ABSTRACT OF THESIS

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Title of Thesis  
The Hemolysins of Staphylococcus aureus

On the basis of chemical and immunologic evidence, most investigators now believe that Staphylococcus aureus may produce at least four distinct hemolytic proteins. These have been designated alpha, beta, gamma and delta hemolysins. The function of these hemolysins in the economy of the Staphylococcus has not been fully elucidated, but some detail is available with regard to their nature and in vitro mode of action. Agreement on the properties of the four hemolysins has nevertheless been difficult to obtain because of wide variation in methods of purification and choice of strains. Moreover, confusion has existed in many laboratories with regard to the identity of the hemolysin under study.

Recent work has permitted a number of generalizations to be made. Amino acid analyses of the hemolysins have revealed that no unusual acids are present. All hemolysins are soluble in water although of somewhat varying stability, and when pure, no carbohydrate, lipid or other accessory materials have been detected. It is also fairly well-established that at least three, the alpha, beta and delta hemolysins, are basic proteins the isoelectric points of which are in the range of 8.5-9.6. Apart from the delta lysin, their molecular weights are less than 100,000 daltons, the majority of observations being in the range of 20,000-50,000 daltons.

The mode of action of the hemolysins has generated some debate, but it is accepted that the beta lysin is an enzyme which degrades sphingomyelin, a phospholipid widely distributed in cell membranes. The view that the delta lysin is also a phospholipase which attacks phosphatidylinositol has frequently been challenged but it must be pointed out that until very recently, workers have not clearly distinguished between gamma and delta lysins. The precise mode of action of the latter is unknown but contemporary work indicates that although gamma lysin has no action on sphingomyelin, phosphatidylinositol or other common phospholipids, nitrogen and phosphorus are released from erythrocyte membranes treated with the lysin. Furthermore, it can be shown that hemolysis is inhibited by the membranes when these are added to lysin-red cell suspensions, and that phospholipids extracted from human erythrocytes competitively inhibit the lytic reaction. The mode of action of the alpha lysin has also been elusive but considerable evidence has been compiled which indicates that the lysin is produced by the Staphylococcus as an inactive protease which degrades membranes that contain an activating protease. It has been observed that hemolytic sensitivity of erythrocyte species to alpha lysin is directly correlated with the level of activator present on the membranes. Apart from this, several investigators have demonstrated that the lysin has surface activity, but it is difficult to reconcile the two points of view.

No agreement has been reached on the biological properties of the beta, gamma and delta hemolysins in view of the difficulties of purification and definition. Most workers agree, however, that the alpha lysin kills mice and rabbits in doses at the microgram level (although it is thousands of times less toxic than botulinum or tetanus toxins) and that it causes tissue necrosis when injected into the skin. In vivo production of the hemolysins in animals and man has been demonstrated by the detection of circulating antibody in normal and diseased subjects, but until the present, controversy existed over whether the delta lysin was in fact capable of eliciting an antibody response, since serum lipoproteins inhibited its lytic activity. The use of homogeneous preparations of the lysin and purified antibody has conclusively demonstrated its immunogenicity.

Use other side if necessary.
THE HEMOLYSINS OF STAPHYLOCOCCUS AUREUS

by

Gordon Marcy Wiseman, M.Sc., Ph.D.

Doctor of Science
University of Edinburgh
1974
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Abstract

On the basis of chemical and immunologic evidence, most investigators now believe that *Staphylococcus aureus* may produce at least four distinct hemolytic proteins. These have been designated alpha, beta, gamma and delta hemolysins. The function of these hemolysins in the economy of the *Staphylococcus* has not been fully elucidated, but some detail is available with regard to their nature and *in vitro* mode of action. Agreement on the properties of the four hemolysins has nevertheless been difficult to obtain because of wide variation in methods of purification and choice of strains. Moreover, confusion has existed in many laboratories with regard to the identity of the hemolysin under study.

Recent work has permitted a number of generalizations to be made. Amino acid analyses of the hemolysins have revealed that no unusual acids are present. All hemolysins are soluble in water although of somewhat varying stability, and when pure, no carbohydrate, lipid or other accessory materials have been detected. It is also fairly well-established that at least three, the alpha, beta and delta hemolysins, are basic proteins the isoelectric points of which are in the range of 8.5-9.6. Apart from the delta lysin, their molecular weights are less than 100,000 daltons, the majority of observations being in the range of 20,000-50,000 daltons.
The mode of action of the hemolysins has generated some debate, but it is accepted that the beta lysin is an enzyme which degrades sphingomyelin, a phospholipid widely distributed in cell membranes. The view that the delta lysin is also a phospholipase which attacks phosphatidylinositol has frequently been challenged but it must be pointed out that until very recently, workers have not clearly distinguished between gamma and delta lysins. The precise mode of action of the latter is unknown but contemporary work indicates that although gamma lysin has no action on sphingomyelin, phosphatidylinositol or other common phospholipids, nitrogen and phosphorus are released from erythrocyte membranes treated with the lysin. Furthermore, it can be shown that hemolysis is inhibited by the membranes when these are added to lysin-red cell suspensions, and that phospholipids extracted from human erythrocytes competitively inhibit the lytic reaction. The mode of action of the alpha lysin has also been elusive but considerable evidence has been compiled which indicates that the lysin is produced by the \textit{Staphylococcus} as an inactive protease which degrades membranes that contain an activating protease. It has been observed that hemolytic sensitivity of erythrocyte species to alpha lysin is directly correlated with the level of activator present on the membranes. Apart from this, several investigators have demonstrated that the lysin has surface activity, but it is difficult to reconcile
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Preface
Interest in the staphylococci arose in the last quarter of the nineteenth century after the germ theory of disease became well-established. At that time, their association with pyogenic infections had been noted and conflicting views were put forward with regard to the manner in which the lesions were initiated. However, in 1872, Klebs postulated a relationship between pathogenicity and toxin production in bacteria, and de Christmas (1888) demonstrated the toxicity of heated broth cultures of staphylococci which had been recovered from lesions.

Since that time, bacteriologists have sought to ascribe a role to the many toxins and "aggressins" produced by the organisms, particularly in relation to the production of disease. One particular event more than any has perhaps served to focus attention on hemolysin production by the staphylococci. In 1928, about 30 children were inoculated with a diphtheria toxin-antitoxin preparation at Bundaberg, Australia. Within 48 hours, most of the children were dead. A strain of Staphylococcus aureus was isolated from the preparation which had stood in the tropical heat for some time. The fluid was highly hemolytic and lethal for laboratory animals and in view of the childrens' terminal symptoms, it was felt that they had been killed by a hemolytic toxin.
This tragedy focused attention on the toxic and invasive properties of the staphylococci in relation to disease, but the nature and mode of action of the exo-cellular products and their significance in the metabolism of the cells producing them has largely been ignored until recently. Beginning with the work of Jackson and his colleagues (1956), accumulation of knowledge of the hemolysins has accelerated to the point where in 1974 a reasonable comparison of their properties may be made. Before this, most work was based upon crude preparations, the use of which produced equivocal results. With the advent of newer techniques in protein chemistry, an idea of the nature of these substances and how they exert their effects on cells has emerged.

