest concentration of mitomycin C was found to
Figure 3.3. The effect of varying concentrations of mitomycin C on the growth of Staphylococcus aureus strain RN25 in yeast diffusate medium.

Aliquots of yeast diffusate medium (50 ml) were inoculated with 0.1 ml of a starter culture of S. aureus strain RN25 and the cultures allowed to grow until they reached the early exponential phase of growth ($O_{540} = 0.4$). The cultures were then treated with various concentrations of mitomycin C, as described in the text. Growth was monitored spectrophotometrically at 540 nm. Control (o), 1 μg.ml$^{-1}$ mitomycin C (o), 2.5 μg.ml$^{-1}$ mitomycin C (□), 5 μg.ml$^{-1}$ mitomycin C (■).
affect the growth of the culture, but did not result in a drop in the cell density of the culture, which would be expected as a result of cell lysis, due to induction of the prophage. This was thought to be due to the growth of the bacteria in the rich yeast diffusate medium, outstripping lysis by the phage. The control culture exhibited a normal pattern of growth, but the final cell density was low. This could have been due to the manipulations of the cells, for example the centrifugation steps may have resulted in excessive clumping of the cells, resulting in low $A_{540}$ readings.

The experiment was repeated in a less rich medium, trypticase soy broth (TSB) in an attempt to produce more pronounced cell lysis by the phage. A concentration of 0.8 $\mu$g.ml$^{-1}$ of mitomycin C was used. Growth was monitored spectrophotometrically at 540 nm and heat-treated samples of the culture supernatants were assayed for haemolytic activity.

The results are shown in Figure 3.4. More pronounced lysis of the bacteria by induced phage was observed, but no surge in delta haemolysin production was apparent. The pattern of growth of the control culture was normal, but the final cell density of the culture was even lower than in the previous experiment. The additional decrease in the final cell density of the control culture was probably due to the less rich medium. Production of delta haemolysin by the control culture in the less rich TSB medium was also reduced.
Two aliquots of TSB medium (50 ml) were inoculated with 0.1 ml of a starter culture of *Staphylococcus aureus* strain RN25 and the cultures allowed to grow until they reached the early exponential phase of growth (\(OD_{540} = 0.4\)). One of the cultures was then treated with 0.8 \(\mu g/ml\) of mitomycin C as described in the text. The other culture was a control and was treated in the same way but without the addition of mitomycin C. Growth was monitored spectrophotometrically at 540 nm, control (\(\odot\)), mitomycin C treated culture (\(\bullet\)). Heat-treated samples of the culture supernatant were assayed for delta haemolysin, control (\(\square\)), mitomycin C treated culture (\(\blacksquare\)).
CHAPTER FOUR
PURIFICATION OF DELTA HAEMOLYSIN

4.1. Comparison of three methods for the purification of delta haemolysin from the culture supernatant of Staphylococcus aureus strain RN25

Delta haemolysin was purified from the culture supernatant of S. aureus strain RN25 by three well documented methods, in order to compare the properties of the products, their purity and the efficiency of the different procedures.

4.1.1. Purification of delta haemolysin by the method of Kantor et al. (1972)

This method involved adsorption of the delta haemolysin to aluminium hydroxide gel (Alumina C gel). Culture supernatant (1 l) was assayed for haemolytic activity and protein concentration and then added to 608 ml of a 10 mg.ml\(^{-1}\) suspension of the alumina gel in water and stirred slowly at room temperature. The mixture had a gel to protein ratio of 1.5 which pilot scale purifications had shown to result in 90% adsorption of the haemolytic activity. Assay of the supernatants of 1 ml samples of the gel mixture for haemolytic activity, revealed that there had been 90% adsorption of the haemolytic activity after 45 min. The mixture was then centrifuged at 150 g for 5 min at room temperature (preparative centrifuge). The supernatant was removed and assayed for residual haemolytic activity and protein concentration. The gel was then washed four times with 0.08M phosphate buffer pH 7.2 by resuspension in 1 l of the buffer and centrifuging as before. Each wash was assayed for haemolytic activity and protein concentration. The adsorbed haemolytic activity was then eluted from the gel by resuspension in 100 ml of 0.5M phosphate buffer pH 7.2 and centrifuging as before. The elution procedure was repeated nine times. The eluates
were assayed for haemolytic activity, pooled accordingly and reassayed. The protein concentration of the eluate could not be determined by the method of Lowry (1951) because of interference due to the high concentration of phosphate. The pooled eluate was dialysed against water at 4°C. A precipitate which appeared was removed by centrifuging at 6,000 g for 10 min at 4°C and then lyophilised. The supernatant was assayed for haemolytic activity and protein concentration and then concentrated using an Amicon filtration system with a UM20E filter (exclusion limit 10,000 - 20,000 daltons, Amicon Corporation, Lexington, Mass., U.S.A.). The concentrate was then lyophilised.

The results of the purification are shown in Table 4.1. The recovery was low, 60% of the haemolytic activity adsorbed to the gel was eluted but large losses took place during the long dialysis and concentration steps. The purification and the specific activity of the product were the lowest of the three purification procedures. The appearance of a precipitate on dialysis was comparable with that reported by Kreger et al. (1971) and referred to as "insoluble" delta haemolysin. The precipitate represented 5% of the initial activity of the supernatant, but only accounted for 20% of the loss of activity during dialysis. The specific activity of the precipitate was slightly higher than that of the product.

4.1.2. Purification of delta haemolysin by the method of Kreger et al. (1971)

This method involved adsorption of the delta haemolysin to hydroxylapatite gel (Biogel HTP). Culture supernatant (1.5 l) was stirred with 30 g of hydroxylapatite gel for 3 h at room temperature and then left to stand overnight at 4°C. Assay of the supernatant of a sample of the gel mixture for haemolytic activity revealed that there had been 95% adsorption of the haemolytic activity to the gel.
Table 4.1. Purification of delta haemolysin from the culture supernatant of *Staphylococcus aureus* strain RN25 by the method of Kantor et al. (1972)

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (HU)</th>
<th>Specific activity (HU.mg(^{-1}))</th>
<th>Recovery (%)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>1 000</td>
<td>4.050</td>
<td>45 000</td>
<td>11.1</td>
<td>100.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Gel supernatant</td>
<td>1 610</td>
<td>3.800</td>
<td>6 762</td>
<td>-</td>
<td>(15.0)</td>
<td></td>
</tr>
<tr>
<td>Adsorbed on gel</td>
<td>-</td>
<td>250</td>
<td>38 238</td>
<td>-</td>
<td>85.0</td>
<td></td>
</tr>
<tr>
<td>Washes 1 - 4</td>
<td>4 375</td>
<td>131</td>
<td>7 438</td>
<td>-</td>
<td>(16.5)</td>
<td></td>
</tr>
<tr>
<td>Adsorbed on gel</td>
<td>-</td>
<td>119</td>
<td>30 800</td>
<td>-</td>
<td>68.4</td>
<td></td>
</tr>
<tr>
<td>Eluted from gel</td>
<td>1 000</td>
<td>-</td>
<td>19 200</td>
<td>-</td>
<td>42.7</td>
<td></td>
</tr>
<tr>
<td>After dialysis</td>
<td>1 051</td>
<td>189</td>
<td>8 093</td>
<td>42.8</td>
<td>18.0</td>
<td></td>
</tr>
<tr>
<td>Precipitate</td>
<td>-</td>
<td>38.7</td>
<td>2 421</td>
<td>62.5</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>After concentration and lyophilisation</td>
<td>-</td>
<td>106.3</td>
<td>5 526</td>
<td>52.0</td>
<td>12.3</td>
<td>4.7</td>
</tr>
</tbody>
</table>
The mixture was then centrifuged at 1,500 g for 5 min at 4°C. The supernatant was removed and assayed for haemolytic activity and protein concentration. The gel was washed by resuspension in 150 ml of 0.01M phosphate buffer pH 6.8, stirring for 1 h at room temperature and centrifuging as before. The washing procedure was repeated 5 times and the resulting supernatants assayed for haemolytic activity and protein concentration. The gel was then washed as described before but with 0.4M phosphate buffer pH 6.8. The supernatants were assayed for haemolytic activity, but it was not possible to determine the protein concentration by the method of Lowry (1951) because of interference by the high concentration of phosphate. The haemolytic activity was eluted from the gel by washing as described before but with 1.0M phosphate buffer pH 7.4. The eluates were assayed for haemolytic activity, pooled accordingly and then reassayed. Again it was not possible to determine the protein concentration. The pooled eluate (900 ml) was then dialysed against water at 4°C. The resulting precipitate was removed by centrifuging at 6,000 g for 10 min at 4°C and then lyophilised. The supernatant was concentrated by dialysis against a 20% (w/v) solution of polyethylene glycol 20,000 at 4°C and then lyophilised.

The results of the purification are shown in Table 4.2. The recovery was better than that of the method of Kantor et al. (1972), however, using this method of purification there were also great losses of haemolytic activity occurring in the long dialysis and concentration steps. The appearance of a precipitate in the dialysis step was previously reported by Kreger et al. (1971) and was described as "insoluble" delta haemolysin. They reported that it represented 35% of the total haemolytic activity purified and had a specific activity of 2 times that of the "soluble" delta haemolysin product. The precipitate which appeared in the dialysis step of this study
Table 4.2. Purification of delta haemolysin from the culture supernatant of *Staphylococcus aureus* strain RN25 by the method of Kreger et al. (1971)

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (HU)</th>
<th>Specific activity (HU.mg(^{-1}))</th>
<th>Recovery (%)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>1 500</td>
<td>6 075</td>
<td>73 200</td>
<td>12.0</td>
<td>100.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Gel supernatant</td>
<td>1 500</td>
<td>4 740</td>
<td>5 625</td>
<td>-</td>
<td>(7.7)</td>
<td></td>
</tr>
<tr>
<td>Adsorbed on gel</td>
<td>-</td>
<td>1 335</td>
<td>67 575</td>
<td>-</td>
<td>92.3</td>
<td></td>
</tr>
<tr>
<td>Pooled washes (1)</td>
<td>900</td>
<td>279</td>
<td>0</td>
<td>-</td>
<td>(0.0)</td>
<td></td>
</tr>
<tr>
<td>Adsorbed on gel</td>
<td>-</td>
<td>1 056</td>
<td>67 575</td>
<td>-</td>
<td>92.3</td>
<td></td>
</tr>
<tr>
<td>Pooled washes (2)</td>
<td>900</td>
<td>-</td>
<td>8 100</td>
<td>-</td>
<td>(11.1)</td>
<td></td>
</tr>
<tr>
<td>Adsorbed on gel</td>
<td>-</td>
<td>-</td>
<td>59 475</td>
<td>-</td>
<td>81.2</td>
<td></td>
</tr>
<tr>
<td>Eluted from gel</td>
<td>900</td>
<td>-</td>
<td>54 000</td>
<td>-</td>
<td>73.8</td>
<td></td>
</tr>
<tr>
<td>After dialysis</td>
<td>1 011</td>
<td>313</td>
<td>24 770</td>
<td>79.1</td>
<td>33.8</td>
<td></td>
</tr>
<tr>
<td>Precipitate</td>
<td>-</td>
<td>51</td>
<td>4 551</td>
<td>89.1</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>After concentration and lyophilisation</td>
<td>-</td>
<td>318</td>
<td>21 260</td>
<td>66.9</td>
<td>29.0</td>
<td>5.6</td>
</tr>
</tbody>
</table>
represented 21% of the total haemolytic activity purified and had a specific activity of 1.3 times that of the "soluble" delta haemolysin product. The "insoluble" delta haemolysin only accounted for 16% of the loss of activity after the dialysis step. The purification and specific activity of the product were also higher than achieved by the method of Kantor et al. (1972).


This method involved precipitation of delta haemolysin from an acidified, heat-treated culture supernatant by the addition of chloroform (1.5% v/v). The haemolysin was then extracted from the washed precipitate into a mixture of chloroform and methanol (2:1 v/v) and purified by transfer between the 2 phases of a chloroform, methanol, water mixture (10:5:3 by volume). In this mixture the haemolysin should distribute mainly in the lower phase under neutral conditions and in the upper phase under acid conditions (Heatley, 1971).

Culture supernatant (2.65 l) was assayed for haemolytic activity and protein concentration and then heated to 60°C and maintained at that temperature with occasional stirring for 1 h. The resulting precipitate was removed by centrifuging at 16 000 g for 45 min at 20°C. The supernatant was removed, assayed for haemolytic activity and protein concentration and then adjusted to pH 5.3 by the addition of 5M H₃PO₄. Chloroform (37.5 ml) was added to the supernatant and the mixture stirred slowly overnight at room temperature. The dark grey precipitate that appeared was collected by centrifuging as before but at 4°C and then washed by resuspension in 120 ml of 0.01M phosphate buffer pH 6.8, saturated with chloroform, and collected by centrifuging as before. The precipitate was then resuspended in 270 ml of a chloroform, methanol mixture (2:1 by volume) and allowed to stand overnight. The precipitate was removed by centrifuging as before and
the clear yellowish supernatant collected after filtration through Whatman No1 filter paper. Phosphate buffer, 0.1M pH 6.8 (50 ml), was added to the filtrate in a 500 ml separating funnel, the contents mixed thoroughly and left to stand for a few hours to allow the 2 phases to separate. The lower layer (expected to contain the delta haemolysin) was removed and the upper layer extracted a second time with 170 ml of a chloroform, methanol, water mixture (86:14:1 by volume). The lower layer was again removed and pooled with that of the first extraction. The pooled lower layers were then transferred to a clean separating funnel and sequentially extracted five times with 20 ml of an acidified chloroform, methanol, water mixture (3:48:47 by volume) containing respectively 100, 300, 200, 200 and 200 μl of 1M H₃PO₄. The 20 ml acid extractions now expected to contain the delta haemolysin were then treated with 125 ml of acetone to precipitate the protein. The precipitates were collected by centrifuging as before and washed by resuspension in a further 125 ml of acetone, collected as before and then dried under vacuum overnight.

The results of the purification are outlined in Table 4.3. This method resulted in the highest recovery and specific activity of the product of the three purification methods investigated. The method was also simpler and quicker than the other 2 methods described. In further purifications of delta haemolysin this method was used.

4.2. Assessment of the purity and characterisation of the products of the three purification procedures

The products of the three purification procedures were subjected to SDS PAGE, native PAGE, amino acid analysis, N-terminal sequence analysis and silica gel thin layer chromatography. In each system the properties of the products were compared, in order to determine which method resulted in the purest product. The properties of the
Table 4.3. Purification of delta haemolysin from the culture supernatant of *Staphylococcus aureus* strain RN25 by the method of Heatley (1971)

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (HU)</th>
<th>Specific activity (HU mg⁻¹)</th>
<th>Recovery (%)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>2 650</td>
<td>10 733</td>
<td>119 250</td>
<td>11.1</td>
<td>100.0</td>
<td>1.0</td>
</tr>
<tr>
<td>After heat-treatment</td>
<td></td>
<td></td>
<td>78 705</td>
<td>-</td>
<td>66.0</td>
<td></td>
</tr>
<tr>
<td>Final product</td>
<td></td>
<td>368</td>
<td>54 855</td>
<td>149.0</td>
<td>46.0</td>
<td>13.4</td>
</tr>
</tbody>
</table>
products of the three purification procedures were also compared with those of delta haemolysin purified by Dr. N.G. Heatley, by his method (Heatley, 1971, 1976) from the culture supernatant of *S. aureus* strain 186X. It was hoped that this would determine whether any differences in the properties of the products were due to the different methods of purification used or were differences in the delta haemolysins of the different strains of *S. aureus*. The delta haemolysin purified by Dr. N.G. Heatley is referred to as "reference" delta haemolysin.

4.2.1. **Polyacrylamide gel electrophoresis (PAGE)**

4.2.1.1. **Demonstration of the difficulty of staining delta haemolysin in polyacrylamide gels containing SDS**

Initial attempts to stain delta haemolysin in polyacrylamide gels containing SDS (5 - 10 μg of protein per sample) were unsuccessful. It was found to be necessary to greatly overload the gels (250 μg of protein per sample) in order to visualise the delta haemolysin. Plates 4.1.a, b) and c) show 20% (w/v) acrylamide gels which were loaded with 2 samples of "reference" delta haemolysin containing 250 and 50 μg of protein. The gels were electrophoresed in the presence of SDS as described in Section 2.2.7.1. The gels were stained by three different methods as described in Section 2.2.7.5.

Plate 4.1.a shows a gel stained by method B, a quick stain for proteins. It can be seen that the sample containing 250 μg of delta haemolysin was faintly stained, but that the sample containing 50 μg of delta haemolysin was not stained at all. Plate 4.1.b shows a gel stained by method D, a stain which uses formaldehyde as a fixative, developed by Steck et al. (1979) specifically for low molecular weight peptides and basic proteins. This method stained the delta haemolysin a little better than the last method although the sample containing
Plate 4.1.a Polyacrylamide gel electrophoresis, in the presence of SDS, of "reference" delta haemolysin

The samples were electrophoresed in a 20% (w/v) acrylamide gel in the presence of SDS and stained and destained by method B (Section 2.2.7.5.).

Track 1, 250 µg "reference" delta haemolysin.
Track 2, 50 µg "reference" delta haemolysin.
Track 3, molecular weight markers (5 µg of each), bovine serum albumin (67 000), ovalbumin (43 000), chymotrypsinogen A (23 200) and cytochrome C (13 400).
Plate 4.1.b  Polyacrylamide gel electrophoresis, in the presence of SDS, of "reference" delta haemolysin

The samples were electrophoresed in a 20% (w/v) acrylamide gel in the presence of SDS and stained and destained by method D (Section 2.2.7.5.).
Track 1, 250 µg "reference" delta haemolysin.
Track 2, 50 µg "reference" delta haemolysin.
50 μg of delta haemolysin was only faintly stained. The gel shown in Plate 4.1.c was stained by method C which contained trichloroacetic acid to fix the protein. This method was found to be the most successful method of staining delta haemolysin. The smearing of the delta haemolysin was at first thought to be due to overloading the gel with protein, but it can be seen from the gels that a lighter loading (50 μg) only results in a fainter smear. The smearing of the delta haemolysin is now thought to be due to the small size and hydrophobic character of the delta haemolysin molecule.

Figure 4.1. shows a plot of the logarithms of the molecular weights of the protein standards from the gel shown in Plate 4.1.a) against their Rf values. From the graph the molecular weight of delta haemolysin was estimated to be between 4 000 and 8 500.

4.2.1.2. SDS PAGE of the products of the three purification procedures

Plate 4.2. shows the results of SDS PAGE of the products of the three purification procedures in a 17.5% (w/v) acrylamide gel. Each sample contained 250 μg of protein. Molecular weight standards (10 μg of each protein) were also electrophoresed. The gel was stained by method C as described in Section 2.2.7.5.

It can be seen from the gel that delta haemolysin purified by the methods of Kantor et al. (1972) and Kreger et al. (1971), tracks 1 and 2 respectively, exhibited multiple additional bands of higher molecular weight as well as the major low molecular weight delta haemolysin component. Tracks 3 and 4 contained samples of delta haemolysin purified by the method of Heatley (1971, 1975) from the culture supernatant of S. aureus strain RN25, extractions 1 and 2 respectively. These samples showed no additional bands and were identical to "reference" delta haemolysin in track 5.

From a plot of the logarithms of the molecular weights of the
Plate 4.1.c Polyacrylamide gel electrophoresis, in the presence of SDS, of "reference" delta haemolysin

The samples were electrophoresed in a 20% (w/v) acrylamide gel in the presence of SDS and stained and destained by method C (Section 2.2.7.5.).

Track 1, 250 µg "reference" delta haemolysin.

Track 2, 50 µg "reference" delta haemolysin.
The standards used were bovine serum albumin (67 000), ovalbumin (43 000), chymotrypsinogen A (23 200) and cytochrome C (13 400). Their \( R_f \) values were measured from the gel shown in Plate 4.2. From the \( R_f \) value of delta haemolysin its molecular weight was estimated to be between 4 000 and 8 500.
The samples were electrophoresed in a 17.5\% (w/v) acrylamide gel in the presence of SDS and stained and destained by method C (Section 2.2.7.5.).

Track 1, 250\( \mu \)g of the product of the purification procedure of Kantor et al. (1972).

Track 2, 250\( \mu \)g of the product of the purification procedure of Kreger et al. (1971).

Track 3, 250\( \mu \)g of the product of the purification procedure of Heatley (1971 and 1976) extraction 1.

Track 4, 250\( \mu \)g of the product of the purification procedure of Heatley (1971 and 1976) extraction 2.

Track 5, 250\( \mu \)g "reference" delta haemolysin.

Track 6, molecular weight standards (5\( \mu \)g of each), bovine serum albumin (67 000), ovalbumin (43 000), chymotrypsinogen A (23 200) and cytochrome C (13 400).

Track 7, 250\( \mu \)g melittin.

Track 8, 250\( \mu \)g "insoluble" delta haemolysin from the method of Kantor et al. (1972).

Track 9, 250\( \mu \)g "insoluble" delta haemolysin from the method of Kreger et al. (1971).
Plate 4.2. Polyacrylamide gel electrophoresis, in the presence of SDS, of the products of the different purification procedures and "reference" delta haemolysin.

...
protein standards against their Rf values measured from the gel, the molecular weight of delta haemolysin was estimated to be 6400.

Tracks 8 and 9 contain samples of the "insoluble" delta haemolysin which precipitated in the dialysis steps of the methods of Kantor et al. (1972) and Kreger et al. (1971) respectively. The major low molecular weight delta haemolysin band can be seen, but again, as with the "soluble" delta haemolysin from these methods of purification, additional higher molecular weight bands were observed and in greater numbers.

It can be seen that only delta haemolysin purified by the method of Heatley (1971, 1976) had been purified to homogeneity, as determined by SDS PAGE, and that differences in the preparations of delta haemolysin appear to be due to the methods employed for its preparation.