This dissertation encompasses contributions from the author’s laboratory to the chemistry and properties of the hemolysins of S. aureus and includes work performed over a period of 10 years.

The Hemolysins of Staphylococcus aureus

It has been generally agreed that S. aureus produces three antigenically distinct hemolysins, designated alpha, beta and delta in the order of their discovery. A fourth hemolysin, gamma, had been described by Smith and Price in 1938, but its existence was controversial until quite recently.
Before the present picture emerged, there was some confusion regarding the number and properties of the hemolysins produced by the Staphylococcus. Morgan and Graydon (1936) claimed that the alpha hemolysin consisted of two distinct lytic substances which they called "alpha-1" and "alpha-2". This suggestion arose when it was observed that different end points were obtained if two alpha hemolysins containing no beta hemolysin were neutralized with an antiserum. All culture filtrates contained alpha-1 and about two thirds of them contained small amounts of alpha-2. Both alpha-1 and alpha-2 hemolysins were antigenic and the antiserum for each hemolysin were partially separable.

The gamma hemolysin of Smith and Price (1938) was said to be less stable to heat when compared to alpha and beta lysins, being completely destroyed by heating for 30 min at 55°C. They produced a gamma lysin which contained no alpha and only small amounts of beta lysin. The hemolytic spectrum of gamma lysin was wide; it was dermonecrotic for guinea-pigs and rabbits and lethal for the latter but not for guinea-pigs and mice. Furthermore, the hemolysin was antigenic. Antiserum selected for a high gamma/alpha ratio also had a high alpha-2/alpha-1 ratio, leading them to suggest that their gamma hemolysin might be identical with the alpha-2 hemolysin of Morgan and Graydon.

Elek and Levy (1950) studied the distribution of hemolysins on rabbit and sheep blood agar plates, finding
that the observed patterns were consistent with the existence of three distinct hemolysins; alpha, beta and delta. In their view, seven combinations were possible in strains of *Staphylococcus*. These combinations were alpha, beta, delta, alpha-beta, alpha-delta, beta-delta and alpha-beta-delta, all of which they eventually observed in their study of a number of strains. The recognition of these lysins was based on a test in which antitoxin-soaked filter paper was immersed in melted blood agar which was allowed to solidify. The organisms were inoculated at right angles to the strip and the plate was incubated at 37°C for 48 h in an atmosphere of CO₂ and air. The method depends upon a different rate of diffusion through the agar and a different degree of inhibition of the various lysins adjacent to the growth streak and antitoxin strip. Elek and Levy claimed that each lysin was recognizable by its characteristic pattern of lysis on sheep and rabbit blood agar and by the degree to which it was neutralized on these agars by commercial antiserum. No more than three basic patterns were observed with coagulase-positive strains, which led them to suggest that only alpha, beta and delta lysins or combinations thereof were produced by the *Staphylococci*. Their feeling was that the alpha-2, gamma and delta lysins were in all probability identical.

Marks (1951) agreed that the alpha-2 hemolysin is identical with the delta lysin, but argued for the separate
existence of the gamma lysin. Delta lysin was shown to react in the same manner with antisera as did the alpha-2 lysin. Morgan and Graydon found that most of their culture filtrates contained a small amount of alpha-2 lysin which obscured the end point of hemolytic titrations by producing a "tail" of minimal lysis. The alpha-2 lysin also acted upon rabbit and sheep erythrocytes. All these findings were in accordance with those for delta lysin. Though Marks was not convinced of the identity of the gamma lysin with the alpha-2 and delta lysins, it is noteworthy that the lytic spectra of the three hemolysins were more or less identical. More recently, Jackson (1962) claimed that the thermolabile factor of his delta lysin preparation is possibly identical with the gamma lysin on the basis of heat stability and behaviour in the presence of mild reducing and oxidizing agents.

Obviously there is considerable difficulty in assessing the validity of these earlier claims with regard to the existence of various hemolysins, since such a great variety of techniques was used to demonstrate their presence. Elek (1959) stated that discrepancies between plate and tube titrations were due to three factors; bacterial variation, interactions between hemolysins, and the different sensitivities of the tube and plate methods. He felt that the tube test was inferior to the plate test because it does not allow more than one lysin to diffuse out as in the
plate method and reveal its presence, all the red cells in the tube test being simultaneously subjected to the action of whatever lysins are present. He suggested that serial dilution will result in the same relative proportions acting together in the tube test. Nevertheless, the qualitative plate test also has its difficulties of interpretation, and certainly for quantitative studies it is not very accurate and is rather laborious because of the problem of measurement of the hemolytic zones. Wiseman (1961), in a quantitative study of coagulase, alpha and delta lysins, dermonecrotic and lethal factors, was unable to correlate the plate and tube methods of demonstrating coagulase and hemolysin production. Other bacteriologists have criticized Elek and Levy’s method because they felt that the test was too subjective. An important objection is that animal erythrocytes are notoriously variable in their sensitivity to the hemolysins and this fact makes it a problem to standardize such a test. However, there is some evidence to show that the substitution of hemolysin-impregnated paper strips for the streak of growth used in the Elek test yields the same pattern of hemolysis. Wiseman (unpublished observation), for example, found that paper soaked with partially purified beta lysin gave the characteristic pattern of lysis that is observed with beta lysin-producing strains which have grown on agar. Apart from its shortcomings, the Elek method might play a useful role in
initial gross screening of strains were it not for the fact that gamma lysin is not detected because of its inhibition by agar.
The Alpha Hemolysin
The Alpha Hemolysin

The hemolytic action of filtrates of *S. aureus* on rabbit erythrocytes was noted years ago by Van de Velde (1894) and Kraus and Clairmont (1900), before the multiplicity of staphylococcal hemolysins was realized. This lysin eventually became known as the alpha hemolysin, or toxin.

Production

Probably the best complex media yet devised are those of Walbum (1921a,b; 1922) and Dolman and Wilson (1940). The former contains meat extract, peptone and MgSO$_4$ buffered at pH 6.8. Dolman-Wilson medium is semi-defined, containing proteose peptone and a solution of calcium and magnesium salts buffered at pH 7.4. The original formulation required the addition of 0.3% agar. Parker *et al.* (1926) obtained good yields by incubating cultures in 10% CO$_2$ in air, and Burnet (1930) achieved reliably high yields of hemolysin by combining incubation in CO$_2$ and air with the addition of 0.3% agar to the medium. Variations of Burnet's method have been used by various investigators, including Kumar and Lindorfer (1962), Robinson and Thatcher (1963) and Lominski *et al.* (1963).