It is interesting to note that melittin (track 7 in the gel shown in Plate 4.2.) also runs as a smear. Melittin has a similar molecular weight to that of delta haemolysin and also shares many of its surface-active properties. This would suggest that the smearing effect observed when these lytic proteins are electrophoresed in the presence of SDS may be due to their small size and unusual properties.

4.2.1.3. Polyacrylamide gradient gel electrophoresis of the products of the three purification procedures in the presence of SDS

Polyacrylamide gradient gels with SDS were used in an attempt to improve the resolution of the delta haemolysin band. The gels were constructed from linear gradients of 10 - 30% (w/v) acrylamide in the presence of SDS as described in Section 2.2.7.2. and stained by method C (Section 2.2.7.5.). An example of the resolution achieved is shown in Plate 4.3. The delta haemolysin still ran as a diffuse band.
The samples were electrophoresed in a gel constructed from a linear gradient of 10 - 30% (w/v) acrylamide in the presence of SDS and stained and destained by method C (Section 2.2.7.5.).

Track 1, myoglobin molecular weight markers (16 900, 14 400 and 6 000 - 8 000).

Track 2, 250 µg of the product of the purification procedure of Kantor et al. (1972).

Track 3, 250 µg of the product of the purification procedure of Kreger et al. (1971).

Track 4 and 5, 5 µg bovine serum albumin (67 000).

Track 6, 250 µg of the product of the purification procedure of Heatley (1971 and 1976).

Track 7, 250 µg "reference" delta haemolysin.
Plate 4.3. Polyacrylamide gradient gel electrophoresis, in the presence of SDS, of the products of the different purification procedures and "reference" delta haemolysin.
4.2.1.4. Polyacrylamide gel electrophoresis of the products of the three purification procedures under native conditions

Electrophoresis of the preparations of delta haemolysin was attempted in alkaline systems without SDS as described in Section 2.2.7.3. The protein did not enter 10% (w/v) acrylamide gels, but remained at the origin whether electrophoresis was towards the anode or the cathode.

4.2.1.5. Native polyacrylamide gel electrophoresis of the products of the three purification procedures in the acidic system of Reisfeld et al. (1962)

Samples of the preparations of delta haemolysin were subjected to electrophoresis in the acidic system of Reisfeld et al. (1962) as described in Section 2.2.7.4. Two samples of each preparation of the toxin (each containing 250 µg of protein) were loaded, one on each half of the gel. After electrophoresis half of the gel was stained by method C (Section 2.2.7.5.) and is shown in Plate 4.4. Each sample resulted in a diffuse protein band. The sample prepared by the method of Heatley (1971 and 1976) and "reference" delta haemolysin behaved identically. The products of the purification procedures of Kantor et al. (1972) and Kreger et al. (1971) had slightly lower mobilities.

The other half of the gel was cut into tracks and each track cut into 5 mm sections. These were pulverised in 0.2 ml of PBS1 and eluted overnight at 37°C. The supernatants were assayed for haemolytic activity. The results are shown in Figure 4.2. The activity in each track was found to coincide with the diffuse bands visualised on the other half of the gel. The differences in the levels of haemolytic activity detected in the gel reflect the differences in the specific activities of the preparations.
The samples were electrophoresed in a 15% (w/v) acrylamide gel towards the cathode. The gel was stained and destained by method C (Section 2.2.7.5.).

Track 1, 250 µg of the product of the purification procedure of Kantor et al. (1972).

Track 2, 250 µg of the product of the purification procedure of Kreger et al. (1971).


Track 4, 250 µg "reference" delta haemolysin.
Plate 4.4. Native polyacrylamide gel electrophoresis of the products of the different purification procedures and "reference" delta haemolysin in the acidic system of Reisfeld et al. (1962)
Samples were electrophoresed in a 15% (w/v) acrylamide gel towards the cathode. One half of the gel was stained with protein stain and is shown in Plate 4.4. The other half was cut into tracks and each track sliced into 5 mm segments. These were eluted in 0.2 ml of PBS1 overnight at 37°C and the eluent assayed for haemolytic activity.

The product of the purification procedure of Kantor et al. (1972), 250 μg, (○).

The product of the purification procedure of Kreger et al. (1971), 250 μg, (○).

The product of the purification procedure of Heatley (1971 and 1976), 250 μg, (■).

"Reference" delta haemolysin, 250 μg, (□).
Figure 4.2. Native polyacrylamide gel electrophoresis of the products of the different purification procedures and "reference" delta haemolysin in the acidic system of Reisfeld et al. (1962); location of haemolytic activity in the gel.
4.2.2. Amino acid analysis of the products of the different purification procedures

The products of the three purification procedures were subjected to amino acid analysis as described in Section 2.2.8. and compared with the amino acid analysis of "reference" delta haemolysin.

Samples were hydrolysed for 24, 48 and 72 h and the results normalised by the method of Ambler (1975). The results are summarised in Table 4.4. The analyses of all the samples were similar. They all lacked proline, tyrosine, histidine and arginine. There were slight differences in the levels of some of the other amino acids in each of the preparations, however, the analysis of the product of the method of Heatley (1971, 1976) was very similar to that of "reference" delta haemolysin. This also suggests that differences in the preparations are due to the methods of purification employed.

4.2.3. N-terminal sequence analysis of the different preparations of delta haemolysin

Samples of delta haemolysin were subjected to N-terminal sequence analysis as described in Section 2.2.9. The products of the purification procedures of Kantor et al. (1972) and Heatley (1971, 1976) both yielded N-terminal sequences which agreed with that found for "reference" delta haemolysin. These sequences also agreed with the whole sequence of delta haemolysin, worked out by Fitton et al. (1980). The results are outlined in Figure 4.3.

Repeated attempts to determine the N-terminal sequence of the product of the method of Kreger et al. (1971) were unsuccessful. This suggested the presence of an N-terminal blocking group. Fitton et al. (1980) reported that delta haemolysin purified from the supernatant of S. aureus strain 186X by the method of Heatley (1971, 1976) ("reference" delta haemolysin) contained a high proportion of protein containing
Table 4.4. Amino acid analyses of the products of the different purification procedures and "reference" delta haemolysin

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Kantor et al. (1972)</th>
<th>Kreger et al. (1971)</th>
<th>Heatley (1971 and 1976)</th>
<th>&quot;Reference&quot; delta haemolysin</th>
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<td>ASX (4)</td>
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<td>3.0</td>
</tr>
<tr>
<td>THR (3)</td>
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<td>2.4</td>
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</tr>
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</tr>
<tr>
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<td>0.0</td>
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<td>1.8</td>
</tr>
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<td>-</td>
</tr>
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<td>1.6</td>
<td>1.0</td>
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<td>-</td>
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</tr>
<tr>
<td>ARG (0)</td>
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Amino acid analyses were performed as described in Section 2.2.8. The numbers in parentheses represent the number of amino acids predicted from the sequence worked out by Pitton et al. (1980).
Figure 4.3. N-terminal sequence analyses of the products of the different purification procedures and "reference" delta haemolysin

<table>
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</tr>
<tr>
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<tr>
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<td></td>
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<tr>
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<tr>
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</tr>
<tr>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COOH</td>
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</tbody>
</table>

X = no PTH amino acid detected. Lysine residues and the N-terminal residue are not usually seen as the attachment of the proteins to the glass support involves their amino groups (Section 2.2.9.).
N-formyl methionine at the N-terminus. The product of the method of Kreger et al. (1971) was therefore treated with 1M HCl in methanol for 1 h at room temperature, in an attempt to remove the blocking group. The preparation was then sequenced, and was found to yield a sequence for the first 10 amino acids which was in agreement with those observed for the other preparations of delta haemolysin (Figure 4.3.).

4.2.4. Silica-gel thin layer chromatography (TLC) of the different preparations of delta haemolysin

From the results of N-terminal sequence analysis of the products of the different purification procedures, and from Pitton et al. (1980), it appears that there are at least two forms of delta haemolysin, a blocked form and an unblocked form. As these were not separated by polyacrylamide gel electrophoresis in the presence of SDS, silica-gel TLC was used in an attempt to separate the two forms of delta haemolysin, and as an alternative, for the assessment of the purity of the products of the different purification procedures. TLC was performed as described in Section 2.2.10.

Figures 4.4.a and b illustrate the separation achieved by this method. Figure 4.4.a is a tracing of a heavily loaded TLC plate; 500 μg of each of the preparations of delta haemolysin in 50 μl of water was spotted along the origin. In this case the solvent used was not fresh. It was subsequently found that the best separation was achieved using solvent made up immediately before use. It appeared that on standing the butanol and the acetic acid in the solvent mixture (Section 2.2.10.) reacted to produce a certain amount of the ester. This appeared to alter the properties of the solvent. It can be seen, however, from the trace in Figure 4.4.a that all of the samples were heterogeneous. The preparations from the methods of Kantor et al. (1972) and Kreger et al. (1971) contained additional spots to those observed
Samples of delta haemolysin (500 μg) in 50 μl of water were spotted as bars across the origin and chromatographed for 4 h in n-butanol, water, acetic acid (45:30:25 by volume). The plate was stained with ninhydrin and then traced. The intensity of the spots is indicated by the number to the left of the spot.

1, the product of the purification procedure of Kantor et al. (1972).

2, the product of the purification procedure of Kreger et al. (1971).

3, the product of the purification procedure of Heatley (1971 and 1976).

4, "reference" delta haemolysin.
Figure 4.4.a  Silica-gel thin layer chromatography of the products of the different purification procedures and "reference" delta haemolysin
Samples of delta haemolysin (50 μg) in 10 μl of water were spotted along the origin and chromatographed for 4 h in n-butanol, water, acetic acid (45:30:25, by volume). The plate was stained first with ninhydrin (solid outlines) and then with Erlich's stain for tryptophan-containing peptides (dotted outlines). The intensity of the spots is indicated by the number to the left of the spot.

1, delta haemolysin purified by the method of Kantor et al. (1972).

2, delta haemolysin purified by the method of Kreger et al. (1971).


4, "reference" delta haemolysin.
Figure 4.4.b Silica-gel thin layer chromatography of the products of the different purification procedures and "reference" delta haemolysin
for delta haemolysin prepared by the method of Heatley (1971, 1976) and "reference" delta haemolysin, which appeared to be identical. The two most densely stained spots in each sample were believed to be the blocked and unblocked forms of delta haemolysin, the latter more polar species running behind the blocked form. It can be seen that the product of the method of Kreger et al. (1971) appeared to contain less of the unblocked species than the other preparations.

Figure 4.4.b illustrates a less heavily loaded TLC plate; 50 µg of each sample in 10 µl of water was spotted along the origin. The trace shows the better separation achieved with freshly made solvent. Again the products of the methods of Kantor et al. (1972) and Kreger et al. (1971) can be seen to differ from each other and from the product of the method of Heatley (1971, 1976) and "reference" delta haemolysin. In all the samples the two main spots believed to be the two forms of delta haemolysin were the only spots to be stained by Erlich's stain for tryptophan-containing peptides.

In summary, all the preparations of delta haemolysin were found to be heterogeneous, but to varying degrees. The differences between the preparations of delta haemolysin appeared, in this study, to be a consequence of the methods of purification employed and not due to possible strain-specific differences.

4.3. Further purification of delta haemolysin

As silica-gel thin layer chromatography revealed that all the preparations of delta haemolysin were heterogeneous, it was deemed necessary to attempt further purification of the delta haemolysin. It was hoped that this would provide a homogeneous preparation of delta haemolysin which could then be used in further studies of the effects of chemical modification on delta haemolysin.

Nolte and Kapral (1979) reported the purification of delta
haemolysin using hydrophobic affinity chromatography on octyl and phenyl sepharose. They found that delta haemolysin eluted from these materials in 40 - 50% (v/v) ethanol.

Chromatography on octyl sepharose was used in an attempt to further purify the "best" preparations of delta haemolysin, those prepared by the method of Heatley (1971 and 1976).

4.3.1. **Further purification of delta haemolysin prepared by the method of Heatley (1971 and 1976) using chromatography on octyl sepharose**

The procedure used for further purification of preparations of delta haemolysin is described in Section 2.2.11. Figure 4.5.a illustrates the elution profile obtained for the product of the purification procedure of Heatley (1971, 1976), and Figure 4.5.b that obtained for "reference" delta haemolysin. It can be seen that chromatography of both preparations resulted in one peak of haemolytic activity, each with a leading shoulder which might represent the more polar unblocked species.

For each of the purifications, fractions were pooled as described in the legends to Figures 4.5.a and b. The pooled fractions were lyophilised, taken up in water and assayed for protein concentration (Section 2.2.6.) and haemolytic activity (Section 2.2.5.).

This purification procedure was carried out routinely and the specific activity of the delta haemolysin (pooled fractions from the main part of the haemolytic peak) was always found to have decreased to 50 - 60 HU.mg⁻¹.

4.3.2. **Silica-gel thin layer chromatography of the pooled fractions after chromatography of "reference" delta haemolysin on octyl sepharose**

Samples of known protein concentration (as determined by amino acid analysis), 10 nmol, from each of the poolings (Section 4.3.1.),
Figure 4.5.a Further purification of the product of the purification procedure of Heatley (1971 and 1976) using chromatography on octyl sepharose.

Protein was estimated spectrophotometrically at 280 nm (•).

The fractions were assayed for haemolytic activity (○). Fractions 60 - 73 were taken to be delta haemolysin peak and pooled.
Figure 4.5.b Further purification of "reference" delta haemolysin using chromatography on octyl sepharose

Protein was estimated spectrophotometrically at 280 nm (●). The fractions were assayed for haemolytic activity (○). Fractions 40 - 60, 61 - 80 and 81 - 160 were pooled.
were lyophilised and taken up in a mixture of chloroform and methanol (2:1 by volume). The samples were then spotted along the origin of a TLC plate. Chromatography was performed as described in Section 2.2.10. The results are illustrated in Figure 4.6. Sample 1, which represented the leading shoulder of the peak of haemolytic activity (described in Section 4.3.1.), can be seen to contain the two spots thought to represent the two forms of delta haemolysin. Sample 2, which represented the main part of the haemolytic peak, also contains these two spots, but appears to contain much more of the blocked form of the delta haemolysin than sample 1, and slightly less of the unblocked form. This would support the suggestion that the leading shoulder of the peak of haemolytic activity might represent the more polar unblocked species of delta haemolysin.

4.3.3. Comparison of the amino acid analyses of "reference" delta haemolysin, before and after chromatography on octyl sepharose

Figure 4.7. compares the amino acid analysis of "reference" delta haemolysin (from Section 4.2.2.) with the analysis of "reference" delta haemolysin after further purification using chromatography on octyl sepharose. It can be seen that the amino acid analysis of delta haemolysin after further purification agrees much more closely with the numbers of amino acids predicted from the sequence, elucidated by Fitton et al. (1980), than the analysis of "reference" delta haemolysin before further purification.

4.3.4. Estimation of the native molecular weight of delta haemolysin using gel filtration on Sephadex G-150

Delta haemolysin purified by the method of Heatley (1971 and 1976) and further purified using chromatography on octyl sepharose was used in this experiment.
Samples of the pooled fractions (30 nmol, estimated by amino acid analysis) in 10 μl of water were spotted along the origin. The plate was chromatographed 4 h in n-butanol, water, acetic acid (45:30:25, by volume) and then stained first with ninhydrin (solid outlines and then with Erlich's stain for tryptophan-containing peptides (dotted outlines). The intensity of the spots is indicated by the number to the left of the spot.

1, protein from the pooled fractions 40 - 60.
2, protein from the pooled fractions 61 - 80.
3, protein from the pooled fractions 81 - 160.
Figure 4.6. Thin layer chromatography of pooled fractions after chromatography of "reference" delta haemolysin on octyl sepharose.
Figure 4.7. Comparison of the amino acid analyses of "reference" delta haemolysin before and after chromatography on octyl sepharose

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>&quot;Reference&quot; delta haemolysin before chromatography on octyl sepharose</th>
<th>&quot;Reference&quot; delta haemolysin after chromatography on octyl sepharose</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASX (4)</td>
<td>3.0</td>
<td>3.8</td>
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</tr>
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<td>ARG (0)</td>
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</tr>
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</table>

Amino acid analyses were performed as described in Section 2.2.8. The numbers in parentheses represent the number of amino acids predicted from the sequence elucidated by Fitton et al. (1980).
Sephadex G-150 (dry bead diameter 40 – 120 μm) was used, which has a molecular weight fractionation range of 5 000 – 300 000. The column (1.5 x 90 cm, bed volume 159 ml) was equilibrated in 0.05M phosphate buffer pH 7.5, and calibrated using molecular weight standards for gel filtration; ribonuclease A (13 700), chymotrypsinogen A (25 000), ovalbumin (43 000), albumin (67 000) and aldolase (158 000). The standards (two at a time) were taken up in 1.5 ml of the buffer, so that both proteins were at a concentration of 5 mg ml\(^{-1}\), and applied directly to the surface of the gel. The flow rate was 15 ml h\(^{-1}\) and the operating pressure was 30 cm of water. The eluent was monitored using a continuous-flow spectrophotometer at 280 nm. A calibration curve was constructed for the column by plotting the logarithms of the molecular weights of the protein standards against \(K_{av}\) for each protein (Figure 4.8.).

\[
K_{av} = \frac{V_e - V_0}{V_t - V_0}
\]

where \(V_e = \) the elution volume of the protein
\(V_0 = \) the column void volume (the elution volume for blue dextran 2 000)
\(V_t = \) the total bed volume.

The molecular weight of the delta haemolysin was estimated from the calibration curve after gel filtration of a sample of 4.36 mg of the protein. The delta haemolysin was taken up in 1 ml of buffer and gel filtration performed as described for the protein standards. From the value for \(K_{av}\) obtained for the toxin, the native molecular weight of delta haemolysin was estimated at 160 000.

Comparison of this estimate with the value obtained from the results of polyacrylamide gel electrophoresis in the presence of SDS, would suggest that the toxin exists as a large multimer under native conditions at pH 7.5. This would support Kantor et al. (1972), whose
Figure 4.8. Estimation of the native molecular weight of delta haemolysin using gel filtration on Sephadex G-150

The molecular weight standards were aldolase (158 000), albumin (67 000), ovalbumin (43 000), chymotrypsinogen A (25 000) and ribonuclease A (13 700). After gel filtration of delta haemolysin, its molecular weight was estimated from the calibration curve to be 160 000.
molecular weight estimates for delta haemolysin range from 5 200 to 195 000. They suggested that the toxin exists as a multimeric assembly of identical sub-units.
THE EFFECTS OF CHEMICAL MODIFICATION ON DELTA HAEMOLYSIN

All modifications were carried out on delta haemolysin purified by the method of Heatley (1971 and 1976) and further purified by chromatography on octyl sepharose.

5.1.1. The blocked N-terminus of delta haemolysin

The first indication that the N-terminus of delta haemolysin was blocked was the failure to sequence the N-terminal end of the molecule as described in Section 4.2.3. Subsequently Fitton et al. (1980) reported that mass spectrometry of delta haemolysin purified by the method of Heatley (1971) from the culture supernatant of S. aureus strain 186X ("reference" delta haemolysin) revealed two species of delta haemolysin, one unblocked and one with an N-terminal formyl methionine block. Fitton et al. (1980) estimated that in the preparation of delta haemolysin described above, 80% was blocked and 20% unblocked.

5.1.2. Treatment to remove the N-formyl block from the N-terminus of delta haemolysin

The N-formyl group blocking the N-terminal methionine of proteins can be removed by treating the protein with 1M HCl in methanol for 90 min at room temperature, under these conditions there should be no serious cleavage of peptide bonds and more severe conditions are required for methylation (Glazer et al., 1975).

Two samples of delta haemolysin (2.5 mg, calculated from the amino acid analysis) were lyophilised and taken up in 1.1 ml of methanol. To one sample 100 μl of 12M HCl was added with mixing, and to the other (the control) was added 100 μl of water. The samples were incubated at room temperature for 90 min and then lyophilised. The
samples were taken up in 1 ml of water and lyophilised several times to remove any traces of acid. Finally the samples, in 1 ml of water, were assayed for haemolytic activity and subjected to amino acid analysis and silica-gel thin layer chromatography. The results of 4 experiments are shown in Table 5.1. The specific activities of the products are expressed as percentages of the control samples as the specific activities of different batches of delta haemolysin varied.

It can be seen from the results that after treatment to unblock the N-terminus of the molecule, the specific activity of the delta haemolysin appeared to increase by an average value of 23% of the control samples.

Figure 5.1. illustrates the results of silica-gel thin layer chromatography of the products of experiments 3 and 4. It can be seen that in each case the control sample consists of a main spot believed to be the blocked delta haemolysin and a less densely stained spot believed to be the more polar unblocked species. After treatment to unblock the delta haemolysin it can be seen that the spot thought to represent the unblocked delta haemolysin has increased in intensity, while the spot thought to represent the blocked form had decreased in intensity.

5.2. Removal of the N-terminal methionine residue from delta haemolysin by treatment with cyanogen bromide

Delta haemolysin, 0.5 mg and 2.0 mg (determined by amino acid analysis) was lyophilised and taken up in 70% (w/v) formic acid containing 1mM dithiothreitol, 50 μl and 200 μl respectively. To the sample containing 2.0 mg of protein was added 4 mg of cyanogen bromide (100 mg.ml⁻¹ in 70% (w/v) formic acid containing 1mM dithiothreitol). Both samples were flushed with nitrogen and incubated in the dark at room temperature. Samples of 50 μl were taken from the sample treated
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Specific activity of the product (expressed as a percentage of the controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>112</td>
</tr>
<tr>
<td>2</td>
<td>124</td>
</tr>
<tr>
<td>3</td>
<td>115</td>
</tr>
<tr>
<td>4</td>
<td>139</td>
</tr>
</tbody>
</table>
1. delta haemolysin from experiment 3 (control).
2. delta haemolysin from experiment 3, treated to remove the N-formyl block.
3. delta haemolysin from experiment 4 (control).
4. delta haemolysin from experiment 4, treated to remove the N-formyl block.
Figure 5.1. Silica-gel thin layer chromatography of delta haemolysin treated to remove the N-formyl block from the N-terminus.
with cyanogen bromide after 6, 12 and 24 h. The control sample (not treated with cyanogen bromide) was incubated for 24 h. These samples (containing 0.5 mg of protein) were diluted with 10 volumes of water and lyophilised.