The effect of CO$_2$ is not entirely explained by its buffering action, since control of pH by alternative means has not been completely successful. Ganczarski (1962)
labelled the alpha hemolysin with $^{14}$CO$_2$, suggesting in view of his results that CO$_2$ fixation might play a role in the formation of a key amino acid essential for lysin production. Enhancement of production by the addition of small amounts (0.3%) of agar, however, has been explained on the basis of its adsorption of an unidentified inhibitor (see Arbuthnott, 1970). The addition of agar is by no means essential, since acceptable yields have been obtained in liquid media (Bernheimer and Schwartz, 1963; Coulter, 1966).

As stated by Arbuthnott, empirical rules exist which, if adhered to, will ensure good yields. Nevertheless, relatively little information is available with regard to nutritional factors which affect production. Gladstone (1938) used a defined liquid medium which contained 16 amino acids, glucose, vitamin B, and nicotinamide. Important amino acids appeared to be arginine and to a lesser extent glycine and proline, although some variation was met with in different strains of S. aureus. He also showed that oxygen was essential. Dalen (1973a) found that arginine, glycine, serine and histidine increased the yield of alpha lysin in a defined medium, in partial agreement with Gladstone. Arginine was also required for growth. Histidine in Gladstone's experiments was without effect, probably because of the small inocula used and prolonged incubation, in Dalen's view. Dalen felt that since histidine caused such a rapid and early production of alpha lysin, it probably
was an inducer of the lysin. He also thought that the stimulating effect of CO₂, serine and glycine was related to the fact that they are precursors of histidine in *S. aureus*. Dalen (1973b) in another paper found that induction of hemolysin by histidine could be repressed by actinomycin D, chloramphenicol, sodium azide and sodium nitrite. The addition of histidine (metabolized to N-acetylhistamine) inhibited growth and lysin production, but this effect was reversed by histidine and histidinol.

Duncan and Cho (1972) have shown that the optimal concentration of glucose for toxin production is 0.2%. Concentrations above or below this level depressed lysin formation. Impairment of the biosynthesis of nucleic acid and protein in staphylococci results in poor yields, as shown by de Repentigny and his co-workers (Sonea *et al.*, 1965; Turgeon *et al.*, 1966; Leboeuf-Trudeau *et al.*, 1967). They found that purine and pyrimidine antagonists, 8-azaguanine and 5-fluorouracil reduced growth and virulence of strain Wood 46, and that the tryptophan analogues, 4- and 5-methyltryptophans and 7-azatryptophan abolished toxin production in the strain. Reversal of inhibition could be achieved with L-tryptophan, indole or anthranilic acid.

The alpha hemolysin is produced during the logarithmic phase of growth when autolysis of cells is not important. Most investigators (see Duncan and Cho, 1972) have found that toxin is released by intact cells, as indicated by
low levels of DNA in the supernatant fluid at a time of maximal production. The usual finding (Arbuthnott, 1970) is that toxin production begins in early logarithmic growth and proceeds at a constant rate until the late log or early stationary phase is reached. McNiven and Arbuthnott (1971) found that less than 1% of toxin is cell-associated, and this form of toxin reached its maximal level at the onset of the stationary phase of growth. Coulter and Mukherjee (1971) located the toxin on the membrane of disrupted staphylococci by means of ferritin-labelled antibody. In this connection Dalen (1973a) found that histidine added to cultures of the organisms induced lysis formation intracellularly within 10 min and extracellularly within 15 min.

Purification

Numerous methods for the purification of the alpha hemolysin are available and each has its advocates. Many of the procedures have yielded valuable information with regard to the characteristics of the lysis. In Arbuthnott’s view, several factors are important. These are that one should start with toxin of high titre and that concentration of toxin protein should be effected early in the procedure. This can conveniently be done with ammonium sulphate or with methanol. Furthermore, the use of defined media facilitates purification. Advantage of the toxin’s pI of 8.6 can be taken with the use of preparative electrophoresis and ion exchange chromatography on DEAE, CM cellulose or DEAE
Sephadex. Arbuthnott (1970) has extensively reviewed the purification of alpha toxin and the essential features of the methods are shown in Table I. Wiseman's laboratory (Fackrell, 1973; Wiseman et al., 1974) has developed a method which is outlined in Fig. 1. The degree of purification achieved is about 308-fold with a recovery of 40%.

**Properties**

**Physicochemical characteristics:** Purified alpha hemolysin is a protein (Arbuthnott, 1970) and claims that it contains carbohydrate (Goshi et al., 1963) are most likely the result of contamination since few have confirmed this finding. Amino acid analyses have been performed by several investigators (Coulter, 1966; Bernheimer and Schwartz, 1963; Fackrell and Wiseman, 1974c; Six and Harshman, 1973a,b) and are shown in Table II. Three of the analyses shown, those of Bernheimer and Schwartz, Coulter, and Six and Harshman, are similar but there are a few differences. The preparation of Fackrell and Wiseman contains a higher concentration of proline, glycine and alanine than those of the other investigators. Minimal molecular weights, for what they are worth, are given as follows: Fackrell and Wiseman, 17,330 daltons (based on 1.4 S lysin); Coulter, 42,000 (2.8 S lysin); Bernheimer and Schwartz, 44,000 (3.0 S lysin); McNiven et al. (1972), 36,000 daltons. The 3.0 S

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<td>D,C,A,B</td>
<td>4.2x10^4</td>
</tr>
<tr>
<td>Kumar et al. (1962)</td>
<td>&quot;</td>
<td>A,A</td>
<td>?</td>
</tr>
<tr>
<td>Bernheimer and Schwartz (1963)</td>
<td>&quot;</td>
<td>D,A,A,E</td>
<td>1.9x10^4</td>
</tr>
<tr>
<td>Goshi et al. (1963)</td>
<td>?</td>
<td>D,D,B,B</td>
<td>8x10^4</td>
</tr>
<tr>
<td>Lominski et al. (1963)</td>
<td>Wood 46</td>
<td>D,C,B,D,B</td>
<td>1.2x10^5</td>
</tr>
<tr>
<td>Jackson (1963a)</td>
<td>209-60</td>
<td>D,D,B</td>
<td>?</td>
</tr>
<tr>
<td>Robinson and Thatcher (1963)</td>
<td>?</td>
<td>D,D,B,A</td>
<td>?</td>
</tr>
<tr>
<td>Cooper et al. (1966)</td>
<td>Wood 46</td>
<td>D,C,A,B,B</td>
<td>1.2x10^6</td>
</tr>
<tr>
<td>Coulter (1966)</td>
<td>&quot;</td>
<td>D,C,A</td>
<td>10^4</td>
</tr>
<tr>
<td>Arbuthnott et al. (1967)</td>
<td>&quot;</td>
<td>D,A,A,E,F</td>
<td>2x10^4</td>
</tr>
<tr>
<td>Packrell (1973) cited by Wiseman et al. (1974)</td>
<td>&quot;</td>
<td>D,D,C,D,B</td>
<td>1.3x10^5</td>
</tr>
</tbody>
</table>