The protein in the samples was purified by gel filtration on Sephadex G-25 in 5% (v/v) acetic acid. Each sample was taken up in 0.5 ml of 5% (v/v) acetic acid and loaded onto a column of Sephadex G-25 (bed volume, 25 ml) which had been equilibrated in 5% (v/v) acetic acid. Fractions of 1 ml were collected and their protein content estimated spectrophotometrically at 280 nm. The fractions containing the protein peak were pooled and lyophilised. Each sample was taken up in 1 ml of water and assayed for haemolytic activity, protein concentration and subjected to amino acid analysis. The absorption spectra of the samples were also recorded.

Table 5.2 illustrates the results. It can be seen that 88% of the methionine was removed after only 6 h and that the specific activity had fallen to 80% of the control. Longer incubation of the samples with the cyanogen bromide did not appear to remove any more methionine or alter the specific activity further. The absorption spectra recorded for the samples were identical with spectra recorded for untreated delta haemolysin, the OD$_{280}$,mg$^{-1}$ ranging from 1.06 - 1.45.

5.3. Acetylation of delta haemolysin using acetic anhydride

Samples of delta haemolysin (2 - 4 mg) were lyophilised and taken up in 0.8 ml of 0.5M phosphate buffer pH 7.2. To samples for acetylation, 20μl of acetic anhydride in 80μl of redistilled methanol was added. To control samples 100μl of redistilled methanol was added. The reactions were allowed to proceed at room temperature for 15 min and then the samples were frozen at -86°C in a mixture of solid carbon dioxide and acetone. The samples were stored at -20°C.
Table 5.2. Removal of the N-terminal methionine residue from delta haemolysin by treatment with cyanogen bromide

<table>
<thead>
<tr>
<th>Sample</th>
<th>Methionine remaining (expressed as a percentage of the control)</th>
<th>Specific activity (expressed as a percentage of the control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100.0</td>
</tr>
<tr>
<td>After 6 h incubation</td>
<td>12</td>
<td>80.5</td>
</tr>
<tr>
<td>After 12 h incubation</td>
<td>11</td>
<td>70.7</td>
</tr>
<tr>
<td>After 24 h incubation</td>
<td>15</td>
<td>81.4</td>
</tr>
</tbody>
</table>

The amount of methionine remaining in the samples after treatment with cyanogen bromide was determined by comparison of the amino acid analyses of the samples with that of the control.
The protein was purified from other reaction products by gel filtration on G-50 in 0.1M ammonium acetate buffer pH 4.5. The fractions constituting the protein peaks, estimated spectrophotometrically at 280 nm, were pooled and lyophilised. The samples were taken up in 1 ml of water and then assayed for haemolytic activity and protein concentration. The samples were also subjected to amino acid analysis. The extent of acetylation was estimated by comparison of the reaction of acetylated samples and their control samples with ninhydrin reagent as described in Section 2.2.12.

The results are outlined in Table 5.3. It can be seen that acetylation of delta haemolysin does lead to reduction of its specific activity. From the three experiments carried out it seems that three amino groups are very susceptible to acetylation and result in a reduction of the specific activity to 40 - 80% of the control samples. Acetylation of a fourth group as observed in experiment 1 resulted in reduction of the specific activity of delta haemolysin to 20% of the control.

Figure 5.2. illustrates the results of silica-gel thin layer chromatography of acetylated delta haemolysin and a control sample. After staining with ninhydrin, which reacts with amino groups, as expected the acetylated delta haemolysin only shows up as a faint spot running ahead of the control sample. After staining with Erlich's reagent for tryptophan-containing peptides, the spot believed to be acetylated delta haemolysin shows up more strongly.

The absorption spectrum of the acetylated delta haemolysin was the same as that recorded for the control sample.
Table 5.3. Acetylation of delta haemolysin using acetic anhydride

<table>
<thead>
<tr>
<th>Sample</th>
<th>Specific activity (expressed as a percentage of the controls)</th>
<th>Acetylation (expressed as a percentage of the controls)</th>
<th>Estimated number of groups modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>88</td>
<td>4.4</td>
</tr>
<tr>
<td>2</td>
<td>42</td>
<td>67</td>
<td>3.4</td>
</tr>
<tr>
<td>3</td>
<td>79</td>
<td>68</td>
<td>3.4</td>
</tr>
</tbody>
</table>

The extent of acetylation was estimated by reaction of samples with ninhydrin (Section 2.2.12.).
Protein concentration was estimated from amino acid analyses of the samples.

1. delta haemolysin (control), 10 nmol, after staining with ninhydrin.

2. delta haemolysin (10 nmol) after treatment with acetic anhydride and staining with ninhydrin.

3. as in 1 after staining with Erlich's stain for tryptophan-containing peptides.

4. as in 2 after staining with Erlich's stain for tryptophan-containing peptides.
Figure 5.2. Thin layer chromatography of acetylated delta haemolysin
5.4. Maleylation of delta haemolysin using maleic anhydride

Maleylation was first attempted using unlabelled maleic anhydride, and subsequently with radioactively labelled maleic anhydride in an attempt to more accurately determine the extent of modification.

5.4.1. "Cold" maleylation of delta haemolysin

Two samples of delta haemolysin (3.78 mg, determined by amino acid analysis) were lyophilised and taken up in 1 ml of 50 mM carbonate, bicarbonate buffer pH 9.2. To each sample 10 μl of phenolphthalein, 1% (w/v) in ethanol, was added. To one sample solid maleic anhydride, 195.5 mg (275 molar excess over the number of amino groups, assuming there to be five per molecule) was added in small aliquots over a period of 15 min at room temperature with stirring. Using the phenolphthalein as an indicator, the pH was maintained by the addition of small aliquots of solid sodium carbonate. After the additions were complete the reaction mixture was left to stand for 30 min at room temperature. The reaction mixture was then frozen at -86°C in a mixture of solid carbon dioxide and acetone. The second sample was a control and was treated in the same way except for the addition of maleic anhydride and sodium carbonate. The samples were stored at -20°C.

The protein in the samples was purified by gel filtration on Sephadex G-50 in 50 mM carbonate, bicarbonate buffer pH 9.2. The eluent was monitored for protein content using a continuous-flow spectrophotometer at 280 nm. The fractions constituting the protein peaks were pooled and lyophilised. The samples were then taken up in 1 ml of water and dialysed against 50 mM carbonate, bicarbonate buffer pH 9.2 overnight. The volumes of the samples were measured and the samples then assayed for haemolytic activity and subjected to amino acid analysis.
The results are shown in Table 5.4. It is clear that maleylation resulted in a complete loss of haemolytic activity. The number of maleyl groups was estimated from the difference spectrum of the reacted delta haemolysin and the unreacted control delta haemolysin (Figure 5.3.). The two protein samples were of equal concentration. Knowing that for maleylated amino groups $\varepsilon_{250} = 3360$ and $\varepsilon_{280} = 308$ and that for maleic acid plus amino groups $\varepsilon_{250} = 939$ and $\varepsilon_{280} = 59$, it was possible to calculate the concentration of maleylated groups using simultaneous equations (Butler and Hartley, 1972). In this way it was found that 49% of the amino groups (assuming the number of amino groups per molecule to be 5) were modified.

Figure 5.4 illustrates the results of silica-gel thin layer chromatography of the maleylated delta haemolysin and the control sample. The control sample gave rise to 2 main spots, the most densely stained spot running ahead was believed to be N-formyl blocked delta haemolysin and the second spot, unblocked delta haemolysin. The maleylated protein, after staining with ninhydrin, which reacts with amino groups, gave rise to a much fainter spot than the control which chromatographed ahead of the control. This spot was stained more densely with Erlich's stain for tryptophan-containing peptides.

5.4.2. Maleylation of delta haemolysin using radioactively labelled maleic anhydride

Delta haemolysin was modified with radioactively labelled maleic anhydride in an attempt to more accurately determine the number of maleyl groups bound to the delta haemolysin after reaction with maleic anhydride. A range of concentrations of maleic anhydride was used in the hope that a varying number of groups would be modified, and that the effect of this on the activity of delta haemolysin could be observed. A sample of delta haemolysin which had been treated to
<table>
<thead>
<tr>
<th>Sample</th>
<th>Total protein (mg)</th>
<th>Recovery (%)</th>
<th>Specific activity (H.U.mg(^{-1}))</th>
<th>Specific activity (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.76</td>
<td>73</td>
<td>66.6</td>
<td>100</td>
</tr>
<tr>
<td>Maleylated delta haemolysin</td>
<td>2.70</td>
<td>71</td>
<td>0.0</td>
<td>0</td>
</tr>
</tbody>
</table>

Protein was estimated by amino acid analysis.
Figure 5.3. The difference spectrum of maleylated and unmaleylated delta haemolysin was measured using equal concentrations of protein, estimated by amino acid analysis.
Each sample contained 10 nmol of protein (estimated by amino acid analysis).

1, control after staining with ninhydrin.
2, delta haemolysin after treatment with maleic anhydride and staining with ninhydrin.
3, control after staining with Erlich's stain for tryptophan-containing peptides.
4, delta haemolysin after treatment with maleic anhydride and staining with Erlich's stain for tryptophan-containing peptides.
Figure 5.4. Silica-gel thin layer chromatography of delta haemolysin after treatment to maleylate the amino groups of the molecule.
remove the N-formyl block from the protein was also modified. Samples of delta haemolysin (0.5 mg) were lyophilised and taken up in 0.25 ml of 50mM carbonate, bicarbonate buffer pH 9.2. (1,4-\textsuperscript{14}C) Maleic anhydride (250 μCi, 32.6 mCi.mmol\textsuperscript{-1}) was taken up in 0.5 ml of acetonitrile containing 492.3 μmol of unlabelled maleic anhydride, resulting in a specific activity of the maleic anhydride of 0.5 mCi.mmol\textsuperscript{-1}. The range of molar excesses used (assuming the number of amino groups to be 5 per molecule) is outlined in Table 5.5. Phenolphthalein, 5 μl (1% (w/v) in ethanol), was added to each sample, and the maleic anhydride added in small aliquots (20 μl or less) over a period of 15 min. The pH was maintained using the phenolphthalein as an indicator, by the addition of small aliquots of a saturated solution of sodium carbonate. An equivalent amount of acetonitrile to that used in the sample treated with the highest concentration of maleic anhydride was added in aliquots to the control sample. The reaction mixtures were left to stand for 30 min at room temperature and then frozen at -86°C in a mixture of solid carbon dioxide and acetone.

The samples were first dialysed against 50mM carbonate, bicarbonate buffer pH 9.2 in an attempt to remove the other reaction products from the protein. However after exhaustive dialysis, scintillation counting of small aliquots of the samples revealed that there was up to 9 times as much radioactivity present than could be accounted for by 100% recovery of 100% labelled protein. Gel filtration on Sephadex G-10 in 50mM carbonate, bicarbonate buffer pH 9.2; on Sephadex G-25 in 50mM carbonate, bicarbonate buffer pH 9.2 containing 6M guanidine hydrochloride; and on Sephadex G-10 in 50mM carbonate, bicarbonate buffer pH 9.2 containing 6M urea; all failed to completely remove the excess radioactivity. Finally the excess radioactivity was separated from the protein by gel filtration on Sephadex G-25 in
Table 5.5. Maleylation of delta haemolysin with radioactively labelled maleic anhydride

<table>
<thead>
<tr>
<th>Sample</th>
<th>Molar excess of maleic anhydride over the amino groups of delta haemolysin</th>
<th>Volume of radioactively labelled maleic anhydride (0.5 mCi.mmol⁻¹) in acetonitrile (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>200</td>
<td>166.6</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>83.3</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>16.7</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>4.2</td>
</tr>
<tr>
<td>Unblocked delta</td>
<td>200</td>
<td>166.6</td>
</tr>
</tbody>
</table>

The number of amino groups per molecule of delta haemolysin was assumed to be five. Acetonitrile (166.6 µl) was also added to the control sample.
25mM carbonate, bicarbonate buffer pH 9.2, containing 50% (v/v) ethanol. The fractions containing the protein were found by scintillation counting of small aliquots of the fractions. The protein eluted in the void volume of the column. The fractions were pooled and lyophilised. The samples were taken up in 0.5 ml of water and aliquots assayed for haemolytic activity and subjected to scintillation counting and amino acid analysis.

The results are outlined in Table 5.6. Gel filtration on Sephadex G-25 in 25mM carbonate, bicarbonate buffer pH 9.2, containing 50% (v/v) ethanol was repeated for sample 1 and found to have no further effect on the radioactive specific activity of the sample. All the samples eluted from the column in the same volume as the control. It can be seen from Table 5.6, that even the sample treated with the lowest concentration of maleic anhydride lost all its activity. The table also shows the estimated number of groups modified assuming all the radioactivity present to be bound as maleyl groups.

Further studies involving reaction of delta haemolysin with radioactive reagents would best be followed by the addition of a large excess of "cold" reagent to dilute out unreacted radioactively labelled reagent.
Table 5.6. The effect of maleylation of delta haemolysin using radioactively labelled maleic anhydride

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage labelling of the number of amino groups (%)</th>
<th>Estimated amino groups modified</th>
<th>Specific activity (HU.mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0.0</td>
<td>54.4</td>
</tr>
<tr>
<td>1</td>
<td>92</td>
<td>4.6</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>71</td>
<td>3.6</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>78</td>
<td>3.9</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>49</td>
<td>2.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Unblocked delta</td>
<td>111</td>
<td>5.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The number of amino groups was assumed to be five per molecule of delta haemolysin.
6.1. Production of in vivo radioactively labelled delta haemolysin

It was thought that in vivo radioactive labelling of delta haemolysin would be more efficient if the strain of *S. aureus* used was auxotrophic for the radioactively labelled amino acid used. For this reason several auxotrophic strains of *S. aureus* were investigated.

6.1.1. Comparison of the growth of auxotrophic strains of *Staphylococcus aureus* and their production of delta haemolysin

Table 6.1 lists the strains of *S. aureus* investigated and their nutritional requirements. Their growth and production of delta haemolysin was compared with strain RN25.

Small scale cultures (10 ml) were grown up and the growth estimated spectrophotometrically at 540 nm. Production of delta haemolysin was estimated by the assay of heat-treated samples of the supernatants for haemolytic activity.

The results are outlined in Table 6.1. Strain P392 was chosen for further investigation as its growth and production of delta haemolysin were adequate and the strain was auxotrophic for the amino acid lysine, which could easily be removed from the rich diffusate medium using the enzyme lysine decarboxylase. It was hoped that removal of most of the lysine would result in more efficient incorporation of added radioactively labelled lysine into the proteins of the strain.

6.1.2. Removal of lysine from yeast diffusate medium

Lysine decarboxylase (10 mg.ml⁻¹, 2.5 units.ml⁻¹, in 50 mM acetate buffer pH 6.0) was added to 5 ml aliquots of sterile yeast diffusate medium as outlined in Table 6.2. The samples of media were then incubated at 37°C. At various time intervals 100 µl samples were
Table 6.1. Comparison of the growth of auxotrophic strains of *S. aureus* and their production of delta haemolysin with that of strain RN25

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Genotype</th>
<th>Growth ($A_{540}$)</th>
<th>Production of delta haemolysin (HU.ml$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN25</td>
<td></td>
<td>18.08</td>
<td>45.2</td>
</tr>
<tr>
<td>P391</td>
<td>thr$^-$</td>
<td>8.04</td>
<td>100.0</td>
</tr>
<tr>
<td>P392</td>
<td>lys$^-$</td>
<td>5.72</td>
<td>13.0</td>
</tr>
<tr>
<td>P363</td>
<td>trp$^-$, his$^-$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P484</td>
<td>thr$^-$, ilv$^-$, thy$^-$, pur$^-$</td>
<td>3.82</td>
<td>6.0</td>
</tr>
</tbody>
</table>

The growth of the strains (10 ml cultures) was estimated spectrophotometrically at 540 nm. Production of delta haemolysin was measured by assaying heat-treated samples of the culture supernatants for haemolytic activity.
removed, made up to 1 ml with water and 50 μl of the dilutions subjected to amino acid analysis. The samples were not hydrolysed so that the levels of free amino acids were measured. The results are shown in Table 6.2. It can be seen that after 4 h incubation at 37°C with 0.5 units of lysine decarboxylase 96% of the free lysine had been removed from 5 ml of yeast diffusate medium.

Subsequently all medium used for labelling cultures with radioactive lysine were first treated with lysine decarboxylase, 1 unit per 10 ml of medium, for 4 h at 37°C, and then autoclaved to destroy the enzyme.

6.1.3. Comparison of the growth of *S. aureus* strains RN25 and P392 in yeast diffusate medium with and without treatment to remove lysine

Lysine decarboxylase (1.0 unit) was added to two 10 ml aliquots of sterile medium in 100 ml Erlenmeyer flasks. These were incubated for 4 h at 37°C with two control flasks each containing 10 ml of medium. The samples were then autoclaved to destroy the enzyme. One control sample and one sample treated with lysine decarboxylase were inoculated with the strain RN25 and the other two samples inoculated with strain P392. The cultures were grown overnight as described in Section 2.2.4.4. The growth of the cultures was estimated spectrophotometrically at 540 nm.

The results are shown in Table 6.3. Surprisingly the growth of the auxotroph was not drastically reduced in medium which had been treated with lysine decarboxylase to remove the lysine, suggesting that it was able to obtain lysine from some other source in the rich yeast diffusate medium, perhaps from small peptides broken down by proteases produced by the strain. However the growth of the auxotroph was reduced to a greater extent than that of strain RN25 in treated medium. For this reason the auxotroph was expected to exhibit more
Table 6.2. Removal of lysine from the yeast diffusate medium using lysine decarboxylase

<table>
<thead>
<tr>
<th>Sample</th>
<th>Units of lysine decarboxylase</th>
<th>Duration of incubation (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>0.00</td>
<td>3.92</td>
</tr>
<tr>
<td>1</td>
<td>0.25</td>
<td>1.76 (45)</td>
</tr>
<tr>
<td>2</td>
<td>0.50</td>
<td>0.32 (8)</td>
</tr>
</tbody>
</table>

The results are expressed as the concentration of lysine in the medium (mM), the figures in parentheses express the amount of lysine in the medium as a percentage of the control. The units of lysine decarboxylase are defined as that amount of enzyme which would release 1.0 μmol of CO₂ from L-lysine per min at pH 6.0 at 37°C.

Table 6.3. Comparison of the growth of S. aureus strains RN25 and P392 in yeast diffusate medium with or without treatment with lysine decarboxylase

<table>
<thead>
<tr>
<th>Sample</th>
<th>Growth (A₅₄₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated medium</td>
</tr>
<tr>
<td>RN25</td>
<td>18.08</td>
</tr>
<tr>
<td>P392</td>
<td>6.4</td>
</tr>
</tbody>
</table>

The growth of the strains (10 ml cultures) was estimated spectrophotometrically at 540 nm. Treated medium was incubated with 1.0 unit of lysine decarboxylase for 4 h at 37°C and autoclaved prior to inoculation with the strain. Figures in parentheses express the growth as a percentage of the control.
efficient uptake of radioactively labelled lysine and produce radioactively labelled delta haemolysin with a higher radioactive specific activity.

6.1.4. Production and purification of in vivo radioactively labelled delta haemolysin

Sterile medium (25 ml) prepared with or without prior treatment with lysine decarboxylase was inoculated with 0.1 ml of a starter culture of the desired strain and growth monitored using a Klett-Summerson photoelectric colorimeter. In the early exponential stage of growth 312.5 µl of L-(U-14C) lysine monohydrochloride (50 µCi.ml⁻¹) was added. After 17 h the cells were removed by centrifuging at 1600 g for 15 min at room temperature, and 0.5 ml of PMSP (1 mg.ml⁻¹) added to the supernatant. The delta haemolysin was purified by the method of Heatley (1971) as described in Section 4.1.3. except that the volumes involved were scaled down in proportion to the small volume of radioactively labelled supernatant.

Radioactively labelled delta haemolysin was purified from the culture supernatants of strain P392 grown in lysine decarboxylase-treated medium, and of strain RN25 grown in untreated medium. The results are compared in Table 6.4.

It can be seen that the radioactive specific activity of the delta haemolysin purified from the culture supernatant of the auxotroph grown in lysine decarboxylase-treated medium was not much higher than that of the delta haemolysin purified from the culture supernatant of strain RN25 grown in untreated medium. Also because of the poorer growth of the auxotroph, the final yield of radioactive delta haemolysin was only half of that from the culture supernatant of strain RN25. This suggests that radioactively labelled delta haemolysin would best be produced from strain RN25 using lysine decarboxylase-treated medium.
Table 6.4. Production of radioactively labelled delta haemolysin

<table>
<thead>
<tr>
<th></th>
<th>Strain P392 grown in medium treated with lysine decarboxylase</th>
<th>Strain RN25 grown in untreated medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total activity in the supernatant due to delta haemolysin (HU)</td>
<td>450</td>
<td>1169</td>
</tr>
<tr>
<td>Total activity purified (HU)</td>
<td>66.5</td>
<td>164</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Protein purified, estimated by amino acid analysis (mg)</td>
<td>0.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Specific activity (HU mg⁻¹)</td>
<td>95</td>
<td>91</td>
</tr>
<tr>
<td>Total radioactivity purified (nCi)</td>
<td>105</td>
<td>211</td>
</tr>
<tr>
<td>Radioactive specific activity (mCi mmol⁻¹)</td>
<td>0.45</td>
<td>0.36</td>
</tr>
</tbody>
</table>
in an attempt to obtain delta haemolysin of a higher radioactive specific activity. This procedure was used in subsequent production of radioactively labelled cultures.