^1A=electrophoresis, B=ion exchange chromatography, C=gel filtration (molecular sieve), D=precipitation, E=gradient fractionation, F=treatment with heat and urea.
CRUDE ALPHA LYSIN

METHANOL
(35% v/v)

PREcipitate

buffer, dialysis

AMMONIUM SULPHATE
(0-40% sat)

PREcipitate

SUPERNATANT

AMMONIUM SULPHATE
(40-60% sat)

SUPERNATANT

PREcipitate

buffer, dialysis

GEL FILTRATION

AMMONIUM SULPHATE
(0-40% sat)

SUPERNATANT

PREcipitate

AMMONIUM SULPHATE
(40-60% sat)

PREcipitate

buffer, dialysis

ION EXCHANGE

PURIFIED ALPHA LYSIN

Fig. 1. Purification Scheme for Staphylococcal Alpha Toxin.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Bernheimer and Schwartz&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Coulter&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Fackrell and Wiseman&lt;sup&gt;2,3&lt;/sup&gt;</th>
<th>Six and Harshman&lt;sup&gt;2&lt;/sup&gt; (A)</th>
<th>Six and Harshman&lt;sup&gt;2&lt;/sup&gt; (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>methionine</td>
<td>10</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>aspartic acid</td>
<td>44</td>
<td>50</td>
<td>20</td>
<td>40</td>
<td>43</td>
</tr>
<tr>
<td>threonine</td>
<td>23</td>
<td>23</td>
<td>10</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>serine</td>
<td>22</td>
<td>20</td>
<td>10</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>21</td>
<td>22</td>
<td>16</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>proline</td>
<td>7</td>
<td>10</td>
<td>14</td>
<td>8</td>
<td>9</td>
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<tr>
<td>glycine</td>
<td>23</td>
<td>24</td>
<td>28</td>
<td>20</td>
<td>24</td>
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<tr>
<td>alanine</td>
<td>12</td>
<td>14</td>
<td>12</td>
<td>11</td>
<td>11</td>
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<td>valine</td>
<td>12</td>
<td>16</td>
<td>8</td>
<td>13</td>
<td>14</td>
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<tr>
<td>isoleucine</td>
<td>13</td>
<td>16</td>
<td>6</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>leucine</td>
<td>15</td>
<td>14</td>
<td>8</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>tyrosine</td>
<td>9</td>
<td>10</td>
<td>6</td>
<td>9</td>
<td>10</td>
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<tr>
<td>phenylalanine</td>
<td>10</td>
<td>10</td>
<td>4</td>
<td>8</td>
<td>8</td>
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<tr>
<td>lysine</td>
<td>23</td>
<td>26</td>
<td>12</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>histidine</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>arginine</td>
<td>10</td>
<td>8</td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>ammonia</td>
<td>71</td>
<td>very high</td>
<td>very high</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>total residues</td>
<td>329</td>
<td>271</td>
<td>166</td>
<td>234</td>
<td>250</td>
</tr>
</tbody>
</table>

<sup>1</sup> Residues were calculated for a molecular weight of 44,000 as determined by the Archibald method.

<sup>2</sup> Histidine residues set at 4.

<sup>3</sup> Based on lysin of $S_{20w} = 1.4$. 
preparations of Six and Harshman were about 28,000 daltons for both (A) and (B) types of toxin.

Coulter (1966) found that his preparation of alpha hemolysin contained two N-terminal amino acids, histidine and arginine. He interpreted this finding to indicate that the toxin consisted of two polypeptide chains. Separation of the postulated chains was attempted by reduction with mercaptoethanol and sulphite but none was achieved. Alpha toxin treated in this way showed arginine only as the N-terminus. Wiseman and Caird (1970\(^1\)) and Wiseman et al. (1974) have repeatedly shown that histidine is the N-terminus. Arginine has never been detected in this position and it is difficult to explain Coulter's results. Noll (1966) has indicated that histidine is not commonly found as the N-terminus of proteins. Perhaps it is also worth mentioning that histidine has been shown to act as an inducer of alpha hemolysin production in *S. aureus* Wood 46 (Dalen, 1973b). The A and B forms of Six and Harshman (1973a) contained alanine as the N-terminus.

Bernheimer and Schwartz (1963) have reported that the alpha hemolysin has a sedimentation coefficient of 2.8-3.1 S in the ultracentrifuge, which agrees with the value obtained by Lominski et al. (1963), Cooper et al. (1966), Six and Harshman (1973a,b), and Coulter (1966). However, another small fast-moving peak of 12-16 S has been reported by Bernheimer and Schwartz, and by Lominski's

\(^1\)Manuscript in Appendix.
group. The 12 S peak seems in fact to be composed of inactive polymerized toxin and can be separated from the 3 S fraction by density gradient centrifugation (Arbuthnott et al., 1967). That the 12 S material is inactive toxin is suggested by the fact that Arbuthnott found that urea would disaggregate the 12 S component, yielding active 3 S toxin. The 12 S component also gave a line of partial identity with the 3 S material in agar gel diffusion tests. Fackrell and Wiseman (1974c) obtained a value of 1.4 S for freshly prepared alpha lysin, but if the preparation was several days old, the value became 2.8 S. A minor component of the 2.8 S preparation of Forlani et al. (1971) had a value of 2.0 S.

Freer et al. (1968) have found that in the electron microscope, negatively stained toxin (12 S) consisted of small rings of 90 Å outside diameter which formed part of a hexagonal arrangement of six subunits each 20-25 Å in diameter. Arbuthnott inactivated alpha hemolysin by heating at 60°C. An insoluble precipitate was observed which could be disaggregated by urea to yield active toxin. It would thus seem that the so-called "Arrhenius" effect (in which hemolysin inactivated at 60°C is reactivated by brief heating at 100°C) can be explained on the basis of formation of 12 S toxin from 3 S preparations. Further heating to 100°C, or treatment with urea, disaggregates the insoluble 12 S component, reforming the active 3 S fraction.

Bernheimer and Schwartz subjected purified alpha toxin
to sucrose density gradient centrifugation, finding that three or four peaks were present which were electrophoretically distinct but biologically similar. Wadstrom (1968) has found that isoelectric focusing of the toxin separated it into components of different isoelectric points (pI). The main component comprised about 80% of the hemolytic activity of the strains producing it and had a pI of 8.5. The several components apparently were interconvertible, but their relation to the alpha 3 S-alpha 12 S interconversion suggested by the Arbuthnott-Bernheimer groups is not clear. Since all four of Wadstrom's components were hemolytically active, they must be varieties of alpha 3 S, since alpha 12 S is biologically inactive. The existence of these closely related species of toxin is not surprising and may help to explain the molecular weight variations which have been encountered in different laboratories.

McNiven et al. (1972) have also found that a main component of the toxin of pI 8.55 ± 0.12 accounted for about 90% of their recovered hemolytic activity, but three additional minor peaks were noted (Table III). There are some similarities between the data of McNiven and Wadstrom. Alpha A, the main component of McNiven, has a pI of 8.55 which closely corresponds to that of the alpha I of Wadstrom, the pI of which is 8.5. This fraction alpha I of Wadstrom's is also his main component. Alpha B (McNiven) and alpha IV (Wadstrom) have similar pI values and each is claimed to show inter-
conversion with its main component alpha A and alpha I respectively. Fackrell and Wiseman (1974c) have also obtained a pI of 8.5 for their purified alpha lysin. A summary of some properties of alpha lysin is given in Table IV.

Mode of action: The action of alpha toxin on artificial membranes has been investigated by Weissman et al. (1966) and others. These artificial membranes, sometimes referred to as spherules or liposomes, can vary in composition, but those used with alpha toxin were often composed of phosphatidylcholine, cholesterol and dicetylphosphate. If these membranes are prepared in salt solutions, ions are trapped between the layers. Weissman et al. showed that alpha toxin released ions from spherules that were treated with it, and that incubation of the toxin with antitoxin neutralized the effect. Weissman claimed that alpha toxin interacted with the phospholipid moiety of the membranes and that the interaction might involve hydrophobic moieties of the toxin. Freer et al. (1968) also showed that alpha toxin disrupted spherules. The disruption of the membranes was accompanied by the appearance of ring structures which resembled 12 S toxin, and adsorption of the hemolytic activity. Again, it was suggested that a hydrophobic interaction between toxin and phospholipid was involved, which resulted in the polymerization of 3 S toxin to the 12 S form. While these experiments undoubtedly shed some light
Table III
Components of the Alpha Toxin of *Staphylococcus aureus*
Separated by Isoelectric Focusing

<table>
<thead>
<tr>
<th>Component</th>
<th>( pI )</th>
<th>Component</th>
<th>( pI )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha A(^1)</td>
<td>8.55±0.12</td>
<td>Alpha I(^2)</td>
<td>8.5</td>
</tr>
<tr>
<td>Alpha B</td>
<td>9.15±0.07</td>
<td>Alpha IV</td>
<td>9.2</td>
</tr>
<tr>
<td>Alpha C</td>
<td>7.36±0.03</td>
<td>Alpha II</td>
<td>7.0</td>
</tr>
<tr>
<td>Alpha D</td>
<td>6.28±0.11</td>
<td>Alpha III</td>
<td>5.0</td>
</tr>
</tbody>
</table>

\(^1\)Alpha A \( \neq \) Alpha B
\(^2\)Alpha I \( \neq \) Alpha IV
### Table IV

Some Characteristics of Staphylococcal Alpha Hemolysin

<table>
<thead>
<tr>
<th>Reference</th>
<th>Strain</th>
<th>Sed. Coeff. (S_{\text{20w}})</th>
<th>Mol. Wt.</th>
<th>pI</th>
<th>N-terminus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kumar et al. (1962)</td>
<td>Wood 46</td>
<td>1.45</td>
<td>1-1.5(\times10^4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bernheimer and Schwartz (1963)</td>
<td>&quot;</td>
<td>3.0(^a)</td>
<td>2.5(\times10^4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lominski et al. (1963)</td>
<td>&quot;</td>
<td>3.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cooper et al. (1966)</td>
<td>&quot;</td>
<td>2.8</td>
<td>2.1(\times10^4)</td>
<td>-</td>
<td>histidine, arginine</td>
</tr>
<tr>
<td>Coulter (1966)</td>
<td>&quot;</td>
<td>2.8</td>
<td>2.1(\times10^4)</td>
<td>-</td>
<td>histidine, arginine</td>
</tr>
<tr>
<td>Arbuthnott et al. (1967)</td>
<td>&quot;</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wadstrom (1968)</td>
<td>Wood 46</td>
<td>V 8, M 18</td>
<td>-</td>
<td>-</td>
<td>alpha 1 8.0-8.7(^a)</td>
</tr>
<tr>
<td>Wiseman and Caird (1970)</td>
<td>Wood 46</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>histidine</td>
</tr>
<tr>
<td>Forlani et al. (1971)</td>
<td>?</td>
<td>2.8</td>
<td>3.3(\times10^4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>McNiven et al. (1972)</td>
<td>Wood 46</td>
<td>-</td>
<td>3.6(\times10^4)</td>
<td>alpha A 8.55(^a)</td>
<td>-</td>
</tr>
<tr>
<td>Six and Harshman (1973a,b)</td>
<td>(A) 3.0</td>
<td>2.8(\times10^4)</td>
<td>7.2</td>
<td>alanine</td>
<td>alanine</td>
</tr>
<tr>
<td></td>
<td>(B) 3.0</td>
<td>2.6(\times10^4)</td>
<td>8.4</td>
<td>alanine</td>
<td>alanine</td>
</tr>
<tr>
<td>Fackrell and Wiseman (1974c)</td>
<td>&quot;</td>
<td>1.4(^c)</td>
<td>4.5(\times10^4)</td>
<td>8.5</td>
<td>histidine</td>
</tr>
</tbody>
</table>

\(^a\)Major component.  
\(^b\)Inactive aggregate.  
\(^c\)Unstable. After standing several days, \(S_{\text{20w}}=2.8\).
on the interaction of alpha toxin with artificial membranes, the latter are far removed in character from natural membranes.

Wiseman and Caird (1970, 1972) have also investigated the action of alpha toxin on erythrocyte membranes. Bernheimer and Schwartz (1963) and Robinson and Thatcher (1963) had shown that highly purified preparations would not degrade casein. Coulter could not demonstrate phospholipase activity with phosphatidylcholine as substrate, nor could Wiseman (Table V). In spite of the apparent interaction of alpha toxin with the phospholipid of artificial membranes, it seemed that the toxin’s action was not that of a phospholipase. Doery and North (1961) some time ago showed that the hemolytic activity of the toxin was inhibited by ganglioside, an observation which has been confirmed by Wiseman (unpublished data). Wiseman further studied the interaction of toxin and ganglioside by difference spectral techniques in the ultraviolet region of the spectrum. He noted that the absorption peak of toxin underwent a small shift to the red end of the UV spectrum, but that a similar phenomenon took place if toxin was incubated with albumin or casein. Thus it appeared that interaction of alpha toxin with ganglioside was nonspecific. It is noteworthy that van Heyningen and Mellanby (1971) have observed that ganglioside inhibits tetanus toxin.