6.2. Experiments using radioactively labelled delta haemolysin

6.2.1. Polyacrylamide gel electrophoresis in the presence of SDS and fluorography of radioactively labelled delta haemolysin

It was thought that the difficulty of staining delta haemolysin after SDS PAGE, described in Section 4.2.1.1., might be due to diffusion of the protein within and out of the gel during the staining and destaining procedures. This would account for the need to load large amounts of protein onto the gel in order to visualise the delta haemolysin and explain the smearing phenomenon observed. In an attempt to reduce the diffusion of the delta haemolysin, SDS PAGE of the radioactively labelled protein was followed immediately by fluorography. It was hoped that this would lead to a better estimation of the molecular weight of the molecule.

Radioactively labelled delta haemolysin, purified from the culture supernatant of strain P392 grown in lysine decarboxylase-treated medium was used for electrophoresis. Proteins of known molecular weights were electrophoresed on one half of the gel and 5 µg of radioactively labelled delta haemolysin (0.15 nCi/µg⁻¹) electrophoresed on the other half. The gel was 20% (w/v) acrylamide and was electrophoresed in the presence of SDS as described in Section 2.2.7.1. Immediately after electrophoresis, the half of the gel containing the radioactively labelled delta haemolysin was prepared for fluorography and applied to film as described in Section 2.2.7.6. The other half of the gel was stained using method B (Section 2.2.7.5.).

Figure 6.1 is a tracing of the developed film. It can be seen that
Radioactively labelled delta haemolysin (5 μg, 0.15 nCi μg⁻¹) was electrophoresed in a 20% (w/v) acrylamide gel in the presence of SDS. The gel was prepared for fluorography immediately after electrophoresis and applied to film. The film was exposed at -70°C for 5 days.
the smearing of the delta haemolysin has been reduced although not eliminated. The reduction in the smearing of the protein may have been due to the fact that much less protein was loaded onto the gel.

Figure 6.2. illustrates the estimation of the molecular weight of the delta haemolysin. This was found to be between 3 300 and 4 700, the closest estimate from SDS PAGE to the value calculated from the amino acid sequence elucidated by Fitton et al. (1980).

6.2.2. Estimation of the number of molecules of delta haemolysin bound to the erythrocyte membrane at saturation with the toxin

The binding of radioactively labelled delta haemolysin was estimated using the standard assay procedure described in Section 2.2.5., but with a total assay volume of 0.5 ml. Assays were set up using $^{14}$C-delta haemolysin (0.15 nCi/μg) as shown in Table 6.5. The samples were incubated at 37°C for 15 min, with mixing, and then centrifuged at 12 000 g for 1.5 min at room temperature to remove cell debris and unlysed cells. Samples of 0.25 ml were removed from the supernatants and added to 1.25 ml of 0.1% (w/v) Na$_2$CO$_3$ for estimation of haemolysis (Section 2.2.5.). Samples of 0.1 ml were removed from the supernatants for scintillation counting as described in Section 2.2.2.

It can be seen from Figure 6.3. that the higher concentrations of delta haemolysin resulted in 100% haemolysis. The amounts of delta haemolysin bound to the erythrocyte membranes in each sample were calculated from the results of scintillation counting of the samples, knowing the radioactive specific activity of the delta haemolysin added. The amount of delta haemolysin bound to the erythrocyte membranes at saturation with the toxin was calculated from a reciprocal plot of the amount of delta haemolysin bound to the erythrocyte membranes against the amount of delta haemolysin added to
Figure 6.2. Estimation of the molecular weight of delta haemolysin

Molecular weight standards were bovine serum albumin (67 000), ovalbumin (43 000), chymotrypsinogen A (25 000) and cytochrome C (13 400). The molecular weight of delta haemolysin was estimated to be between 3 300 and 4 700.
Table 6.5. Estimation of the number of molecules of delta haemolysin bound to the erythrocyte membrane at saturation with the toxin

<table>
<thead>
<tr>
<th>Tube number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
<tr>
<td>Delta haemolysin (0.5 mg.mL⁻¹) (μl)</td>
</tr>
<tr>
<td>PBS1 (μl)</td>
</tr>
<tr>
<td>Assay erythrocyte suspension (μl)</td>
</tr>
</tbody>
</table>

Assays were treated as described in Section 2.2.5. except that the total volume of the assay mix was halved.
Figure 6.3. Estimation of the number of molecules of delta haemolysin bound to the erythrocyte membrane at saturation with the toxin; estimation of haemolysis in the samples.

Haemolysis was estimated as described in Section 2.2.5.
the samples (Figure 6.4.), and was found to be 40 μg.

The number of molecules of delta haemolysin bound per erythrocyte, at saturation with the toxin, was calculated as shown in Figure 6.5., and found to be $1.3 \times 10^3$. In comparison, taking the amount of delta haemolysin required to produce 50% haemolysis in the standard assay (Section 2.2.5.) as 10 μg, the number of molecules of delta haemolysin per erythrocyte in this case was calculated in the same way and found to $1.6 \times 10^7$. 
Figure 6.4. Estimation of the number of molecules of delta haemolysin to the erythrocyte membrane at saturation with the toxin

The amount of delta haemolysin bound to the erythrocyte membranes in the assay, at saturation with the toxin, was estimated from this plot to be 40 \( \mu \text{g} \).
Figure 6.5. Calculation of the number of molecules of delta haemolysin bound to the erythrocyte membrane at saturation with the toxin

The amount of delta haemolysin bound to the erythrocyte membranes in the assay = 40 μg

The number of moles of delta haemolysin bound = 40 \times 10^{-6}

The number of molecules of delta haemolysin bound = \frac{40 \times 10^{-6} \times 6.023 \times 10^{23}}{3 \, 000}

The number of erythrocytes per ml of whole blood = 6 \times 10^9

The number of erythrocytes in the assay mix = \frac{6 \times 10^9 \times 0.25}{25}

The number of molecules of delta haemolysin bound per erythrocyte at saturation with the toxin = \frac{40 \times 10^{-6} \times 6.023 \times 10^{23} \times 25}{3 \, 000 \times 6 \times 10^9 \times 0.25}

= 1.3 \times 10^8

The molecular weight of delta haemolysin was assumed to be 3 000 (Pitton et al., 1980). Avogadro's number = 6.023 \times 10^{23}. The number of erythrocytes per ml of whole blood was assumed to be 6 \times 10^9.
CHAPTER SEVEN
BIOSYNTHESIS OF DELTA HAEMOLYSIN

The small size of delta haemolysin (26 amino acids) and the existence of the N-formyl methionine containing species suggested that the toxin might not be the total gene product, but the N-terminal portion of a larger gene product. In addition, the hydrophobic character of the molecule suggested that it might be a signal peptide, as discussed in Section 1.6.

Procaine and cerulenin have been used to inhibit extracellular protein production, resulting in a build up of the precursors of exported proteins and their identification (Fishman et al., 1980 and Lazdunski et al., 1979). It was hoped that either using procaine or cerulenin, or by arresting growth before delta haemolysin appeared in the culture supernatant, it might be possible to identify any precursor of delta haemolysin, using radioactively labelled cell cultures and techniques of immunoprecipitation.

7.1. The effect of inhibitors of extracellular protein production, procaine and cerulenin, on the growth of S. aureus strain RN25, its production of delta haemolysin and total extracellular protein

The effect of varying concentrations of the inhibitors of extracellular protein production, procaine and cerulenin, on the growth of S. aureus strain RN25 and its production of delta haemolysin were investigated. The effects of the inhibitors on total extracellular protein production were examined using polyacrylamide gel electrophoresis, in the presence of SDS, of concentrated samples of the culture supernatants after treatment with the inhibitors.
7.1.1. The effects of various concentrations of procaine on the growth of *S. aureus* strain RN25, its production of delta haemolysin and total extracellular protein

Sidearm flasks containing 25 ml of sterile yeast diffusate medium were inoculated with 0.1 ml of a starter culture of *S. aureus* strain RN25. Growth was monitored using a Klett-Summerson colorimeter. When the cultures had reached the early exponential phase of growth (60 - 70 Klett units) various concentrations of procaine (200 mg/ml$^{-1}$, sterilised using a filter) were added to the flasks as shown in Table 7.1.

Growth was monitored until the cultures reached the stationary phase and is illustrated in Figure 7.1. It can be seen that the two lowest concentrations of procaine had virtually no effect on the growth of strain RN25, the differences between these curves and the control curves were no greater than differences seen between control curves in other experiments. A concentration of 1.5% (w/v) procaine did affect growth in that the generation time of the culture appeared to be longer, although the final density of the culture was the same as the controls. The highest concentration of procaine resulted in complete inhibition of growth.

The effects of the various concentrations of procaine on the final levels of production of delta haemolysin by strain RN25 are shown in Table 7.1. It can be seen that increasing concentrations of procaine, increasingly reduced the amounts of delta haemolysin produced by the cultures. A concentration of 1.5% (w/v) of procaine resulted in reduction of the amount of delta haemolysin produced to 5% of the control.

Plate 7.1. illustrates the effect of various concentrations of procaine on the production of extracellular protein by strain RN25. The supernatants of cultures treated with various concentrations of
Table 7.1. The effect of procaine on the production of delta haemolysin by *S. aureus* strain RN25

<table>
<thead>
<tr>
<th>Flask number</th>
<th>Concentration of procaine (% w/v)</th>
<th>Production of delta haemolysin (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>97</td>
</tr>
<tr>
<td>4</td>
<td>0.55</td>
<td>73</td>
</tr>
<tr>
<td>5</td>
<td>1.5</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>2.0</td>
<td>0</td>
</tr>
</tbody>
</table>

Cultures of *S. aureus* strain RN25 (25 ml), in the early exponential phase of growth, were treated with various concentrations of procaine, as shown above. The cultures were allowed to grow to stationary phase and the production of delta haemolysin measured by assaying heat-treated samples of the culture supernatants for haemolytic activity.
Figure 7.1. The effect of various concentrations of procaine on the growth of *Staphylococcus aureus* strain RN25.

Aliquots of yeast diffusate medium were inoculated with 0.1 ml of a starter culture of *S. aureus* strain RN25. Growth was monitored using a Klett-Summerson colorimeter. In the early exponential phase of growth various concentrations of procaine (200 mg.ml$^{-1}$ in water) were added to the flasks. Control (●), control plus water (○), 0.2% (w/v) procaine (■), 0.55% (w/v) procaine (□), 1.5% (w/v) procaine (▲) and 2.0% (w/v) procaine (△).
Plate 7.1. The effect of various concentrations of procaine on the production of extracellular protein by Staphylococcus aureus strain RN25

Cultures of S. aureus strain RN25 (25 ml) were treated with various concentrations of procaine in the early exponential phase of growth. The cultures were grown to stationary phase and then 50 \( \mu l \) samples of 10-fold concentrated culture supernatants were loaded onto a 15\% (w/v) acrylamide gel in SDS. The concentrations of procaine used were Track 1, control; Track 2, 0.75\% (w/v) procaine; Track 3, 1.0\% (w/v) procaine; Track 4, 1.25\% (w/v) procaine; Track 5, 1.75\% (w/v) procaine. Track 6 contained molecular weight standards, bovine serum albumin (67 000), 10 \( \mu g \) and cytochrome C (13 400), 10 \( \mu g \).
procaine as shown in Plate 7.1., were concentrated 10-fold using
Aliquots of 50 μl of each sample were then electrophoresed in a
15% (w/v) acrylamide gel in the presence of SDS as described in
Section 2.2.7.1. The gel was stained by method B (Section 2.2.7.5.).
In this experiment a narrower range of concentrations of procaine was
used. It can be seen that increasing the concentration of procaine
in the cultures resulted in some decrease in extracellular protein
production.

7.1.2. The effects of various concentrations of cerulenin on the
growth of S. aureus strain RN25, its production of delta haemolysin and
total extracellular protein

Various concentrations of cerulenin (10 mg.ml⁻¹ in ethanol,
sterilised using a filter) were added to 20 ml aliquots of sterile
yeast diffusate medium in sidearm flasks as outlined in Table 7.2.a.
The flasks were then inoculated with 0.1 ml of a starter culture of
S. aureus strain RN25 and growth monitored using a Klett-Summerson
colorimeter.

The effects of this treatment on the growth of strain RN25 are
shown in Figure 7.2.a. The control treated with the largest volume of
ethanol used showed normal exponential growth although the final
density of the culture was reduced to 84% of the untreated control.
The growth of strain RN25 was unaffected by the lowest concentration of
cerulenin added, however a concentration of cerulenin of 31.25 μg.ml⁻¹
affected the exponential phase of growth and reduced the final density
of the culture to 84% of the untreated control. The highest
concentration of cerulenin used severely affected the growth of strain
RN25 reducing it to 36% of the untreated control (45% of the control
treated with the same amount of ethanol).
Table 7.2.a The effect of cerulenin on the production of delta haemolysin by *S. aureus* strain RN25

<table>
<thead>
<tr>
<th>Flask</th>
<th>Concentration of cerulenin (µg.ml(^{-1}))</th>
<th>Ethanol added (µl)</th>
<th>Production of delta haemolysin (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>0.0</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>200.0</td>
<td>92</td>
</tr>
<tr>
<td>3</td>
<td>3.75</td>
<td>7.5</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>31.25</td>
<td>62.5</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>100.0</td>
<td>200.0</td>
<td>3</td>
</tr>
</tbody>
</table>

Cerulenin (10 mg.ml\(^{-1}\) in ethanol) was added to 20 ml aliquots of sterile yeast diffusate medium as shown above. The media were then inoculated with *S. aureus* strain RN25 and the cultures allowed to grow until they reached stationary phase. The production of delta haemolysin was measured by assaying heat-treated samples of the culture supernatants for haemolytic activity (Section 2.2.5.).
Figure 7.2.a The effect of various concentrations of cerulenin on the growth of *Staphylococcus aureus* strain RN25

Aliquots of yeast diffusate medium (20 ml) were treated with various concentrations of cerulenin (10 mg.ml\(^{-1}\) in ethanol) and then inoculated with 0.1 ml of a starter culture of *S. aureus* strain RN25. Growth was monitored using a Klett-Summerson colorimeter. Control (o), control plus ethanol (●), 3.75 µg.ml\(^{-1}\) cerulenin (□), 31.25 µg.ml\(^{-1}\) cerulenin (■) and 100 µg.ml\(^{-1}\) cerulenin (Δ).
The effects of various concentrations of cerulenin on the production of delta haemolysin by strain RN25 is shown in Table 7.2.a. It can be seen that the production of delta haemolysin by the ethanol-treated control was reduced to 92% of the untreated control. However, even the lowest concentration of cerulenin added reduced delta haemolysin production to 17% of the untreated control and the two higher concentrations of cerulenin reduced delta haemolysin production to 3% of the untreated control.

Plate 7.2.a illustrates the results of polyacrylamide gel electrophoresis in the presence of SDS of the extracellular proteins of the cultures. Samples of the culture supernatants after treatment with cerulenin were concentrated 10-fold using Minicon concentrators and 50 μl of each sample was electrophoresed in a 15% (w/v) acrylamide gel in the presence of SDS as described in Section 2.2.7.1. The gel was stained by method B (Section 2.2.7.5.). It can be seen that increasing concentrations of cerulenin result in considerable reduction of extracellular protein production.

From these results it can be seen that cerulenin was more efficient in cutting down delta haemolysin and total extracellular protein production than procaine, without affecting the growth of the bacteria. Consequently, the effects of cerulenin were investigated in more detail, over a smaller range of concentration.

Cultures were set up as before but containing the concentrations of cerulenin outlined in Table 7.2.b. It can be seen from Figure 7.2.b that none of the concentrations of cerulenin used seriously affected the growth of strain RN25. The effects of the various concentrations of cerulenin on the production of delta haemolysin are outlined in Table 7.2.b. In this experiment the cerulenin was added as a 1 mg.ml⁻¹ solution in ethanol; it is interesting to note that the largest volume of ethanol used, when added alone to a control sample, caused a
Cultures of *Staphylococcus aureus* strain RN25 (20 ml) were grown in various concentrations of cerulenin to stationary phase and then 50 μl samples of 10-fold concentrated culture supernatants were loaded onto a 15% (w/v) acrylamide gel in SDS.

Track 1, control.

Track 2, plus 200 μl of ethanol.

Track 3, 3.75 μg.mL⁻¹ cerulenin.

Track 4, 31.25 μg.mL⁻¹ cerulenin.

Track 5, 100 μg.mL⁻¹ cerulenin.

Track 6, molecular weight standards, bovine serum albumin (67 000), 10 μg and cytochrome C (13 400), 10 μg.
Table 7.2.b The effect of cerulenin on the production of delta haemolysin by *S. aureus* strain RN25

<table>
<thead>
<tr>
<th>Flask</th>
<th>Concentration of cerulenin (µg.ml⁻¹)</th>
<th>Ethanol added (µl)</th>
<th>Production of delta haemolysin (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>625</td>
<td>48</td>
</tr>
<tr>
<td>3</td>
<td>3.75</td>
<td>75</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>7.50</td>
<td>150</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>18.75</td>
<td>375</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>31.25</td>
<td>625</td>
<td>4</td>
</tr>
</tbody>
</table>

Cerulenin (1 mg.ml⁻¹ in ethanol) was added to 20 ml aliquots of sterile yeast diffusate medium as shown above. The media were then inoculated with *S. aureus* strain RN25 and the cultures allowed to grow until they reached stationary phase. The production of delta haemolysin was measured by assaying heat-treated samples of the culture supernatants for haemolytic activity (Section 2.2.5.).
Figure 7.2.b The effect of various concentrations of cerulenin on the growth of *Staphylococcus aureus* strain RN25

Aliquots of yeast diffusate medium (20 ml) were treated with various concentrations of cerulenin (1 mg.ml⁻¹ in ethanol) and then inoculated with 0.1 ml of a starter culture of *S. aureus* strain RN25. Growth was monitored using a Klett-Summerson colorimeter. Control (○), control plus ethanol (●), 3.75 μg.ml⁻¹ cerulenin (□), 7.50 μg.ml⁻¹ cerulenin (■), 18.75 μg.ml⁻¹ cerulenin (△) and 31.25 μg.ml⁻¹ cerulenin (▲).
reduction in delta haemolysin to 48% of the untreated control. However, with reference to the previous experiment in which the ethanol concentration (1% by volume) was not interfering, a concentration of 18.75 μg.ml⁻¹ was chosen for use in experiments using cerulenin as an inhibitor of extracellular protein production. The cerulenin was subsequently added as a 10 mg.ml⁻¹ solution in ethanol, keeping the concentration of ethanol below 1% (v/v). This concentration of cerulenin severely inhibited the production of delta haemolysin as shown in Table 7.2.b, and considerably reduced the total extracellular protein production of S. aureus strain RN25 as shown in Plate 7.2.b without affecting the growth of the bacteria, Figure 7.2.b.

7.2. **Search for a possible precursor of delta haemolysin**

It was hoped that using cultures of S. aureus strain RN25 labelled in vivo with radioactively labelled lysine, it would be possible to identify any precursors of delta haemolysin present in the cell lysate or the culture supernatant, using techniques of immunoprecipitation. Treatment of the cultures with cerulenin, and harvesting the cultures in the mid-exponential phase of their growth were both tried in an attempt to produce a build up of any precursors of delta haemolysin.

7.2.1. **Preparation of antiserum to delta haemolysin from rabbits**

Preimmune and immune sera from 4 rabbits was provided by Dr. W.H. Turner of the Wellcome Research Laboratories, Beckenham, Kent. The rabbits were immunised against delta haemolysin purified by the method of Heatley (1971 and 1976) from the culture supernatant of S. aureus strain 186X ("reference" delta haemolysin), using a schedule described by Turner (1978).

Plate 7.3. illustrates the results of Ouchterlony double diffusion
Cultures of *S. aureus* strain RN25 (20 ml) were grown in various concentrations of cerulenin to stationary phase and then 50 μl samples of 10-fold concentrated culture supernatants were electrophoresed in a 15% (w/v) acrylamide gel in SDS.

Track 1, control.
Track 2, control plus ethanol.
Track 3, 3.75 μg.ml⁻¹ cerulenin.
Track 4, 7.50 μg.ml⁻¹ cerulenin.
Track 5, 18.75 μg.ml⁻¹ cerulenin.
Track 6, 31.25 μg.ml⁻¹ cerulenin.
Track 7, molecular weight standards, bovine serum albumin (67 000), 10 μg and cytochrome C (13 400), 10 μg.
The centre wells at the top contained 5 µg of delta haemolysin and at the bottom, 10 µg. The delta haemolysin was purified by the method of Heatley (1971 and 1976) from the culture supernatant of S. aureus strain RN25 and further purified using octyl sepharose chromatography.

Wells 1 - 4 A and C contained 10 µl of preimmune serum from rabbits 1 - 4.

Wells 1 - 4 B and D contained 10 µl of immune serum from rabbits 1 - 4.

Wells 5 A and C contained 10 µl of human serum.

Wells 6 A and C contained 10 µl of immunoglobulin fraction prepared from the preimmune serum of rabbit 2.

Wells 6 B and D contained 10 µl of immunoglobulin fraction prepared from the immune serum of rabbit 2.
Plate 7.3. Ouchterlony double diffusion tests

The method used to describe in Section 7.3.3. It was noted that antibody 1 and 2 appeared to have the highest titres.

It has been reported that sera from rabbits form a precipitin line with homologous sera (Bentley, 1977). This could not be seen with sera from the rabbits, although there was some stained material immediately surrounding the cells. However a precipitin line

(Section 6.1.2.), with or without carboxylase (10, 75, 150 μl) and autologous labelled with 200 μl of L-(8-3H) lysine (specific activity 40 uCi/μmol, 140 Ci/mmol). As no other samples were obtained from immunoprecipitation studies, autologously labelling the cultures with 14C-labeled lysine was mandatory for a higher specific activity (1 Ci/mmol, 370 Ci/mmol) for treatment in the table below.

Each sample was treated with a 5 ml 20% alanolamine solution, which had previously been heated with lysine concentrates (Section 4.1.2.) was used in this experiment. One sample was treated with carboxylase, 1.5 μl of a 76 mCi/μl solution in ethanol and then both
tests of the sera against "reference" delta haemolysin. The method used is described in Section 2.2.13. It can be seen that rabbits 3 and 4 appeared to have the highest titres.

It has been reported that delta haemolysin forms a precipitin line with unimmune sera (Heatley, 1977). This could not be seen with preimmune sera from the rabbits, although there was some stained material immediately surrounding the wells. However a precipitin line could be seen with unimmune human serum. For this reason immunoglobulin (Ig) fractions were prepared from the sera as described in Section 2.2.14. It can be seen from Plate 7.3. that the Ig fraction prepared from the preimmune serum of rabbit 2 resulted in no stainable material immediately surrounding the wells. The Ig fraction prepared from the immune serum of rabbit 2 still gave a precipitin line. In subsequent immunoprecipitation reactions the Ig fractions prepared from immune sera were used.

7.2.2. Immunoprecipitation from radioactively labelled cultures of S.aureus strain RN25

Cultures of S.aureus strain RN25 (20 ml), were grown up in yeast diffusate medium, previously treated with lysine decarboxylase (Section 6.1.2.), with or without cerulenin (18.75 μg.ml⁻¹) and radioactively labelled with 250 μl of L-(U⁻¹⁴C) lysine monohydrochloride (50 μCi.ml⁻¹, 340 mCi.mmol⁻¹). As no clear results were obtained from immunoprecipitation studies, radioactively labelling the cultures with L-(4-5⁻³H) lysine monohydrochloride of a higher specific activity (1 mCi.ml⁻¹, 108 Ci.mmol⁻¹) was attempted as described below.

Yeas diffusate medium, 2 x 5 ml in 50 ml Erlenmeyer flasks, which had previously been treated with lysine decarboxylase (Section 6.1.2.) was used in the experiment. One sample was treated with cerulenin, 7.5 μl of a 10 mg/ml⁻¹ solution in ethanol, and then both
samples were inoculated with 25 μl of a starter culture of *S. aureus* strain RN25. Growth was monitored in 20 ml of yeast diffusate medium, previously treated with lysine decarboxylase (Section 6.1.2.), which was inoculated with 0.1 ml of a starter culture of *S. aureus* strain RN25 at the same time as the 5 ml cultures. The cultures were grown at 37°C shaking at 180 rpm.

In the early exponential phase of growth 50 μl of L-(4-5-3H) lysine monohydrochloride (1 mCi.ml⁻¹, 108 Ci.mmol⁻¹) was added to each 5 ml culture. The cultures were then left to grow overnight. The cells were harvested by centrifuging at 1 600 g for 15 min at room temperature. The culture supernatants were removed, treated with PMSF (20 μg.ml⁻¹) and stored at -20°C. The bacteria were then washed by resuspension in 5 ml of 0.01M Tris-HCl buffer pH 7.5, containing 0.15M NaCl, and recovered by centrifuging as before. The bacteria were then taken up in 1 ml of 0.01M Tris-HCl buffer pH 7.5, containing 0.15M NaCl, for the preparation of cell lysates. Lysostaphin, 30 μl of a 1 mg.ml⁻¹ solution, was added to each of the samples, which were then incubated at 37°C for 30 min. This was followed by the addition of DNAase, 10 μl of a 1 mg.ml⁻¹ solution, to each of the samples with incubation at 37°C for a further 30 min. The samples were then referred to as "cell lysates". Samples of 0.5 ml of the cell lysates were centrifuged at 12 000 g for 5 min at room temperature. The supernatants were removed and referred to as "cleared cell lysates". The lysates were treated with PMSF (20 μg.ml⁻¹) and stored at -20°C.

Initially immunoprecipitation studies were carried out using the protein A-bearing Cowan I strain of *Staphylococcus aureus* as described by Kessler (1975). This technique is based on the substitution of the protein A-bearing strain of *S. aureus* for the second or anti-Ig antibody in indirect immune precipitations. Protein A binds specifically and strongly to the Fc regions of many mammalian Ig subclasses (Kessler,
1975).

Samples of the cell lysates (100 µl), cleared cell lysates (100 µl) and the culture supernatants (1 ml) were taken and treated with 200 µl of the immunoadsorbent staphylococci, prepared as described by Kessler (1975). The samples were incubated at 4°C for 30 min and then centrifuged at 12 000 g for 30 s at room temperature to remove the staphylococci. It was hoped that this treatment would remove any protein from the samples which bound non-specifically to the immunoadsorbent staphylococci. The supernatants from the samples were removed and the pellets stored at 4°C. The supernatants were then treated with 100 µl of the Ig fraction prepared from the preimmune serum of rabbit 3 (Section 7.2.1.) and incubated at 37°C for 30 min. This was followed by the addition of 200 µl of the immunoadsorbent staphylococci to each sample with further incubation at 4°C for 30 min. The samples were then centrifuged at 12 000 g for 30 s at room temperature. It was hoped that this treatment would also remove non-specifically bound protein from the samples. The supernatants were removed and the pellets stored at 4°C.

The supernatants were finally treated with 100 µl of the Ig fraction prepared from the immune serum of rabbit 3 (Section 7.2.1.) and incubated at 37°C for 30 min. This was followed by the addition of 200 µl of the immunoadsorbent staphylococci to each sample with further incubation at 4°C for 30 min. The samples were then centrifuged as before and the supernatants removed and discarded. The pellets, expected to contain proteins bound specifically to the antibodies raised against delta haemolysin, were stored at 4°C.

The pellets from the three stages of the immunoprecipitation were washed twice by resuspension in 1 ml of 0.05% (v/v) Nonidet P-40 in NaCl-EDTA-Tris (NET) buffer pH 7.4 (150mM NaCl, 5mM EDTA, 50mM Tris), containing 0.02% (w/v) sodium azide, and centrifuging at 12 000 g for
30 s at room temperature. The pellets were then resuspended in 50 µl of sample buffer for polyacrylamide gel electrophoresis in the presence of SDS (Section 2.2.7.1.) and heated to 100°C for 5 min. The samples were then centrifuged as before to remove the immunoadsorbent staphylococci. The supernatants, now expected to contain the protein which had bound to the immunoadsorbent staphylococci, were then electrophoresed in two 15% (w/v) acrylamide gels in the presence of SDS with proteins of known molecular weight (Section 2.2.7.1.). The gels were stained using method B (Section 2.2.7.5.) and the migration of the proteins of known molecular weight measured. The gels were then prepared for fluorography and applied to film as described in Section 2.2.7.6. The films were exposed at -70°C for 1 week and 8 weeks.

Plates 7.4.a and b show the results obtained after exposure of the films for 8 weeks, and illustrate some of the problems encountered in this experimental approach. Plate 7.4.a shows the results obtained from S.aureus strain RN25 grown in the absence of cerulenin. Tracks 1, 2 and 3 show the high level of protein bound non-specifically to the immunoadsorbent staphylococci. Initially the three stages of the immunoprecipitation were carried out with separate samples of the cell lysates and culture supernatants, but the high level of protein bound non-specifically to the immunoadsorbent staphylococci, as shown in Plate 7.4.a Tracks 1, 2 and 3, made interpretation of the results impossible. Increasing the number of washing steps in the treatment of the staphylococcal pellets did not reduce this non-specific binding. Plate 7.4.a Track 3 shows the protein brought down from the culture supernatant of S.aureus strain RN25. The diffuse band at the bottom of the track is believed to be delta haemolysin. The other intense band has an apparent molecular weight of 32 000 and may be alpha haemolysin. It can be seen that delta haemolysin was also precipitated from the culture supernatant by the Ig fraction from preimmune serum and the
Immunoprecipitation using the Cowan I strain of *S. aureus* as described by Kessler (1975). Cell lysates and culture supernatant were prepared from *S. aureus* strain RN25 grown in the absence of cerulenin and radioactively labelled with L-\((4-5^3\text{H})\) lysine monohydrochloride.

Track 1, proteins precipitated from cell lysate by immunoadsorbent staphylococci alone.

Track 2, proteins precipitated from "cleared cell lysate" by immunoadsorbent staphylococci alone.

Track 3, proteins precipitated from culture supernatant by immunoadsorbent staphylococci alone.

Track 4, proteins precipitated from cell lysate by the Ig fraction of preimmune serum plus immunoadsorbent staphylococci.

Track 5, proteins precipitated from "cleared cell lysate" by the Ig fraction of preimmune serum plus immunoadsorbent staphylococci.

Track 6, proteins precipitated from culture supernatant by the Ig fraction of preimmune serum plus immunoadsorbent staphylococci.

Track 7, proteins precipitated from cell lysate by the Ig fraction of immune serum plus immunoadsorbent staphylococci.

Track 8, proteins precipitated from "cleared cell lysate" by the Ig fraction of immune serum plus immunoadsorbent staphylococci.

Track 9, proteins precipitated from culture supernatant by the Ig fraction of immune serum plus immunoadsorbent staphylococci.
Plate 7.4.a  Immunoprecipitation from cell lysates and culture supernatants of *S. aureus* strain RN25 radioactively labelled with L-(4-5-³H) lysine monohydrochloride
Immunoprecipitation using the Cowan I strain of S. aureus as described by Kessler (1975). Cell lysates and culture supernatant were prepared from S. aureus strain RN25 grown in the presence of cerulenin and radioactively labelled with L-(4-5-\(^3\)H) lysine monohydrochloride.

Track 1, proteins precipitated from cell lysate by immunoadsorbent staphylococci alone.

Track 2, proteins precipitated from "cleared cell lysate" by immunoadsorbent staphylococci alone.

Track 3, proteins precipitated from culture supernatant by immunoadsorbent staphylococci alone.

Track 4, proteins precipitated from cell lysate by the Ig fraction of preimmune serum plus immunoadsorbent staphylococci.

Track 5, proteins precipitated from "cleared cell lysate" by the Ig fraction of preimmune serum plus immunoadsorbent staphylococci.

Track 6, proteins precipitated from culture supernatant by the Ig fraction of preimmune serum plus immunoadsorbent staphylococci.

Track 7, proteins precipitated from cell lysate by the Ig fraction of immune serum plus immunoadsorbent staphylococci.

Track 8, proteins precipitated from "cleared cell lysate" by the Ig fraction of immune serum plus immunoadsorbent staphylococci.

Track 9, proteins precipitated from culture supernatant by the Ig fraction of immune serum plus immunoadsorbent staphylococci.
Plate 7.4.b  **Immunoprecipitation from cell lysates and culture supernatants of S. aureus strain RN25 grown in the presence of cerulenin**

and radioactively labelled with L-(4-5\(^3\)H) lysine monohydrochloride

The protein band in lane 3 may have been due to the fact that most of the protein had already precipitated non-specifically in the previous treatments of the sample. It is possible that the predominating character of delta fatty acids might make it susceptible to non-specific binding in the immunoprecipitation assay. Perhaps the bands seen in lanes 1 to 5 in the immunoprecipitation assay from the cell lysate and the culture supernatant are the expression of S. aureus strain RN25 in the presence of cerulenin.

In Track 1 of Plate 7.4.b, precipitation of a higher molecular weight protein is also evident. This protein is also present in Track 1 of Plate 7.4.a and could be a candidate for production of delta fatty acids, one of which has been brought from lane 7. Finally, the immunoprecipitation assay in Track 3, Plate 7.4.a, and 7.
immunoadsorbent staphylococci (Track 6). Delta haemolysin was also
precipitated by the Ig fraction from immune serum and the
immunoadsorbent staphylococci, although by this stage the intensity of
the protein band had decreased; this may have been due to the fact
that most of the protein had already precipitated non-specifically in
the previous treatments of the sample. It is possible that the
hydrophobic character of delta haemolysin might render it susceptible to
non-specific binding to the immunoadsorbent staphylococci, perhaps due
to interaction of the toxin with the outer membranes of the bacteria.
It is likely that any precursor of delta haemolysin would share some
of its hydrophobic properties and also be susceptible to non-specific
binding to the bacteria. This makes interpretation of the results of
immunoprecipitation experiments using the immunoadsorbent staphylococci
difficult.

Plate 7.4.b illustrates the results of immunoprecipitation from
cell lysates and the culture supernatant of \textit{S. aureus} strain RN25 grown
in the presence of cerulenin. It can be seen from Track 3 that total
extracellular protein production has been greatly reduced, and that
no delta haemolysin has been brought down from the culture supernatant
by the immunoadsorbent staphylococci. Tracks 7 and 9 do show some
precipitation of the delta haemolysin by the Ig fraction of immune
serum and the immunoadsorbent staphylococci from the cell lysate and
the culture supernatant. Perhaps growth of \textit{S. aureus} strain RN25 in
the presence of cerulenin simply results in an accumulation of delta
haemolysin within the cell. However in Track 7 of Plate 7.4.b
precipitation of a higher molecular weight protein is also evident.
This protein is also present in Track 7 of Plate 7.4.a and could be
a candidate for a precursor of delta haemolysin, most of which had
been brought down non-specifically by the immunoadsorbent staphylococci
in Tracks 1, Plates 7.4.a and b.
Immunoprecipitation from cell lysates and culture supernatants using Protein A Sepharose as an immunoadsorbent was investigated in an attempt to resolve the problems of non-specific binding of protein to the immunoadsorbent staphylococci. Protein A Sepharose consists of protein A isolated from a strain of *S. aureus* and covalently coupled to Sepharose CL-4B. Immunoprecipitations were carried out in the same way as described before but replacing the addition of immunoadsorbent staphylococci with the addition of 100 µl of Protein A Sepharose, added as a suspension of the gel (70 mg ml⁻¹) in NET buffer containing azide.

Plate 7.4. e Tracks A and B show the non-specific binding of protein from the cell lysate and culture supernatant of *S. aureus* strain RN25 grown in the absence of cerulenin. The film was exposed for 8 weeks. It can be seen that the non-specific binding of delta haemolysin from the culture supernatant appears to have decreased, but that the overall level of non-specifically bound protein remains high.

Immunoprecipitation studies using Protein A Sepharose with cell lysates and culture supernatants from *S. aureus* strain RN25, grown in the absence or presence of cerulenin, also failed to clearly identify a precursor of delta haemolysin. The precipitation of a few high molecular weight proteins from cell lysates by the Ig fraction of immune serum and Protein A Sepharose was observed, but as in Plate 7.4.a and b Tracks 7, these protein bands were very faint. Again this may have been due to most of the protein having been brought down non-specifically by the Protein A Sepharose.

It was thought that any precursor of delta haemolysin might be present in high amounts at the stage of growth of the culture just before delta haemolysin is known to appear in the supernatant. Delta haemolysin was found to be first detectable in the culture supernatant of *S. aureus* strain RN25 in the mid to late exponential phase of growth.
Immunoprecipitation using Protein A Sepharose as an immunoadsorbent.

Track A, proteins precipitated by Protein A Sepharose alone from cell lysate of *S. aureus* strain RN25 grown in the absence of cerulenin and radioactively labelled with L-(4-5-³H) lysine monohydrochloride.

Track b, proteins precipitated by Protein A Sepharose alone from culture supernatant of *S. aureus* strain RN25 grown as described above.

Immunoprecipitation using Protein A Sepharose as an immunoadsorbent from cell lysates and culture supernatants prepared from cultures of *S. aureus* strain RN25, grown in the absence or presence of cerulenin and harvested in the mid exponential phase of growth.

Track 1, proteins precipitated from cell lysate (- cerulenin) by Protein A Sepharose alone. Track 2, proteins precipitated from culture supernatant (- cerulenin) by Protein A Sepharose alone. Track 3, proteins precipitated from cell lysate (+ cerulenin) by Protein A Sepharose alone. Track 4, proteins precipitated from culture supernatant (+ cerulenin) by Protein A Sepharose alone.

Track 5, proteins precipitated from cell lysate (- cerulenin) by the Ig fraction of preimmune serum plus Protein A Sepharose. Track 6, proteins precipitated from culture supernatant (- cerulenin) by the Ig fraction of preimmune serum plus Protein A Sepharose. Track 7, proteins precipitated from cell lysate (+ cerulenin) by the Ig fraction of preimmune serum plus Protein A Sepharose. Track 8, proteins precipitated from culture supernatant (+ cerulenin) by the Ig fraction of preimmune serum plus Protein A Sepharose. Tracks 9 - 12 as described for Tracks 5 - 8 but using the Ig fraction of immune serum.
Plate 7.4.c  Immunoprecipitation from cell lysates and culture supernatants of *S. aureus* strain RN25 using Protein A Sepharose.

Cultures of *S. aureus* strain RN25 in pyridoxine-deficient (1 × 10^8 cfu) were grown, in the absence of presence of serum, as described before. In this experiment the radioactive label added to

![Image of gel electrophoresis](image.png)

A and B show the presence of proteins. A few of the proteins bands are more intense in Track 1 and 3 than in Track 5 and 7, which show protein bands seen nonspecifically by the Ig fraction of *S. aureus* and Protein A Sepharose. In conclusion, however, the existence of a protein of 25kDa was observed, and its identification remains to be demonstrated.
For this reason immunoprecipitation studies using Protein A Sepharose were carried out with cell lysates and culture supernatants from cultures radioactively labelled with L-(U-\(^{14}\)C) lysine monohydrochloride and harvested in the mid-exponential phase of their growth.

Cultures of *S. aureus* strain RN25 in yeast diffusate medium (2 x 5 ml) were grown, in the absence or presence of cerulenin, as described before. In this experiment the radioactive label added to each culture was 150 \(\mu\)l of L-(U-\(^{14}\)C) lysine monohydrochloride (50 \(\mu\)Ci.ml\(^{-1}\), 340 mCi.mmol\(^{-1}\)) and the cultures were harvested in the mid-exponential phase of their growth. The culture supernatants and cell lysates were prepared and stored as described before. Immunoprecipitation was also carried out as described before, but with 50 \(\mu\)l of the cell lysates and 500 \(\mu\)l of the culture supernatants, and 100 \(\mu\)l of Protein A Sepharose suspension replacing the immunoadsorbent staphylococci.

The results are shown in Plate 7.4.c Tracks 1 - 12. Again the non-specific binding of protein to the Protein A Sepharose (Tracks 1 - 4) is high. It can be seen from Tracks 9 and 11 that several proteins have been precipitated by the Ig fraction from immune serum and Protein A Sepharose, from cell lysates of cultures grown in the absence and presence of cerulenin. A few of the protein bands are more intense in Tracks 9 and 11 than in Tracks 5 and 7, which show protein brought down non-specifically by the Ig fraction of preimmune serum and Protein A Sepharose. In conclusion, however, the existence of a precursor of delta haemolysin and its identification remains to be demonstrated.
CHAPTER EIGHT
DISCUSSION

8.1. Production of delta haemolysin by *Staphylococcus aureus*

The production of different haemolysins and total extracellular protein by several strains of *S. aureus* was investigated (Sections 3.1. and 3.2.). The aim was to choose a strain of *S. aureus* with high production of delta haemolysin and relatively low production of other proteins and haemolysins. It was hoped that this would minimise problems of contamination in purification studies.

In the course of this investigation it was found that the production of normal levels of haemolysins by the strains of *S. aureus* appeared to be related to the presence of prophage, as in strains RN1, RN25, RN27 and A003. Strain RN450, which has been "cured" of prophages, was found to have no detectable haemolytic activity in the culture supernatant. That the normal production of haemolysins may be linked in some way to the presence of prophages is supported by the fact that haemolytic activity is present in the culture supernatants of strains RN451 and RN740. These strains are derived from strain RN450 and have been reinfected with phages.

It was thought that if the ability to produce haemolysins was directly linked to the presence of prophage in a strain, for example if the haemolysins were phage encoded proteins, then induction of the prophage might result in a surge in production of the toxins. The effect of induction of the prophage in strain RN25 on its production of delta haemolysin was investigated using mitomycin C (Section 3.5.). Conditions were established under which lysis of the bacteria was observed, but no surge in the production of delta haemolysin was detected. The nature of the relationship between the presence of prophage and the production of delta haemolysin remains obscure.

The cell lysates of strains RN25, 186X and RN450 were assayed for
haemolytic activity (Section 3.3.), in an attempt to find out if the inability of strain RN450 to produce delta haemolysin was due to an inability to secrete the toxin, resulting in an intracellular build up of the protein. Haemolytic activity was not detected in any of the cell lysates, even when they had been prepared in the presence of PMSF to prevent degradation by proteases. The cell lysates were in fact found to have an inhibitory effect on delta haemolysin. This effect and the failure to detect haemolytic activity within the bacteria, may have been due to the presence of membrane fragments which would be expected to bind the membrane-active toxin.

Strain RN25 was chosen for further study as it exhibited fairly high production of delta haemolysin with lower production of alpha and beta haemolysins when compared with the other strains of S. aureus investigated. The growth of this strain and its production of delta haemolysin were investigated in more detail (Section 3.4.). It was found that delta haemolysin is first detectable in the supernatant in the late exponential phase of growth.

8.2. Purification and properties of delta haemolysin

Three methods for the purification of delta haemolysin were compared using culture supernatant from S. aureus strain RN25. It was hoped that this would reveal whether differences in the properties of delta haemolysin reported by the original authors (Section 1.3.2.) were due to different strains of S. aureus being used or due to the different purification procedures employed.