^1Published papers in Appendix.
Table V

Toxin-Induced Liberation of Non-esterified Fatty Acids from Selected Substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Toxin</th>
<th>Fatty acid (milliequivalents)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phospholipids (rabbit erythrocytes)</td>
<td>alpha</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>phospholipase A (snake venom)</td>
<td>$16 \times 10^{-4}$</td>
</tr>
<tr>
<td>phosphatidylcholine</td>
<td>alpha</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>phospholipase A (snake venom)</td>
<td>$8 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

1Wiseman (unpublished observations).
Though the toxin did not release acid-soluble phosphorus from the erythrocytes and their ghosts (ghosts are membranes free of hemoglobin), we noted that nitrogen appeared in the supernatant fluid and increased proportionally with respect to time (Wiseman and Caird, 1970). The same phenomenon was observed with lipid-free membrane protein, the composition of which is given in Table VI. In particular, the ability of toxin to release nitrogen from red cells was directly correlated with their hemolytic sensitivity to the toxin. The toxin had no effect upon hemoglobin and several other proteins tested but when incubated together with hemoglobin and a ghost preparation, more nitrogen was released from the proteins than from the ghosts alone. It thus appeared that the toxin was activated in some way by the ghost enzymes and there seemed to be more force to this argument when it was shown that the proteolytic activity of the red cell ghosts was also directly correlated with their hemolytic sensitivity.

A great deal of additional evidence has been obtained which supports these conclusions. It has been possible to immunize mice against rabbit erythrocyte protease (Wiseman and Caird, 1972). When rabbit red cells, which had been incubated with antiprotease, were treated with the hemolysin, the latter was unable to degrade azocoll, presumably because the antiprotease blocked the activating enzyme. However, rates of hemolysis were in the presence of toxin unaffected
Table VI
Comparison of Chemical Composition of Rabbit Erythrocytes and Erythrocyte Protein Free of Lipid

<table>
<thead>
<tr>
<th>Substance</th>
<th>% composition of Ghosts</th>
<th>Lipid-free membrane protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>46.5</td>
<td>89.3</td>
</tr>
<tr>
<td>Lipid phosphorus</td>
<td>30.8</td>
<td>3.1</td>
</tr>
<tr>
<td>Total lipid</td>
<td>42.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>11.8</td>
<td>0</td>
</tr>
<tr>
<td>Neutral sugar</td>
<td>4.4</td>
<td>3.2</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>Neuraminic acid</td>
<td>3.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Total carbohydrate (as glucose)</td>
<td>8.7</td>
<td>6.8</td>
</tr>
</tbody>
</table>

1Wiseman (unpublished observations).
by treatment of the rabbit red cells with antiprotease, although the prelytic phase was increased with respect to the controls.

The protease of rabbit erythrocyte membranes, whatever its natural function, is not under in vitro conditions firmly bound to the membranes. This was shown by an experiment in which ghosts were incubated at 37°C for 30 min with samples being taken and assayed for proteolytic activity at intervals of 1-10 min. An increasing amount of proteolytic activity was found in the supernatant fluid as incubation progressed. The hemolytic activity of alpha hemolysin was greatly reduced when it was added to supernatant fluid which contained the proteolytic activity. This suggests that the toxin is either inhibited by something in the fluid or that it might form a complex with the activating enzyme. If EDTA were added to the fluid, some hemolytic activity was recovered.

It was necessary to isolate activated toxin apart from protoxin and activating enzymes. Toxin-ghost supernatant fluid treated with EDTA was passed through a column of Sephadex G-75. The hemolytic fraction taken from the column was also proteolytic, and both hemolytic and proteolytic activities were neutralized by alpha antitoxin.

What probably happens is that alpha hemolysin incubated with intact red cells or ghosts combines with activating enzyme (protease) on the membrane surface and in the sur-
rounding fluid. The complex formed must be unstable (we do not know for how long it may remain a complex) and the activated toxin then attacks the membrane protein, liberating nitrogen. In the case of intact red cells, release of hemoglobin would follow. This may be summarized in the following equations:

\[ (1) \, I^1 + P^2 \rightleftharpoons IP \rightleftharpoons T^3 + P \]
\[ (2) \, T + S^4 \rightleftharpoons TS \rightarrow \text{lysis} + T \]

An analysis of toxin-treated ghosts further revealed that protein content was reduced (Table VII).

An observation which at first appeared puzzling was the fact that alpha antitoxin reduced the proteolytic activity of rabbit erythrocyte ghosts. This inhibitory activity could be removed by treatment of the antitoxin with a heavy suspension of ghosts. It is safe to say that the toxin is activated when injected in sublethal doses into a rabbit because of the effects it has on the animal. It would seem that antibodies are formed against a complex of activated toxin and activating enzyme.

The most recent finding (Wiseman et al., 1974) is that the hemolysin is activated by trypsin and that it degrades tosyl arginine methylester (TAME). By coupling trypsin to carboxymethylcellulose (CMC) in the presence of \( N,N^1\)-dicyclohexylcarbodiimide, we were able to activate

| 1 | inactive toxin |
| 2 | protease |
| 3 | active toxin |
| 4 | membrane substrate |
Table VII
Analysis of Rabbit Erythrocyte Membranes Treated with Staphylococcal Alpha Hemolysin

<table>
<thead>
<tr>
<th>Assay</th>
<th>Untreated membranes</th>
<th>Membranes treated with alpha hemolysin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipid</td>
<td>1.19</td>
<td>1.20</td>
</tr>
<tr>
<td>Total lipid</td>
<td>1.45</td>
<td>1.50</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.33</td>
<td>0.34</td>
</tr>
<tr>
<td>Total carbohydrate</td>
<td>0.26</td>
<td>0.27</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>0.14</td>
<td>0.14</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>Pentose (as ribose)</td>
<td>0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>0.50</td>
<td>0.39</td>
</tr>
<tr>
<td>Nitrogen as protein</td>
<td>3.10</td>
<td>2.42</td>
</tr>
<tr>
<td>Protein</td>
<td>1.84</td>
<td>1.66</td>
</tr>
</tbody>
</table>

1 Wiseman (unpublished observations).
2 Mean of three determinations.
3 Nitrogen concentration multiplied by 6.25.
4 Determined by the Lowry reaction.
the hemolysin and keep it separated from the trypsin. Toxin activated in this way hydrolyzed TAME. It was further ascertained that the $K_m$ of toxin was different from that of trypsin although they had the same $V_{max}$ when TAME was the substrate for both.