The three methods investigated included that of Kantor et al. (1972) (Section 4.1.1.). This method involved adsorption of the delta haemolysin to aluminium hydroxide gel, washing the gel in 0.08M phosphate buffer pH 7.2, eluting the toxin from the gel in 0.5M phosphate buffer pH 7.2 and dialysis and concentration of the product.
The method of Kreger et al. (1971) (Section 4.1.2.) involved adsorption of the toxin to hydroxyapatite gel, washing the gel in 0.01M and 0.4M phosphate buffer pH 6.8, elution of the toxin in 1.0M phosphate buffer pH 7.4 and dialysis and concentration of the product. The method of Heatley (1971 and 1976) (Section 4.1.3.) involved precipitation of delta haemolysin from an acidified, heat-treated culture supernatant by the addition of chloroform (1.5%). The haemolysin was then extracted from the washed precipitate in a mixture of chloroform and methanol (2:1 by volume) and purified by transfer between the two phases of a chloroform, methanol, water mixture (10:5:3 by volume). In this mixture the toxin should distribute mainly in the lower phase under neutral conditions and in the upper phase under acidic conditions (Heatley, 1971). The product was finally precipitated with acetone and dried under vacuum.

The results of these methods of purification are shown in Tables 4.1., 4.2. and 4.3. and are summarised in Table 8.1. It can be seen that the method of Heatley (1971 and 1976) resulted in the highest recovery and purification of delta haemolysin and yielded the product with the highest specific activity. This method was also less laborious than the other two methods described and was used for all subsequent purifications of delta haemolysin.

The method of Kreger et al. (1971) resulted in a recovery of 29%, which was comparable with that observed by the original authors (Table 1.3.). The greatest loss of activity occurred during the lengthy dialysis step necessary to reduce the high salt content of the eluent. A precipitate appeared during the dialysis step, a phenomenon also reported by Kreger et al. (1971) and referred to as "insoluble" delta haemolysin. This was reported to represent 35% of the total activity purified and have a specific activity twice that of the "soluble" delta haemolysin. In this study the precipitate represented
Table 8.1. Comparison of three methods for the purification of delta haemolysin

<table>
<thead>
<tr>
<th>Reference</th>
<th>Specific activity (HU.mg(^{-1}))</th>
<th>Recovery (%)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kantor et al. (1972)</td>
<td>52.0</td>
<td>12.3</td>
<td>4.7</td>
</tr>
<tr>
<td>Kreger et al. (1971)</td>
<td>66.9</td>
<td>29.0</td>
<td>5.6</td>
</tr>
<tr>
<td>Heatley (1971 and 1976)</td>
<td>149.0</td>
<td>46.0</td>
<td>13.4</td>
</tr>
</tbody>
</table>

Detailed tables of purification are shown in Section 4.1.
18% of the total activity purified and had a specific activity of 1.3 times that of the "soluble" delta haemolysin. However this precipitate only accounted for 16% of the loss of activity observed during the dialysis step.

The method of Kantor et al. (1972) resulted in the lowest recovery (12%) and purification of delta haemolysin. In contrast the original authors reported a recovery of 93%, however this value was estimated before dialysis and concentration of the product. As in the method of Kreger et al. (1971) the greatest loss of activity occurred during the long dialysis step during which a precipitate appeared which was not reported by Kantor et al. (1972). The precipitate represented 30% of the total activity purified and had a specific activity of 1.2 times that of the "soluble" delta haemolysin product. However, the precipitate only represented 20% of the loss of activity observed during the dialysis step.

The losses of activity observed during the dialysis steps of the methods of Kreger et al. (1971) and Kantor et al. (1972) could have been due to inactivation of the delta haemolysin, although this seems unlikely as the protein is usually observed to be quite stable (Section 1.2.4.). Another explanation for the losses of activity could be that the delta haemolysin escaped through the dialysis bags into the diffusates, where its activity was undetectable in the large volumes. However this also seems unlikely as subsequent gel filtration studies suggest that delta haemolysin exists as a high molecular weight aggregate under neutral conditions. This has also been observed by Pitton (1981). Another alternative is that a proportion of the delta haemolysin might bind to the dialysis membranes.

The products of the three purification procedures were subjected to polyacrylamide gel electrophoresis, amino acid analysis and silica-gel thin layer chromatography in an attempt to characterise and assess the purity of the products.
The products were initially examined using polyacrylamide gel electrophoresis (Section 4.2.1.). Initial attempts to stain delta haemolysin in gels, after polyacrylamide gel electrophoresis in the presence of SDS, were unsuccessful. Using delta haemolysin purified by Dr. N.G. Heatley, using his method, from the culture supernatant of \textit{S. aureus} strain 186X (referred to as "reference" delta haemolysin), the efficacy of three different stains was investigated (Section 4.2.1.1.).

It was found to be necessary to load large amounts of delta haemolysin onto the gels in order to visualise the toxin. It can be seen from Plate 4.1.a) that after staining with a general protein stain (method B, Section 2.2.7.5.), 250 \( \mu \text{g} \) of delta haemolysin appeared as a faint diffuse band. In contrast, the protein standards, of which only 5 \( \mu \text{g} \) of each protein was loaded onto the gel, were visualised as clearly defined, densely stained bands. A stain for basic proteins and small peptides (Steck et al., 1980, Section 2.2.7.5.) was found to be only slightly more effective in visualising the toxin, Plate 4.1.b). The stain which was found to be most effective in staining delta haemolysin contained trichloroacetic acid as a fixative for the protein (method C, Section 2.2.7.5.). After using this stain 250 \( \mu \text{g} \) of delta haemolysin appeared as a fairly dense, although still diffuse, band (Plate 4.1.c)). It was thought that the diffuseness of the toxin band might be due to the large amount of protein loaded onto the gel. However, a lighter loading of 50 \( \mu \text{g} \) of the toxin still appeared as a diffuse band but less densely stained (Plate 4.1.c)).

The need to load large amounts of the toxin onto gels in order to visualise the protein and the diffuseness of the delta haemolysin band might be due to the small size of the protein, enabling it to diffuse within and out of the gel during the staining and destaining procedures. From these gels the molecular weight of delta haemolysin was estimated to be between 4 000 and 8 500.
The results of polyacrylamide gel electrophoresis of the products of the three purification procedures in the presence of SDS (Section 4.2.1.2.) are shown in Plate 4.2. It can be seen that samples purified by the method of Heatley (1971 and 1976), from the culture supernatant of *Staphylococcus aureus* strain RN25, appeared identical to "reference" delta haemolysin, purified by the same method from the culture supernatant of *S. aureus* strain 186X. These preparations gave a single diffuse band with an apparent molecular weight of approximately 6 000. The products of the methods of Kantor et al. (1972) and Kreger et al. (1971), and samples of the "insoluble" delta haemolysin from these procedures, were all found to exhibit several higher molecular weight protein bands in addition to the major, diffuse, low molecular weight band believed to be delta haemolysin. It would seem that differences in the products of the three purification procedures are due to the different methods employed for their preparation.

Only the samples prepared by the method of Heatley (1971 and 1976) appeared to be purified to homogeneity, as determined by polyacrylamide gel electrophoresis in the presence of SDS. However, the contaminating bands in the other samples may be exaggerated by the poor staining of the delta haemolysin. In comparison with the intensity of the protein standards, of which 5 µg of each protein was loaded onto the gel, the additional bands observed with delta haemolysin prepared by the methods of Kantor et al. (1972) and Kreger et al. (1971) appeared to constitute 5 - 10 µg of protein, which represents only 2 - 5% contamination of the samples. However, the impurity of these two preparations has also been observed by Lee et al. (1976) when they tested the products for a number of biological activities associated with the culture supernatant of *S. aureus*.

It is also interesting to note the behaviour of a commercial preparation of melittin in polyacrylamide gel electrophoresis in the
presence of SDS. The toxin can be seen to run as a similarly diffuse band just behind the delta haemolysin. It was also necessary to load a large amount of the protein onto the gel in order to visualise the toxin. These phenomena, observed for both delta haemolysin and melittin, might be due to the small size and perhaps the hydrophobic character of the toxins. The resolution of the delta haemolysin bands was not improved by using polyacrylamide gradient gel electrophoresis in the presence of SDS (Section 4.2.1.3).

Polyacrylamide gel electrophoresis of delta haemolysin in the presence of SDS was also performed by Kantor et al. (1972), who reported a single band of molecular weight less than 10,000. The authors' failure to observe additional higher molecular weight bands after electrophoresis of the toxin may have been due to a smaller amount of protein being loaded onto tube gels. The toxin may have been visualised with more ease in tube gels than in slab gels due to less diffusion of the protein out of the thicker gel.

Polyacrylamide gel electrophoresis of the products of the three methods of purification was attempted under non-denaturing conditions in alkaline and acidic gel systems. In alkaline systems (Section 4.2.1.4.) the samples did not enter the gels, but remained at the origin whether electrophoresis was towards the anode or the cathode. This was also observed by Kantor et al. (1972) who reported that no protein bands were observed to enter the gel after electrophoresis under non-denaturing conditions at pH 9.5. Kreger et al. (1971) reported that under the same conditions two bands were observed, the main component hardly entering the gel.

The results of electrophoresis of the samples under non-denaturing conditions in the acidic gel system of Reisfeld et al. (1962) are shown in Plate 4.4. (Section 4.2.1.5.). "Reference" delta haemolysin and the sample prepared by the method of Heatley (1971 and 1976) were
again observed to behave identically. All of the samples yielded very
diffuse bands, the products of the methods of Kantor et al. (1972) and
Kreger et al. (1971) having slightly lower mobilities. In each case
haemolytic activity was found to coincide with the diffuse protein
bands observed (Section 4.2.1.5.). These results also agree with those
observed by Kantor et al. (1972) who reported a single broad band of
protein after electrophoresis of delta haemolysin under the same
conditions. Kreger et al. (1971) reported two protein bands after
electrophoresis of their product under the same conditions.

The results of polyacrylamide gel electrophoresis under non-
denaturing conditions also indicate that differences in the preparations
of delta haemolysin are due to the different methods of purification
employed and not to differences between strains of S. aureus used.

The products of the different purification procedures and
"reference" delta haemolysin were subjected to amino acid analysis
(Section 4.2.2.). The analyses of all the samples were similar
(Table 4.4.) and all lacked the amino acids proline, tyrosine,
histidine and arginine. This agrees with results reported by Kantor
et al. (1972), Kreger et al. (1971) and Heatley (1971). The analyses
of the product of the method of Heatley (1971 and 1976) and "reference"
delta haemolysin were in the best agreement and most closely approached
the number of amino acids predicted from the sequence elucidated by
Fitton et al. (1980).

N-terminal sequence analysis (Section 4.2.3.) revealed that the
products of the methods of Kantor et al. (1972) and Heatley (1971 and
1976) both yielded the same sequence for 24 amino acid residues as
"reference" delta haemolysin. These sequences agreed with the complete
sequence of delta haemolysin worked out by Fitton et al. (1980).
Attempts to determine the N-terminal sequence of the product of the
method of Kreger et al. (1971) were repeatedly unsuccessful suggesting
the existence of an N-terminal blocking group. Fitton et al. (1980) subsequently reported that up to 80% of a preparation of delta haemolysin purified from the culture supernatant of S. aureus strain 186X by the method of Heatley (1971 and 1976), "reference" delta haemolysin, contained N-formyl methionine at the N-terminus which was detected by mass spectrometry. N-terminal sequence analysis of the product of the method of Kreger et al. (1971), after treatment to remove the N-terminal block, yielded the sequence of the first 10 amino acid residues. This sequence also agreed with that worked out for delta haemolysin by Fitton et al. (1980). It seems that the method of Kreger et al. (1971) resulted in a preparation of delta haemolysin with a very high proportion of the protein containing the N-formyl methionine block at the N-terminus.

Differences in the proportions of N-terminally blocked protein in the samples of delta haemolysin would account for the slightly different mobilities of the preparations in the acidic polyacrylamide gel electrophoresis system of Reisfeld et al. (1962). The sample containing the highest proportion of N-formylated toxin would have the lowest mobility.

Silica-gel thin layer chromatography was performed in an attempt to separate the two forms of delta haemolysin and as an alternative method for the assessment of the purity of the products of the different purification procedures (Section 4.2.4.). The separation achieved by this procedure is illustrated in Figures 4.4.a) and b). The results revealed that all the preparations of delta haemolysin were heterogeneous. However, in each case there were only two spots which were also stained by Erlich's stain for tryptophan-containing peptides. As the amino acid sequence of delta haemolysin (Fitton et al., 1980) contains one tryptophan residue, these spots were believed to be the N-terminally blocked and unblocked forms of delta haemolysin. The
products of the purification methods of Kreger et al. (1971) and Kantor et al. (1972) appeared to contain the highest proportions of the N-terminally blocked species of delta haemolysin.

As silica-gel thin layer chromatography revealed that all the preparations of delta haemolysin were heterogeneous, further purification of the toxin was deemed necessary before studies of the effects of chemical modification on the haemolytic activity of the toxin could be carried out. Nolte and Kapral (1979 and 1981) reported hydrophobic interaction chromatography of purified delta haemolysin on octyl and phenyl sepharose. They reported that the toxin eluted from these materials in 40 - 50\% (v/v) ethanol.

The "best" preparations of delta haemolysin, those purified by the method of Heatley (1971 and 1976), were further purified using chromatography on octyl sepharose (Section 4.3.1.). Haemolytic activity was always found to coincide with a major protein peak eluting in 50 - 70\% (v/v) ethanol, with a leading shoulder which may have been due to the more polar unblocked species of delta haemolysin. Silica-gel thin layer chromatography (Section 4.3.2.) of samples from the shoulder and the main peak supported this and also revealed considerable purification of the delta haemolysin. However, the specific activity of the delta haemolysin was always found to have decreased to 50 - 60 HU.mg^{-1} after chromatography on octyl sepharose. The amino acid analysis of delta haemolysin after chromatography (Section 4.3.3.), when compared with that of "reference" delta haemolysin, also indicates further purification of the toxin, agreeing much more closely with the numbers of amino acids predicted from the sequence of Fitton et al. (1980).

The method employed by Nolte and Kapral for the preparation of delta haemolysin, for use in studies on the hydrophobic interaction chromatography of the toxin, is described by Kapral and Miller (1971).
and Nolte and Kapral (1981). The latter authors estimated the molecular weight of delta haemolysin to be 1600 using polyacrylamide gel electrophoresis in the presence of SDS. They used a system developed for the analysis of oligopeptides by Swank and Munkres (1971), who demonstrated that the molecular weight of several peptides could be determined with an error of less than 18%, but indicated that the intrinsic charge and shape of small peptides is more important in determining their mobility than for larger proteins. Nolte and Kapral (1981) suggest that the discrepancy between their estimate for the molecular weight of delta haemolysin and that calculated from the amino acid sequence of the toxin (Fitton et al., 1980) may be due to the anomalous behaviour of delta haemolysin in polyacrylamide gel electrophoresis in the presence of SDS, perhaps due to the hydrophobic character of the toxin.

The native molecular weight of delta haemolysin, purified by the method of Heatley (1971 and 1976) and octyl sepharose chromatography, was estimated to be 160,000 at pH 7.5 using gel filtration on Sephadex G-150. Comparison of this estimate with the results of polyacrylamide gel electrophoresis in the presence of SDS and the molecular weight of delta haemolysin calculated from the amino acid sequence of Fitton et al. (1980) suggests that the toxin exists as a large multimer under native conditions. This agrees with Kantor et al. (1972), whose estimates for the molecular weight of the toxin range from 5200 to 195,000, they suggest that the toxin exists as a multimeric assembly of identical sub-units.

Fitton (1981) observed a native molecular weight for delta haemolysin of 210,000 at pH 6.0 using gel filtration on Sepharose 6B. However, in 0.5% (v/v) acetic acid, 50mM sodium hydroxide, 70% (v/v) methanol or 6M guanidine hydrochloride, using gel filtration on Sephadex G-50, he found the molecular weight of the toxin to have
decreased to 14,000. He suggests that the toxin exists as a tetramer in aqueous solution at extremes of pH and in aqueous alcoholic solution and as a multimeric aggregate, consisting of 16–18 tetramers (64–72 monomers) at neutral pH in aqueous solution. Fitton (1981) also reports that the fluorescence of tryptophan (residue 15) in the toxin indicates that the amino acid is in a hydrophobic environment at pH 6.0 in aqueous solution, possibly buried within the multimeric structure, and in a more polar environment, possibly exposed, when the toxin is in the form of a tetramer.

The wide range of molecular weights reported for delta haemolysin suggest that the toxin might not exist as structures of rigid stoichiometry, but may form large aggregates of molecular weight 150,000–200,000 or small oligomers according to the environment in which it finds itself.

8.3. The effects of chemical modification on delta haemolysin

As discussed in Section 1.2.4, the mechanism of action of delta haemolysin has been compared to that of melittin, the major lytic peptide of bee venom (Section 1.4.). Thelestam and Möllby (1975b) have shown that the lytic effects of these toxins are similar to, but distinguishable from, those of the non-ionic detergent Triton X-100. They reported that treatment with delta haemolysin or melittin resulted in "small" membrane lesions initially, demonstrated by leakage of radioactively labelled nucleotides (M.W. less than 1,000) from lung fibroblasts. In contrast, treatment with Triton X-100 resulted in the rapid release of radioactively labelled RNA (M.W. greater than 200,000) from lung fibroblasts, indicating the formation of "large" lesions in the membrane immediately. It was necessary to prolong incubation with delta haemolysin or melittin or increase the concentrations of the toxins to produce the latter effect.
The amino acid sequence of melittin was elucidated by Habermann and Jentsch (1967) and is compared with that of delta haemolysin (Fitton et al., 1980) in Figure 8.1. It can be seen that both toxins contain a high proportion of hydrophobic amino acids (30 - 35%) and are amphipathic, with 3 or 4 positively charged amino acids at the C-terminus. Using the method of secondary structure analysis of Pasman and Chou (1974) and Pasman et al. (1976), Fitton (1981) predicted that delta haemolysin contains two alpha helical regions, residues 1 - 6 and 11 - 18, joined by a flexible hinge region, residues 7 - 10. This compares well with the secondary structure predicted for melittin by Dawson et al. (1978) (Figure 8.1 and Section 1.4.). Fitton (1981) also reports that a molecular model of delta haemolysin, incorporating the predicted secondary structure of the toxin, reveals regions of hydrophobic and hydrophilic character on opposing faces of the alpha helices, resulting in an amphipathic structure closely comparable with that suggested for melittin by Dawson et al. (1978) shown in Figure 1.3. (Section 1.4.).

A considerable amount of work has been done on the effects of chemical modification on the activity and properties of melittin. It was hoped that similar modifications could be carried out on delta haemolysin and the effects of such modifications compared with studies on melittin. Studies of this kind might reveal the regions of the delta haemolysin molecule important in the mechanism of its lytic action.

Delta haemolysin was first treated as described in Section 5.1.2. to remove the N-formyl block (Section 4.2.3.) from the N-terminus of the molecule. Thin layer chromatography of the delta haemolysin used in these experiments revealed a main spot believed to be the blocked form of the toxin and a fainter spot believed to be the unblocked species. After treatment to remove the N-formyl moiety, the spot thought to represent the unblocked form was observed to have increased
Figure 8.1 Comparison of the amino acid sequences of delta haemolysin and melittin of bee venom

<table>
<thead>
<tr>
<th>Melittin</th>
<th>Delta haemolysin</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₂CHO</td>
<td>NH₂CHO</td>
</tr>
<tr>
<td>1 GLY</td>
<td>1 MET</td>
</tr>
<tr>
<td>ILE (α)</td>
<td>ALA (α)</td>
</tr>
<tr>
<td>GLY</td>
<td>GLN (α)</td>
</tr>
<tr>
<td>ALA</td>
<td>ASP (α)</td>
</tr>
<tr>
<td>5 VAL (α)</td>
<td>5 ILE (α)</td>
</tr>
<tr>
<td>LEU (α)</td>
<td>LEU (α)</td>
</tr>
<tr>
<td>LYS (α)</td>
<td>SER (α)</td>
</tr>
<tr>
<td>VAL (α)</td>
<td>THR hinge region</td>
</tr>
<tr>
<td>LEU (α)</td>
<td>ILE (α) region</td>
</tr>
<tr>
<td>10 THR</td>
<td>10 GLY</td>
</tr>
<tr>
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<td>ASP (α)</td>
</tr>
<tr>
<td>GLY (α)</td>
<td>LEU (α)</td>
</tr>
<tr>
<td>LEU (α) hinge region</td>
<td>VAL (α)</td>
</tr>
<tr>
<td>PRO (α)</td>
<td>LYS (α)</td>
</tr>
<tr>
<td>15 ALA</td>
<td>TRP (α)</td>
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<tr>
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<tr>
<td>SER (α)</td>
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<tr>
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<tr>
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<td>20 VAL (α)</td>
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<tr>
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<td>THR</td>
</tr>
<tr>
<td>25 GLN</td>
<td>25 LYS (α)</td>
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<tr>
<td>GLN</td>
<td>LYS (α)</td>
</tr>
<tr>
<td>CONH₂</td>
<td>COOH</td>
</tr>
</tbody>
</table>

Amino acid sequence of melittin after Habermann and Jentsch (1967), of delta haemolysin after Fitton et al. (1980). Secondary structure predictions for melittin are after Dawson et al. (1978) and for delta haemolysin after Fitton (1981). Hydrophobic amino acids are marked (●), positively charged amino acids (●) and negatively charged amino acids (●).
in intensity, while the spot believed to be the blocked form of the toxin was seen to decrease in intensity. Treatment to remove the N-formyl block from the N-terminus of delta haemolysin resulted in an average increase in the haemolytic activity of the toxin of 23%.

Habermann and Jentsch (1967) observed that a proportion of melittin is blocked at the N-terminus. This was also observed by Kreil and Kreil-Kiss (1967) and the block identified as an N-formyl group, they detected N-formyl glycine. The N-formyl melittin was found to possess about 80% of the haemolytic activity of unsubstituted melittin (Lubke et al., 1971 and Schröder et al., 1971). This agrees well with the results of treatment to remove the N-formyl block from delta haemolysin, which revealed that the N-formylated form of the toxin also had only 80% of the haemolytic activity of the unblocked form. It seems that the N-terminal residues of both toxins are involved in their optimal activity, the masking of the positive charge of the N-terminus resulting in a decrease in the haemolytic potency of the peptides of 20%. It is conceivable that if delta haemolysin sits in the target membrane in a similar way to that suggested by Dawson et al. (1978) for melittin (Section 1.4.), the presence of the N-terminal positive charge might result in the optimum orientation of the molecule in the membrane.

The involvement of the N-terminal residue of delta haemolysin in its mechanism of action is also implicated by the results of removal of the N-terminal methionine residue by cleavage using cyanogen bromide. After treatment with cyanogen bromide, amino acid analysis revealed that 85 - 95% of the methionine had been removed. The treatment resulted in a 20 - 30% reduction of the haemolytic activity of the toxin. The N-terminal methionine might be necessary to maintain the N-terminal alpha helix predicted by Fitton (1981) and so maintain the correct orientation of the toxin in the target membrane.
The amino groups of melittin, which contains three lysine residues, have been modified by several different reagents. Acetic anhydride has been used with the aim of altering the asymmetric charge distribution of the toxin, while the small size of the resulting amide group would minimise the steric effects of the modification on the conformation of the protein. Habermann and Kowallek (1970) exhaustively acetylated melittin achieving 96% modification of the amino groups. The product was observed to have 78% of the haemolytic potency of unmodified melittin.

After treatment of delta haemolysin with acetic anhydride (Section 5.3.) the haemolytic activity of the toxin was also found to have decreased. The activity of the toxin was reduced by 20 - 80% of that of unmodified delta haemolysin, depending on the extent of acetylation. This was determined by reaction with ninhydrin reagent; 60 - 80% acetylation of the amino groups of the toxin was achieved.

It can be seen that treatment with acetic anhydride appears to affect the haemolytic activity of delta haemolysin to a greater extent than that of melittin. This might be due to the fact that extensively acetylated melittin still bears the two positive charges of the arginine residues at the C-terminus (Maulet et al., 1980), which could maintain some of the amphipathic nature of the molecule thought to be necessary for its activity. In contrast, delta haemolysin contains only lysine residues, therefore the amphipathic character of the toxin could be severely disrupted by acetylation of its amino groups.

A detailed investigation of the effect of acetylation of the amino groups of delta haemolysin might involve the use of radioactively labelled acetic anhydride. The effects of a detailed range of acetylation on the activity of the toxin could be studied. In addition, enzymic digestion of the radioactively labelled
derivatives of the toxin might identify which amino groups are modified preferentially, and reveal the relative importance of the different residues in the mechanism of action of the toxin.

The importance of the amino groups of melittin in relation to its activity is also emphasised by the results of increasing the basicity of the molecule by conversion of the lysine residues to homoarginine using S-methyl isothiourea (Habermann and Kowallek, 1970). The resulting derivative, in which 86% of the amino groups were modified, had 3.5 times the haemolytic potency of unmodified melittin. Further studies on the effects of chemical modification on the haemolytic activity of delta haemolysin could include reaction of the toxin with S-methyl isothiourea. The increase in the basicity of the molecule that would be expected to result from this treatment might also result in an increase in the haemolytic activity of the toxin.

Habermann and Kowallek (1970) also introduced negative charges into the melittin molecule by succinylation of the lysine residues. Succinylation of 93% of the amino groups resulted in a reduction of the haemolytic activity of the toxin to less than 5% of that of unmodified melittin. To investigate the effect of introducing negative charges into the delta haemolysin molecule the protein was reacted with maleic anhydride (Section 5.4.1.). The extent of maleylation of the amino groups was estimated spectrophotometrically to be 49%. This was found to result in total loss of detectable haemolytic activity. Delta haemolysin was also modified using a range of concentrations of radioactive maleic anhydride (Section 5.4.2.) in an attempt to study the effects of different extents of maleylation on the activity of the toxin. The extent of modification of the amino groups ranged from 50 - 90%. In all cases detectable haemolytic activity was lost.

The substitution of the positively charged amino groups of
melittin and delta haemolysin with negatively charged groups appears to have a far more deleterious effect on the haemolytic activity of the toxins than substitution with uncharged amide groups by acetylation. The negatively charged groups may actually repel the toxin molecules from the negatively charged phospholipid head groups of the target membranes.

Further chemical modification of delta haemolysin is limited by the fact that the toxin lacks cysteine, histidine, tyrosine and arginine, amino acids which are susceptible to a great range of chemical modifications. Further studies could include acetylation with radioactive acetic anhydride and analysis of the derivatives, and reaction of the toxin with S-methyl isothioura as discussed before. Modification of the carboxyl groups of delta haemolysin, for example by esterification, could be investigated, along with modification of the tryptophan residue of the toxin. It would also be interesting to investigate the effects of cross-linking delta haemolysin molecules on the activity of the toxin, in order to ascertain whether free monomers of the toxin are required for activity.

The limited number of chemical modifications of delta haemolysin performed in this study do reveal remarkable similarities in the characteristics of the toxin with those of melittin. This is an interesting example in which evolutionary unrelated proteins, with little primary sequence homology, may have significant secondary structural homology enabling them to perform similar functions.

8.4. The production of radioactively labelled delta haemolysin

It was thought that in vivo radioactive labelling of delta haemolysin would be more efficient if the strain of S. aureus used was auxotrophic for the radioactively labelled substrate. Several auxotrophic strains of S. aureus were compared with strain RN25 with
regard to the growth of the strains and their production of delta haemolysin (Section 6.1.1.). Strain P392 was chosen for production of radioactively labelled delta haemolysin as the strain is auxotrophic for lysine, an amino acid which could easily be removed from the rich yeast diffusate medium using the enzyme lysine decarboxylase. It was hoped that removal of lysine from the medium would result in more efficient incorporation of the radioactively labelled amino acid by the bacteria. Amino acid analysis of the yeast diffusate medium revealed that 96% of the lysine could be removed by treatment with lysine decarboxylase (Section 6.1.2.).

Production of radioactively labelled delta haemolysin by *S. aureus* strain P392, grown in medium which had been treated with lysine decarboxylase, was compared with that of *S. aureus* strain RN25 grown in untreated medium (Section 6.1.4.). Although the radioactive delta haemolysin produced by strain P392 had a slightly higher specific activity, the recovery of the toxin was only half that from strain RN25. This was probably due to the poorer growth and production of delta haemolysin by strain P392 (Sections 6.1.1. and 6.1.3.). The results suggest that radioactively labelled delta haemolysin would best be produced from strain RN25 grown in medium treated with lysine decarboxylase. This procedure was subsequently used to produce radioactively labelled cultures for use in immunoprecipitation studies (Section 7.2.2.).

Radioactively labelled delta haemolysin purified by the method of Heatley (1971 and 1976) (Section 6.1.4.) was used in an attempt to more accurately determine the molecular weight of the toxin using polyacrylamide gel electrophoresis in the presence of SDS. It was thought that the difficulty of staining delta haemolysin and the diffuseness of the protein band, after polyacrylamide gel electrophoresis in the presence of SDS (Section 4.2.1.1.), might be
due to diffusion of the toxin within and out of the gel during staining and destaining procedures. It was hoped that using a small amount of radioactively labelled delta haemolysin and following electrophoresis immediately by treatment for fluorography, diffusion of the delta haemolysin might be minimised. The diffusion of the toxin band was reduced and the molecular weight of delta haemolysin was estimated to be between 3 300 and 4 700. This was the closest estimate using polyacrylamide gel electrophoresis in the presence of SDS, to the value calculated from the amino acid sequence of Pitton et al. (1980).

The binding of delta haemolysin to the erythrocyte membrane, at saturation with the toxin, was estimated using radioactively labelled delta haemolysin. The number of molecules bound to the erythrocyte membrane at saturation with the toxin was estimated to be $1.3 \times 10^8$. From the amount of delta haemolysin required to produce 50% haemolysis, the number of molecules bound to the erythrocyte membrane during lysis was estimated to be $1.6 \times 10^7$. Nolte and Kapral (1981), using delta haemolysin labelled in vivo with tritiated isoleucine observed 30% of the toxin bound to the erythrocytes between 10 and 80% haemolysis. Even at this value the level of protein bound to the erythrocytes is high and consistent with a detergent-like mode of action of the toxin.

8.5. Biosynthesis of delta haemolysin

The small size of delta haemolysin and the existence of an N-formyl methionine moiety in a high proportion of the purified toxin, led to the suggestion that the toxin might not be the total gene product but the N-terminal region of a larger precursor protein (Section 1.6.). The problem faced by the bacteria of secretion of such a hydrophobic and potentially harmful protein would also suggest the existence of such a precursor. In addition, the hydrophobic character of the toxin and the existence of the N-formyl methionine
residue, suggest that the toxin might be the signal sequence of another extracellular product of *S. aureus*, also secreted after cleavage from the parent protein. The N-formyl group of the toxin would thus be protected from deformylase, which normally removes this group from proteins post-translationally in prokaryotes.

Procaine and cerulenin have been used to inhibit extracellular protein production, resulting in a build up of the precursors of the exported proteins and leading to their identification (Fishman *et al.*, 1980 and Lazdunski *et al.*, 1979) (Section 1.6.). It was hoped that either using procaine or cerulenin, or by arresting the growth of *S. aureus* strain RN25 before delta haemolysin appeared in the culture supernatant, any build up of a precursor of the toxin might enable its identification, using radioactively labelled cultures and techniques of immunoprecipitation.

The effects of varying concentrations of procaine and cerulenin on the growth of *S. aureus* strain RN25 and its production of delta haemolysin and total extracellular protein production were investigated (Section 7.1.). A concentration of 1.5% (w/v) of procaine was found to reduce the production of delta haemolysin by *S. aureus* strain RN25 to 5% of that of the control culture, and to reduce total extracellular protein production slightly. However, the growth of the culture was affected at this concentration of procaine. The generation time of the bacteria appeared to have lengthened, although the final cell density of the culture was the same as that of the control (Section 7.1.1.). Cerulenin was found to be more effective as an inhibitor of extracellular protein production at much lower concentrations (Section 7.1.2.). A concentration of cerulenin of 18.75 μg.ml⁻¹ was found to reduce delta haemolysin production to 4% of that of the control culture and considerably reduce total extracellular protein production with no apparent effect on the growth of the culture.
This concentration of cerulenin was used in subsequent experiments, added to cultures as a 10 mg.ml\(^{-1}\) solution in ethanol. It was noted that when cerulenin was added as a 1 mg.ml\(^{-1}\) solution in ethanol, the largest volume of ethanol used (3% v/v) when added alone to a control culture, caused a reduction in the production of delta haemolysin to 48% of that of the control culture (Section 7.1.2.). Ethanol (8% v/v) has been reported to inhibit the processing of exported proteins of *Escherichia coli* by Enequist et al. (1981) and Palva et al. (1981), who suggest that this inhibition is due to dissipation of the proton motive force by the ethanol.

Preimmune and immune sera from rabbits immunised against "reference" delta haemolysin, using a schedule described by Turner (1978), was provided by Dr. W.H. Turner of the Wellcome Research Laboratories, Beckenham, Kent. The immunoglobulin fractions from the sera were prepared for experimental use (Section 7.2.1.), as delta haemolysin has been reported to form precipitin lines with unimmune sera (Heatley, 1977).

Initially immunoprecipitation studies were carried out using the protein A-bearing Cowan I strain of *S. aureus* as described by Kessler (1975) (Section 7.2.2.). Plates 7.4.a) and b) show results obtained from cultures labelled with L-(4-5-\(^3\)H) lysine monohydrochloride, in the absence or presence of cerulenin, and some of the problems encountered in this experimental approach. A large amount of protein was found to bind non-specifically to the immunoadsorbent staphylococci. This was not reduced by increasing the number of washing steps in the treatment of the immunoadsorbent staphylococci after immunoprecipitation. Precipitation, by the immunoadsorbent staphylococci, of delta haemolysin and another protein (M.W. 32 000), possibly alpha haemolysin, from the culture supernatant of the radioactively labelled bacteria was observed. These proteins were not precipitated from the culture
supernatant of bacteria treated with cerulenin, indicating the inhibition of extracellular protein production.

It is possible that the hydrophobic character of delta haemolysin might render it particularly susceptible to the phenomenon of non-specific binding of protein to the immunoadsorbent staphylococci observed, perhaps due to interaction of the toxin with the cell surface of the bacteria. It is likely that a precursor of delta haemolysin would share some of its hydrophobic properties and also be susceptible to the non-specific binding of protein described. This might result in any precursor of delta haemolysin being removed from the cell lysates or culture supernatants by the immunoadsorbent staphylococci and therefore unavailable for reaction with antibody to delta haemolysin. This makes interpretation of the results of immunoprecipitation experiments using the immunoadsorbent staphylococci difficult.

Precipitation of a high molecular weight protein from the cell lysate of *S. aureus* strain RN25 grown in the presence of cerulenin was observed (Plate 7.4.b) Track 7). This protein was also precipitated from the cell lysate of strain RN25 grown in the absence of cerulenin (Plate 7.4.a) Track 7) and could be a candidate for a precursor of delta haemolysin, most of which is precipitated non-specifically by the immunoadsorbent staphylococci.

Immunoprecipitation from cell lysates and culture supernatants using Protein A Sepharose as an immunoadsorbent was investigated in an attempt to reduce the non-specific binding of protein. Plate 7.4.c) Tracks A and B show the non-specific binding of protein from the cell lysate and culture supernatant of *S. aureus* strain RN25 grown in the absence of cerulenin. Although the non-specific precipitation of delta haemolysin from the culture supernatant appeared to have been reduced, the overall level of non-specifically bound protein from the cell lysate remained high.
It was thought that a precursor of delta haemolysin might be present in high concentration at the stage of growth of the culture just before delta haemolysin appears in the culture supernatant. Delta haemolysin was found to be first detectable in the culture supernatant of *S. aureus* strain RN25 in the mid to late exponential phase of growth (Section 3.4.). Immunoprecipitation using Protein A Sepharose, from cell lysates and culture supernatants of *S. aureus* strain RN25 labelled with L-$(U-^{14}C)$ lysine monohydrochloride and harvested in the mid-exponential phase of growth, was investigated.

An example of the results obtained is shown in Plate 7.4.c). Non-specific binding of protein to the Protein A Sepharose was again high. Tracks 9 and 11 reveal that several high molecular weight proteins were precipitated by the Ig fraction of immune serum and Protein A Sepharose from the cell lysates of strain RN25 grown in the absence or presence of cerulenin. One protein of a similar molecular weight to that observed in Plate 7.4.a) and b) Tracks 7, was more intense in Track 11 (culture grown in the presence of cerulenin) than in Track 9 (culture grown in the absence of cerulenin). This protein band was also more intense in Track 11 than in Tracks 5 and 7, protein precipitated non-specifically by the Ig fraction of preimmune serum and Protein A Sepharose. This protein might then be a precursor of delta haemolysin.

Immunoprecipitation studies which might clearly identify any precursor of delta haemolysin could include the use of higher titre antibody, perhaps using a double antibody precipitation system. Studies might be more successful if dilute samples of the cell lysates were used and possibly fractionated samples of the cell lysates.
REFERENCES


Comparison of Three Methods for the Purification of the Delta Haemolysin of *Staphylococcus aureus*

By GILLIAN M. SMITH and WILLIAM V. SHAW*

Department of Biochemistry, University of Leicester, Leicester LE1 7RH

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Three methods for the purification of *Staphylococcus aureus* delta haemolysin were compared (Kantor *et al.*, 1972; Kreger *et al.*, 1971; Heatley, 1971). The products of these purifications from the culture supernatant of *S. aureus* strain RN25 were compared by electrophoresis, amino acid analysis, amino-terminal sequence analysis and thin-layer chromatography. The method of Heatley (1971) was found to be superior in terms of recovery and purity of the product. Delta haemolysin prepared by the method of Kreger *et al.* (1971) could not be sequenced successfully prior to treatment aimed at the removal of the *N*-formyl group at the amino-terminus. Delta haemolysin appears to exist in two distinct molecular forms, one with *N*-formylmethionine and the other with un-formylated methionine in the amino-terminal position. The former polypeptide species is purified preferentially by the method of Kreger *et al.* (1971). Thin-layer chromatography of the products of each method of purification revealed that they were all heterogeneous, although the major component from the product of the method of Heatley (1971) represented not less than 70% of the product.

**INTRODUCTION**

*Staphylococcus aureus* produces a number of extracellular toxins (Bernheimer, 1974; Rogolsky, 1979). Delta haemolysin is one of several cytolytic toxins produced by the organism and is distinguished by some notable properties (McCartney & Arbuthnott, 1978). It is relatively thermostable and can withstand heating to 80 °C for 15 min; it is hydrophobic in character and is soluble in a 2:1 (v/v) mixture of chloroform and methanol; it has a high proportion of hydrophobic amino acids and is devoid of proline, arginine, tyrosine, cysteine and histidine. It also has a low specific haemolytic activity and exhibits little or no species specificity as regards the cell type used in the assay system. The toxin is inhibited by phospholipids, some fatty acids, normal serum and the alpha and beta globulin fractions of serum. It has been shown to be highly surface-active and its mode of action is thought to be detergent-like.

Delta haemolysin was first claimed to have been purified by Yoshida (1963) and subsequently by Kreger *et al.* (1971), Heatley (1971) and Kantor *et al.* (1972). Amino acid analyses of the products of these purification methods were similar and all showed a gross deficiency or absence of the residues noted above. There were, however, differences in the reported physico-chemical properties of the products, notably in ultracentrifugation studies, isoelectric focusing and polyacrylamide gel electrophoresis. The products of the purification methods of Kantor *et al.* (1972) and Kreger *et al.* (1971) have also been studied by Lee *et al.* (1976) and judged to contain varying but low levels of other toxic or enzymic activities.

The present study was designed to determine whether the reported differences between preparations of delta haemolysin are a consequence of the strains of *S. aureus* used or arise from variations in isolation and purification techniques. The three most recently described
methods were used to purify delta haemolysin from the culture supernatant of one genetically well defined strain and the products were compared directly by electrophoresis, amino acid analysis and partial or complete sequence determination. A preliminary account of these studies has been presented (Smith et al., 1979).

**METHODS**

*Reagents.* Unless otherwise stated reagents were obtained from Fisons Scientific Apparatus (Loughborough, Leics.) and were of analytical grade.

*Bacterial strains.* The study was performed with *Staphylococcus aureus* RN25 (kindly provided by Dr R. Novick). Strain RN25 is a derivative of strain RN1 which is lysogenic for at least three staphylococcal phages (φ11, φ12 and φ13); RN25 appears to harbour only phage φ13 (R. Novick, personal communication).

*Maintenance of the organism.* The strain was maintained on plates of Penassay base agar (Difco) kept at 4 °C, and was subcultured monthly.

*Growth of the organism.* *Staphylococcus aureus* RN25 was grown in the yeast diffusate medium of Gladstone & van Heyningen (1957). Small-scale cultures (10 ml volumes of sterile medium in 100 ml Erlenmeyer flasks) were grown overnight at 37 °C with shaking at 150–180 rev. min⁻¹ on a gyrotary shaker. Large-scale cultures (500 ml volumes of sterile medium in 2 l Erlenmeyer flasks) were inoculated with 3 ml of small overnight cultures and grown as described above. Cells were removed by centrifugation at 13000 g for 15 min at 20 °C. The supernatant was stored for up to a few days at 4 °C or for longer periods at −20 °C.

*Assay for haemolytic activity.* Citrated human blood (type O, Rh positive) from a single donor was washed five times in saline and finally resuspended in phosphate-buffered saline (PBS) pH 6-8 (Heatley, 1971). Blood was prepared fresh each week and stored at 4 °C. Each week a standard curve was constructed using a stock solution of delta haemolysin purified from *S. aureus* strain 186X (supplied by Dr N. G. Heatley).

Samples for assay were made up to 0-5 ml with PBS and mixed with 0-5 ml of 25-fold diluted erythrocyte suspension in PBS. The tubes were incubated for 15 min at 37 °C with mixing and then centrifuged for 1-5 min at 12000 g to remove unlysed cells and cell debris; 0-5 ml samples of the supernatants were removed and added to 2-5 ml portions of 0-1% (w/v) Na₂CO₃ and mixed. A sample substituting water for PBS was taken to represent 100% haemolysis and the absorbance maximum of the resulting haemoglobin solution was taken as the amount of toxin yielding 50% haemolysis in the standard assay. Haemolytic activities of samples were calculated from a standard curve.

*Estimation of protein.* Protein was estimated by the Lowry method. Protein concentration was calculated from a standard curve constructed using Fraction V bovine serum albumin (Sigma).

*Cell growth of strain RN25 and production of delta haemolysin.* Sterile medium (500 ml) in a 2 l Erlenmeyer flask was inoculated with 5 ml of a small overnight culture (A₄₅₀ 16-8). At 30 min intervals 1 ml samples were removed. Part of each sample (0-5 ml) was heat-treated at 60 °C for 1 h to remove any alpha haemolysin; any precipitate that appeared was removed by centrifugation at 12000 g at room temperature for 3 min. The supernatant was assayed for delta haemolysin. The remainder of each sample was used to monitor growth turbidimetrically at 540 nm. Reliable measurement of changes in the total protein in the culture fluid was not possible because of the rich growth medium used.

*Purification of delta haemolysin.* Delta haemolysin was purified from the culture supernatant of *S. aureus* strain RN25 by three different methods.

1. *Selective desorption from alumina Cy gel* (Kantor et al., 1972). This method involved adsorption of delta haemolysin to aluminium hydroxide gel (alumina Cy gel; Sigma). The method of Kantor et al. (1972) was adhered to closely. In our case, however, a higher gel to protein ratio of 1:5 was required to achieve 90% adsorption of the haemolytic activity when the culture supernatant was mixed with the gel. The gel was washed with 0-08 M-phosphate buffer pH 7-2 and then eluted with 0-5 M-phosphate buffer pH 7-2. When the eluted protein was dialysed against distilled water a precipitate appeared; this was not reported by the original authors. The precipitate was removed by centrifuging for 10 min at 6000 g at 4 °C, lyophilized and assayed for haemolytic activity. The supernatant was concentrated as described in the original report and then lyophilized.