The period of exposure of protoxin to trypsin is important. The protoxin is rapidly activated (within 30 sec), but degradation of activated toxin by trypsin commences and after 16 min incubation at 37°C, very little hemolytic activity remains. It should also be pointed out that while protoxin has no proteolytic activity, activated toxin has a high level of such activity which is degraded in parallel with hemolytic activity as incubation proceeds.

Amino acid N-terminal analyses showed that histidine was the N-terminus of protoxin (inactive toxin), whereas the terminal amino acid of activated toxin is either leucine or isoleucine. This additional N-terminus appears only when the protoxin has been activated by trypsin, although several N-termini are probably possible depending on when the toxin-trypsin reaction is stopped. Presumably, activating enzymes of the red cell membrane would attack the protoxin in similar fashion, but we have no evidence to support this assumption. The protoxin and its conversion to active toxin bears some similarity to the trypsinogen-trypsin system found in mammals. Perhaps it is worth noting that the N-terminus of bovine trypsin is isoleucine.
At present, our results cannot be reconciled with those of Six and Harshman (1973a,b) in which alanine was the N-terminus.

Recently, Freer et al. (1973) claimed that they could obtain no evidence in support of our conclusions regarding proteolytic activity of activated alpha toxin. They found that rabbit erythrocyte ghosts treated with alpha toxin showed (1) no alteration in polypeptide patterns assessed by disc gel electrophoresis, (2) no reduction in sedimentable protein, (3) a freeze-etching pattern under the electron microscope unlike that obtained with known proteolytic enzymes, and (4) the presence of phenylmethane sulphonyl fluoride (a protease inhibitor) did not affect the hemolytic titre of the toxin. The fundamental weakness of their study is that they failed to look for protease (activator) activity in their ghost suspensions. It is quite conceivable that this was lost in preparation, or that under the conditions of their testing, the protease was inactive. In this connection, it is well to take note of the work of Dodge et al. (1963) who showed that the method of preparation greatly affected the quality of the ghosts. Without the presence of membrane proteases, no activation of the alpha toxin takes place, which would explain their negative findings. Furthermore, one can still not be certain what a freeze-etch image represents at the molecular level. Speth et al. (1972) have found that phospholipase C (Clostridium perfringens
alpha toxin) yields a freeze-etching pattern for the red cell membrane quite unlike that of phospholipase A, although about 80% of the membrane phosphatides are split. If a common pattern is not obtained among the phospholipases, and there is no reason why there should be, then it may be that a common pattern of etching may not be found among membranes treated with various proteases.

Arbuthnott et al. (1973), in a companion paper to that of Freer et al., have gone on to state that certain lipids induce polymerization of alpha 3 S toxin to alpha 12 S. They specified that a mixture of phosphatidylcholine, cholesterol and dicetylphosphate (70:10:20 molar ratio) was most efficient in the induction of polymerization. Of individual lipids tested, diglyceride was best. These authors concluded that the polar group of phosphatidylcholine is therefore not required for polymerization, and that the alpha toxin can interact hydrophobically with lipids, as concluded earlier by Freer et al. (1968). Doery and North (1961) reported that the hemolytic activity of alpha toxin was inhibited by ganglioside. Buckelew and Colacicco (1971) have demonstrated that the toxin readily spreads as a film on aqueous media and that penetration of toxin into lipid monolayers is related to the structure of the lipid. They found that film penetration was greatest with cholesterol and least with ganglioside.

However, Camejo et al. (1968) have pointed out that
most lipids and proteins display some degree of association when brought together. There is a difficulty in connection with the view that surface activity of alpha toxin and its interaction with membrane lipids is responsible for hemolysis of sensitive erythrocytes and other biological properties attributed to the toxin. This is that it has not been possible to distinguish whether the surface activity of toxin is specific or nonspecific. Is the surface activity of the toxin in fact directly responsible for its toxicity?

At present it is not entirely possible to reconcile the alpha toxin's surface activity with our view that toxin activated by membrane protease is the primary event in toxin-cell interaction. Perhaps the interaction of toxin with membrane lipids might serve to anchor it in a particular conformation which is susceptible to the action of the protease activator.

In summary, our concept is that alpha toxin is secreted by the *Staphylococcus* as a zymogen. This proenzyme is activated by red cell protease(s) and the activated toxin degrades membrane protein which results in leakage of hemoglobin from the cell. Erythrocytes which contain lower concentrations of activator are relatively insensitive to the toxin while those with higher levels, for example, the cells of the rabbit, are particularly sensitive. The same system is no doubt responsible for tissue necrosis caused by the toxin and for its lethal effect on animals, although
the sequence of events which leads from injection to death within minutes (at the appropriate dose) is not known.

Related to the question of the alpha toxin's mode of action on cells is its function in the staphylococci which produce it. Apart from some speculation by various investigators, little information is available. Coulter and Mukherjee (1971) coupled ferritin-labelled antitoxin with disrupted staphylococci treated with toxin. The toxin was located on the cell membrane with the aid of the electron microscope. This finding led them to construct a hypothesis to the effect that the toxin in some way controls cellular permeability. However, no evidence in support of this hypothesis was offered other than the finding of the toxin on the membrane.

Toxic properties of the alpha hemolysin: Various definitions of a "toxin" have been put forward but Bonventre (1970) has defined a toxin as a high molecular weight protein of microbiological origin which is antigenic and causes reversible or irreversible disruption of normal physiological processes in a sensitive animal. The alpha hemolysin fulfills these criteria and thus may be referred to as a toxin.

Both cold-blooded animals and warm-blooded animals are sensitive to the toxin and the severity of its effects is dose-dependent. Fackrell and Wiseman (1974c) have determined that the LD$_{50}$ of the toxin for mice is $0.68 \pm 0.19$ µg or 27-34 µg/kg body weight. This figure agrees well with
the value obtained by Arbuthnott (1970). Lominski et al. (1963) calculated the MLD to be 1 µg toxin. Other values range from 12 LD$_{50}$/µg (Goshi et al., 1963) to 3.9 (Jackson, 1963a). Rabbits which have received intravenously a minimal lethal dose die after a few days. The major pathological findings are kidney necrosis and there may be flaccid paralysis of the hind legs. Larger intravenous doses may cause death in a few minutes, the animal showing unsteadiness, respiratory difficulty and intermittent muscular spasms. However, at death the limbs are flaccid and no histological changes appear evident. Intravascular hemolysis has been noted in animals which have received large doses. Naturally the time to death depends upon the route of injection. Subcutaneous administration of small doses results in what appears to be hemolysis under the skin in the surrounding area which progresses to severe necrosis and sloughing of tissue after several days.