2. *Chromatography on hydroxylapatite* (Kreger et al., 1971). This method involved adsorption of delta haemolysin to hydroxylapatite gel (Biogel HTP; Bio-Rad Laboratories). The gel was mixed with the culture supernatant and then dialysed first with 0-01 M-phosphate buffer pH 6-8 and then with 0-4 M-phosphate buffer pH 6-8. The haemolytic activity was eluted with 1-0 M-phosphate buffer pH 7-4. The precipitate which appeared on dialysis of the eluate against distilled water was removed by centrifuging for 10 min at 6000 g at 4 °C,
lyophilized and assayed for haemolytic activity. The supernatant was concentrated as described in the original report and then lyophilized.

3. Solvent transfer method (Heatley, 1971). The parameters of this method were investigated by Heatley (1971; 1976). It involves precipitation of the haemolysin by the addition of chloroform (final concentration 1·5%, v/v) to an acidified heat-treated culture supernatant. The toxin is then extracted from the precipitate with chloroform/methanol (2:1, by vol.) and purified by transfer between the two phases of chloroform/methanol/water (10:5:3, by vol.). The haemolysin distributes mainly in the lower phase under neutral conditions and in the upper phase under acid conditions (Heatley, 1971).

Supernatant fluid was heated to 60 °C and maintained at this temperature with occasional stirring for 1 h. The resulting precipitate was removed by centrifuging at 16000 g for 45 min at 20 °C. The supernatant was removed and adjusted to pH 5·3 with 5 M-phosphoric acid, chloroform was added (final concentration 1·5%, v/v) and the mixture was stirred overnight at room temperature. The dark grey precipitate which appeared was collected by centrifuging at 16000 g for 45 min at 4 °C and washed by resuspension in 120 ml of 0·01 M-phosphate buffer pH 6·8 saturated with chloroform. The suspension was again centrifuged at 4 °C and the resulting precipitate was resuspended in 270 ml of chloroform/methanol (2:1, by vol.) and left overnight at 4 °C. The precipitate was again collected by centrifugation and the clear, yellowish supernatant fluid was filtered through Whatman no. 1 paper. To this supernatant was added 50 ml of 0·1 M-phosphate buffer pH 6·8 with thorough mixing. The phases were left to separate for about 1 h at room temperature. The lower layer (expected to contain the toxin) was removed and the upper layer was again extracted with 170 ml of chloroform/methanol/water (86:14:1, by vol.). The lower layer was again removed and pooled with that of the first extraction. The pooled lower layers were then extracted five times with 20 ml of acidified chloroform/methanol/water (3:48:47, by vol.), containing sequentially 100, 300, 200, 200 and 200 μl of 1 M-phosphoric acid. Each of the five extracts was then treated with 125 ml acetone to precipitate the protein. A white precipitate appeared in the first and second extracts. The precipitates were centrifuged as before, the supernatants were removed and the precipitates were dried under vacuum overnight.

Polyacrylamide gel electrophoresis. Electrophoresis was performed in gel slabs (15 x 14 x 0·2 cm) in a vertical apparatus at room temperature at constant voltage. Stacking was carried out at 60 V and separation was achieved at 120 V for approximately 3 h.

Electrophoresis in the presence of sodium dodecyl sulphate (SDS). The method used was based on that of Laemmli et al. (1970) using a ratio of acrylamide to bisacrylamide of 77:1 for 10% (w/v) acrylamide gels, 173:1 for 15% (w/v) acrylamide gels and 239:1 for 17·5% (w/v) acrylamide gels. Electrophoresis was towards the anode using bromophenol blue (0·1%, w/v) as the tracking dye.

Gradient gel electrophoresis in the presence of SDS. This was carried out using gels with a linear gradient (10–30%, w/v) of acrylamide under the conditions outlined above.

Electrophoresis under non-denaturing conditions (alkaline pH). The system outlined for SDS electrophoresis was used omitting SDS from all buffers. The sample buffer was replaced by 50 mM-Tris/HCl buffer pH 7·8 containing 0·1 M-2-mercaptoethanol plus glycerol (1 drop in each sample). The gels were electrophoresed as above.

Electrophoresis under non-denaturing conditions (acidic pH). 'Native' electrophoresis was also carried out in the acidic system of Reisfeld et al. (1962). Electrophoresis was towards the cathode using methyl green (0·5%, w/v) as the tracking dye.

Staining and destaining. Gels were stained overnight at room temperature in a solution containing 2 g PAGE blue G-90 and 100 g trichloroacetic acid in 1 litre of 40% (v/v) methanol in distilled water. Gels were destained at room temperature in 7% (v/v) acetic acid.

Amino acid analysis. Amino acid analyses were performed using a single column amino acid analyser by the method of Spackman et al. (1958). Samples were hydrolysed in 6 M-HCl containing 10 μM-phenol for 24, 48 and 72 h at 105 °C in sealed, evacuated tubes. Chromatograms of the samples and standards were integrated automatically and data were normalized by the method of Ambler (1975).

N-terminal amino acid sequence analysis. Automated amino-terminal sequencing of the products of the three purification procedures was performed using an Anachem APS 2400 solid-phase sequencer based on the principles described by Laursen (1971). The samples were coupled to isothiocyanate-glass (Machleidt et al., 1973). Phenylthiodydantoin amino acids were identified by silica gel thin-layer chromatography (Bridgen et al., 1975).

Thin-layer chromatography. Thin-layer chromatography of the products of the three purification procedures was used as an alternative to polyacrylamide gel electrophoresis for assessing their purity. Thin-layer plates (20 x 20 cm; 0·25 mm silica gel; Polygram Sil G, Camlab, Cambridge) were used. Samples (0·5 mg) of each delta haemolysin preparation were dissolved in water and spotted across the origin. The chromatogram was developed in n-butanol/water/acetic acid (45:30:25, by vol.) for about 4 h, air dried and then stained by spraying with cadmium ninhydrin reagent (Glazer et al., 1975). The ninhydrin-positive areas were circled and the chromatogram was then re-stained for tryptophan-containing peptides using Ehrlich's reagent (Glazer et al., 1975).
Fig. 1. Growth of *S. aureus* strain RN25 and production of delta haemolysin. Samples of the culture fluid were taken at the times indicated for measurement of turbidity (○), and for assay of haemolysin (●) after removal of bacteria by centrifugation. Growth was at 37 °C in the medium described in Methods.

### Table 1. Purification of delta haemolysin by three different methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Recovery (%)</th>
<th>Specific activity of final product (haemolytic units mg⁻¹)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kantor <em>et al.</em> (1972)</td>
<td>12.3</td>
<td>52</td>
<td>4.7</td>
</tr>
<tr>
<td>Kreger <em>et al.</em> (1971)</td>
<td>29.0</td>
<td>67</td>
<td>5.6</td>
</tr>
<tr>
<td>Heatley (1971)</td>
<td>46.0</td>
<td>149</td>
<td>13.4</td>
</tr>
</tbody>
</table>

### RESULTS

**Growth of *S. aureus* RN25 and production of delta haemolysin**

Delta haemolysin was produced in the late-exponential phase of growth and reached a constant level in the stationary phase (Fig. 1). All cultures were therefore grown overnight and harvested approximately 15–17 h after inoculation.

**Purification of delta haemolysin**

1. **Selective desorption from alumina *Cy* gel (Kantor *et al.*, 1972).** The results of the purification are shown in Table 1. Recovery was low. Only 60% of the toxin adsorbed to the gel was eluted and losses were considerable during the long dialysis and concentration steps. The purification factor and the specific activity of the product were the lowest of the three purification methods tested. The appearance of a precipitate on dialysis was comparable with that seen in the method of Kreger *et al.* (1971). The precipitate represented 5% of the initial activity but only accounted for 20% of the loss of activity on dialysis. The specific activity of the precipitate was slightly higher than that of the product.

2. **Chromatography on hydroxylapatite (Kreger *et al.*, 1971).** The results of the purification are shown in Table 1. The appearance of a precipitate in the dialysis step was reported previously by Kreger *et al.* (1971) and was described as 'insoluble' delta haemolysin. They reported that it represented 35% of the total activity purified. The precipitate which appeared in the dialysis step in the present study represented 21% of the total activity purified.
Fig. 2. Polyacrylamide gel electrophoresis in the presence of SDS.
(a) In 15% (w/v) acrylamide: track 1, standards – Fraction V bovine serum albumin (mol. wt 65000), chymotrypsinogen (24500), lysozyme (14500) and RNAase (12100); 2, 250 µg delta haemolysin purified by the method of Kantor et al. (1972); 3, 250 µg delta haemolysin purified by the method of Kreger et al. (1971); 4, 250 µg delta haemolysin purified by the method of Heatley (1971); 5, 250 µg delta haemolysin supplied by Dr N. G. Heatley, purified by his method from S. aureus 186X.

(b) In a linear gradient of 10–30% (w/v) acrylamide: track 1, myoglobin standards – mol. wt 16900, 14400 and 6000–8000; 2, 250 µg delta haemolysin purified by the method of Kantor et al. (1972); 3, 250 µg delta haemolysin purified by the method of Kreger et al. (1971); 4 and 5, Fraction V bovine serum albumin (mol. wt 65000); 6, 250 µg delta haemolysin purified by the method of Heatley (1971); 7, 250 µg delta haemolysin supplied by Dr N. G. Heatley, purified by his method from S. aureus 186X.

and had 1-3 times the specific activity of the ‘soluble’ delta haemolysin product. Recovery was better than that obtained by the method of Kantor et al. (1972) (see above), the greatest loss of activity occurring in the dialysis and concentration steps. The precipitate of ‘insoluble’ delta haemolysin only represented 16% of the loss of activity on dialysis. The degree of purification and specific activity of the product were also higher than achieved by the method of Kantor et al. (1972).

3. Solvent transfer method (Heatley, 1971). As indicated in Table 1, this method resulted in the highest recovery and the highest specific activity of delta haemolysin of the three methods used. The method was found to be simple, reproducible and very much quicker than the other two methods.

Polyacrylamide gel electrophoresis

The products of the three purification methods were compared with a sample purified by the method of Heatley (1971) from S. aureus 186X and kindly donated by him.

It was necessary to overload the gels in order to visualize the delta haemolysin. In SDS gels (Fig. 2a) the delta haemolysin ran as a very diffuse band just behind the dye front with an apparent molecular weight of less than 10000. The products of purification by the methods of
Kantor et al. (1972) and Kreger et al. (1971) showed multiple additional bands with higher apparent molecular weights. These could in principle be either impurities or aggregates of delta haemolysin. Delta haemolysin prepared by all three methods did not stain well in spite of heavy loading and care in destaining. This phenomenon is thought to be an inherent property of the molecule which may have led previously to emphasis being given to the additional bands in the preparations obtained by the methods of Kantor et al. (1972) and Kreger et al. (1971).

Polyacrylamide gradient gels in the presence of SDS (Fig. 2b) were run in an attempt to sharpen the delta haemolysin band and estimate more accurately the molecular weight of the toxin polypeptide. The delta haemolysin still ran as a diffuse band with an apparent molecular weight of less than 7000. Additional bands such as were noted previously were again observed in the delta haemolysin purified by the alumina or hydroxylapatite methods. Insulin B chain and melittin, which have molecular weights very similar to that calculated for delta haemolysin from amino acid sequence analysis (Fitton et al., 1980), also ran as diffuse bands under the same conditions (results not shown). This observation suggests that, with the conditions employed for electrophoresis under denaturing conditions, the diffuseness of the delta haemolysin band is a simple consequence of molecular size.

In alkaline systems without SDS the preparations of delta haemolysin did not enter the gel but remained at the origin, whereas in the acidic system of Reisfeld et al. (1962) they ran as a diffuse band (Fig. 3). The preparations obtained by the methods of Kantor et al. (1972) and Kreger et al. (1971) had slightly lower mobilities. Two sets of samples were run in this experiment, one set on each half of the gel. Half of the gel was visualized by staining for protein whereas each track in the other half of the gel was cut into 5 mm strips which were pulverized in 0-2 ml PBS and eluted overnight at 37°C prior to assay for haemolytic activity. Haemolytic activity in each track was localized only to that part of the track which corresponded to the diffuse band visualized by the protein stain.

Amino acid analysis

The amino acid analyses of the products of the three purification procedures were similar in that they all lacked proline, arginine, histidine and tyrosine, although there were slight
differences in the amounts of the other amino acids (Table 2). The amino acid analysis of the product obtained by the purification method of Heatley (1971) agreed very well with that of the delta haemolysin provided by Dr N. G. Heatley and purified from *S. aureus* strain 186X by his method.

### N-terminal amino acid sequence analysis

Analysis of the delta haemolysin prepared by the methods of Kantor *et al.* (1972) and Heatley (1971) gave the following amino-terminal sequence: N H ^-X^-Ala-Gln-Asp-Ile-Ile-Ser-Thr-Ile-Gly-Asp-Leu-Val-X-Trp-Ile-Asp-Thr-Val-Asn-X-Phe-Thr-X-X, where X signifies that no phenyl thiohydantoin amino acid was detected. Lysine residues and the amino-terminal residue are not usually seen due to the method of attachment of the polypeptides to the glass support. This sequence, together with the amino acid analyses of the products, suggested that their amino acid sequences were identical with that found by Fitton *et al.* (1980) for delta haemolysin purified by Dr N. G. Heatley by his method from *S. aureus* 186X, containing N-terminal methionine. Repeated attempts to determine the N-terminal sequence of delta haemolysin purified by the method of Kreger *et al.* (1971) were unsuccessful, suggesting that the terminal amino acid was blocked. Mass spectrometry of delta haemolysin purified by the method of Heatley (1971) from *S. aureus* 186X showed that a proportion of it contained N-formylmethionine (Fitton *et al.*, 1980). When the Kreger preparation was treated with 1 m-HCl in methanol for 1 h at room temperature to unblock the amino-terminus (Fitton *et al.*, 1980), subsequent sequence analysis revealed that the first ten residues were the same as in the other preparations.

### Thin-layer chromatography

An example of the separation achieved using thin-layer chromatography is shown in Fig. 4. Delta haemolysin purified in this study by the method of Heatley (1971) was identical chromatographically with that supplied by Dr N. G. Heatley; however, each preparation was found to be heterogeneous. The most densely stained spot is believed to be delta haemolysin. The preparations of delta haemolysin purified by the methods of Kantor *et al.* (1972) and...
Fig. 4. Thin-layer chromatography of the products of the three delta haemolysin purification procedures. The continuous outlines represent areas stained with ninhydrin and the dashed outlines represent tryptophan-positive areas stained with Ehrlich’s reagent. The intensities of the stained areas are indicated according to the scale: +, faint; 1 to 4, in order of increasing intensity. The solvent system was n-butanol/water/acetic acid (45:30:25, by vol.). Track 1, delta haemolysin purified by the method of Kantor et al. (1972); 2, delta haemolysin purified by the method of Kreger et al. (1971); 3, delta haemolysin purified by the method of Heatley (1971); 4, delta haemolysin supplied by Dr N. G. Heatley, purified by his method from S. aureus 186X.

Kreger et al. (1971) differed in that they exhibited additional spots; however, they also yielded densely staining spots attributable to delta haemolysin. In each case this spot was also the most tryptophan-positive spot when stained to detect tryptophan. When this spot was eluted and assayed for haemolytic activity it was found to be active whereas controls from other regions of each track were negative (data not shown).

**DISCUSSION**

Comparison of the products of the three different purification procedures for delta haemolysin revealed several slight differences between them. Each method gave a different recovery and a different degree of purification. The method of Kantor et al. (1972) gave a very low recovery and purification, with the greatest loss of activity occurring during dialysis and concentration. The method of Kreger et al. (1971) gave recoveries and purification which were only modestly better than those obtained by the method of Kantor et al. (1972). Both methods were mechanically clumsy, labour intensive and time-consuming: large volumes of eluate had to be dialysed and concentrated. Both resulted in the appearance of a white precipitate on dialysis of the eluate which represented 20–40% of the purified haemolytic activity. This was first observed by Kreger et al. (1971) and referred to as ‘insoluble’ delta haemolysin. The method of Heatley (1971) was technically the simplest and quickest and resulted in the highest recovery and overall purification.

Polyacrylamide gel electrophoresis in the presence of SDS revealed differences between the three preparations in that those obtained by the methods of Kantor et al. (1972) and Kreger et al. (1971) exhibited multiple additional high molecular weight bands; these could be due to impurities or, less likely, aggregates of the toxin. Delta haemolysin appeared as a major and
very diffuse band of apparent molecular weight of less than 7000 in all of the preparations. In the acidic system of Reisfeld et al. (1962) without SDS the preparations all ran as diffuse bands with almost identical mobilities. Haemolytic activity was localized over the whole of the diffuse bands. This heterogeneity of the preparations might account for some of the discrepancies which have arisen in connection with previously published studies of delta haemolysin. Differences appear, therefore, to be due to methods of preparation as the strain used in this study was the same for each method. This is supported by the fact that delta haemolysin prepared by the method of Heatley (1971) from different strains appeared to be identical.

Slight differences in the amino acid analyses were observed for the three different preparations of delta haemolysin. However, none contained proline, arginine, tyrosine and histidine. This suggests that the additional stained bands observed after polyacrylamide gel electrophoresis in SDS were either aggregates of the monomeric delta haemolysin or contaminating proteins which were quantitatively unimportant. We favour the latter view since the higher molecular weight species did not give a regular pattern nor did they appear with the preparation purified by the solvent transfer method. The difficulty encountered in staining delta haemolysin by conventional methods suggests that other workers may have drawn attention to bands on SDS gels which were contaminants rather than the delta haemolysin which was certainly present. These contaminants appeared to be more readily excluded from the final preparation by the organic solvent transfer method than by the other techniques which rely on aqueous solvents.

The N-terminal sequence of the preparations purified by alumina adsorption or the solvent transfer method agreed with that found for delta haemolysin prepared by the method of Heatley (1971) from \textit{S. aureus} 186X (Fitton et al., 1980). Mass spectrometry of the latter preparation of delta haemolysin showed that in addition to the unblocked peptide, \textit{N}-formylmethionine was present at the amino-terminus of a proportion of these molecules. Treatment of the product prepared by the method of Kreger et al. (1971) with methanolic HCl yielded a polypeptide which was identical with other preparations for the first ten amino acid residues of the primary structure. It seems likely, therefore, that delta haemolysin exists in culture filtrates in at least two forms, the unblocked polypeptide and the \textit{N}-formyl blocked form, and that the latter is preferentially purified by the method of Kreger et al. (1971).

Thin-layer chromatography revealed that all the preparations consisted of several components of which the delta haemolysin was the most prominent and which represented visually not less than 60\% of the total peptide applied. Sequencing data firmly in agreement with the sequence found by Fitton et al. (1980) also suggest that the delta haemolysin is the major component. However, it is believed that perhaps a family of similar molecules are purified by the methods employed including the two forms of delta haemolysin.

We conclude that the principal component of the \textit{S. aureus} delta haemolysin purified from the same culture supernatant by three different methods is a 26 residue polypeptide which is identical in primary structure to that described recently by Fitton et al. (1980). The single and probably important qualification is that each of the three methods yields a product which contains a variable amount of the \textit{N}-formylated terminal methionyl delta haemolysin. The hydroxylapatite chromatography procedure yields this molecule exclusively, whereas the alumina C\textgamma gel method and the solvent transfer procedure yield mixtures of both molecular species, the formyl-blocked product being the major component in the latter. Studies are in progress to determine whether the \textit{N}-formyl delta haemolysin is the amino-terminal sequence of a unique membrane-associated or secreted protein which is synthesized in large amounts or whether it serves as a leader or signal sequence for a family of such proteins.

We thank Dr N. G. Heatley for his advice and encouragement and for reference samples of delta haemolysin purified by his method. The Science Research Council and The Wellcome Foundation provided support in the form of a C.A.S.E. studentship to Gillian Smith. We are grateful to Dr J. E. Fitton and J. Keyte for assistance with N-terminal sequence and amino acid analyses.
REFERENCES


STUDIES ON THE DELTA HAEMOLYSIN OF
STAPHYLOCOCCUS AUREUS
by GILLIAN MARGARET SMITH B.Sc.

ABSTRACT

The production of haemolysins by several strains of Staphylococcus aureus was investigated and strain RN25 chosen for further study.

Three methods for the purification of delta haemolysin were compared for efficiency and purity of the products. The products were characterised using polyacrylamide gel electrophoresis, amino acid analysis and N-terminal sequence analysis. The method of Heatley (1971 and 1976) was found to be the most efficient and yielded the product of highest purity and specific activity. From polyacrylamide gel electrophoresis in the presence of SDS the molecular weight of delta haemolysin was estimated to be less than 5000. N-terminal sequence analysis of the products of the purification procedures led to the observation that the preparations of delta haemolysin contained a proportion of N-terminally blocked protein.

Thin layer chromatography of the products revealed the three preparations of delta haemolysin to be heterogeneous. The toxin was further purified using hydrophobic affinity chromatography.

The molecular weight of native delta haemolysin was estimated from gel filtration to be 150,000.

The effect of chemical modification of delta haemolysin on its haemolytic activity indicated that the N-terminus of the toxin is involved in its mechanism of action and that the amino groups of the molecule are very important for its activity. The results were closely comparable to those observed for melittin of bee venom.

The binding of delta haemolysin to erythrocyte membranes was estimated using radioactively labelled toxin.

Inhibitors of extracellular protein production, procaine and cerulenin, were found to inhibit the production of delta haemolysin and total extracellular protein. Using cerulenin and harvesting cultures of strain RN25 in the mid-exponential phase of growth, the possibility of the existence of a precursor of delta haemolysin was investigated using techniques of immunoprecipitation.