Arbuthnott (1970) has summarized the main findings. These are that the toxin acts on the peripheral circulation, the heart and the central nervous system. In perfused heart muscle from the rabbit, chicken and cat, the toxin caused constriction of the coronary arteries and systolic arrest (Wiegershausen, 1960,1962). Nelis (1935) also suggested a direct action on the respiratory centre. Samánek and Zajic (1965) and Svihovec et al. (1965) have pointed to a decline in cardiac output in animals which received toxin.
The latter group noted changes in blood pressure which they suggested resulted from an effect of the toxin on smooth muscle of the blood vessels. In keeping with this is the observation of Thal and Egner (1954, 1961) to the effect that the toxin causes vasospasm of the small vessels. As pointed out by Arbuthnott (1970), there is no unified concept of the mode of action of alpha toxin in the animal body. However, our observations (Wiseman and Caird, 1970, 1972; Wiseman et al., 1974), which indicate that the toxin is activated by red cell proteases with subsequent degradation of membrane protein, suggest that it may act in vivo by selective destruction of those cells which have the highest membrane content of activating protease. Certainly this hypothesis may explain the toxin's necrotic effect on skin after subcutaneous or intradermal injection. Nevertheless, it is not at all clear how this proteolytic action could account for the rapid death observed in sensitive animals given large doses of toxin.

Apart from sensitive erythrocytes, alpha hemolysin exerts a toxic effect on a wide variety of cultured cells. Jeljaszewicz et al. (1965) have described cytopathic changes in KB cells with loss of 5-nucleotidase activity. Korbecki and Jeljaszewicz (1965) observed lowered glucose utilization and lactic acid production in several mammalian cell lines treated with toxin, as well as the disappearance of cellular dehydrogenases. Madoff et al. (1963) noted that toxin-treated
Ehrlich ascites cells did not multiply in mice and that free amino acids were rapidly released from such cells. Human amnion cells were somewhat more resistant, however. These findings along with those of Jeljaszewicz’s group were interpreted to indicate a primary action on the membrane rather than cellular metabolism.

Galanti et al. (1968) noted that the ATP content of rabbit kidney and chick embryo cell cultures was decreased in the presence of toxin, and that it was not detected in the supernatant.

The Czechoslovak group (Novak et al., 1970) found that partially purified toxin stimulated ATPase activity in rat liver mitochondria. In later studies, Novak et al. (1971a,b) showed that crude alpha toxin abolished phosphorylation of ADP in mitochondria, but the component of toxin that accomplished this was heat stable. They also observed that partially purified toxin uncoupled oxidative phosphorylation; the phosphorylation itself was inhibited, but electron transport was relatively unaffected. In this connection, Symington and Arbuthnott (1969) noted that purified toxin increased the rate of succinate oxidation by Krebs 2 mouse ascites tumour cells.

These studies, interesting though they may be, are not particularly meaningful in view of the fact that toxin of undefined purity was used. The use of crude or partially purified toxin in metabolic studies is particularly unhelpful.
Other than the author's own work, few studies have been directly concerned with an investigation of the biochemistry of toxin-membrane interactions, most of the evidence pertaining to the action of toxin on membranes having been obtained by electron microscopy (Bernheimer et al., 1972; Klainer et al., 1972) or by detection of changes in membrane properties (Rahal et al., 1967).
The Beta Hemolysin
The Beta Hemolysin

Discovery of the beta hemolysin originates from an investigation of two preparations of alpha toxin by Glenny and Stevens (1935). One of these preparations was neutralized by one equivalent of antitoxin, but the other required 10 equivalents. Incubation of titrations in the presence of sensitive red cells at 37°C, followed by a period of holding at 4°C, resulted in intensification of the hemolysis, hence the origin of the term "hot - cold" hemolysin.

Production

The production of beta hemolysin on solid and in liquid media has been reviewed by the author (Wiseman, 1970). As with alpha toxin, incubation of cultures in the presence of CO₂ in air enhances titres, concentrations of 10-25% having been found satisfactory by most investigators. Beta hemolysin is produced by the organisms during the early logarithmic phase of growth and its rate of production is maximal at that time.

Purification

Methods of purification which have been developed are given in Table VIII.

Properties

Physicochemical characteristics: The beta hemolysin is a protein. Chesbro et al. (1965) reported that its

\footnote{Publication in Appendix, pp. 239-243.}
<table>
<thead>
<tr>
<th>Reference</th>
<th>Strain</th>
<th>Procedures&lt;br&gt; (&lt;sup&gt;1&lt;/sup&gt;)</th>
<th>Sp. Act. (HU/mg protein or nitrogen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Robinson &lt;i&gt;et al.&lt;/i&gt; (1958)</td>
<td>L16</td>
<td>D,B,D,A</td>
<td>?</td>
</tr>
<tr>
<td>Jackson (1963b)</td>
<td>J32a</td>
<td>D,D,B</td>
<td>?</td>
</tr>
<tr>
<td>Chesbro &lt;i&gt;et al.&lt;/i&gt; (1965)</td>
<td>UNH-Donita</td>
<td>B</td>
<td>?</td>
</tr>
<tr>
<td>Wiseman and Caird (1967)</td>
<td>R-1, 252F</td>
<td>B,D,C</td>
<td>?</td>
</tr>
<tr>
<td>Maheswaran &lt;i&gt;et al.&lt;/i&gt; (1967)</td>
<td>T19</td>
<td>D,C,B,B</td>
<td>7x10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gow and Robinson (1969)</td>
<td>MB534</td>
<td>D,C,B,E</td>
<td>5x10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hague and Baldwin (1969)</td>
<td>681</td>
<td>D,B</td>
<td>10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wadstrom and Mollby (1971a)</td>
<td>R-1</td>
<td>B,A&lt;sup&gt;2&lt;/sup&gt;,C</td>
<td>3x10&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>A=electrophoresis, B=ion exchange chromatography, C=gel filtration (molecular sieve), D=precipitation, E=gradient fractionation.

<sup>2</sup>A=electrofocusing.
molecular weight was 59,000 daltons determined according to the method of Martin and Ames (1961). More recently, however, Chesbro and Kucic (1971) reported molecular weights varying from 11,000 to 59,000 depending upon the strain and the method of molecular weight determination used.

Fackrell and Wiseman (1974c) have reported a molecular weight of 26,000 daltons based on gel filtration and a minimum molecular weight of 16,100 daltons calculated from amino acid analyses. The discrepancies are probably due to association of the hemolysin molecule and to methods of preparation and analysis.

The amino acid composition of beta hemolysin as determined by Fackrell and Wiseman is shown in Table IX. Noteworthy is the large number of ammonia residues, the absence of proline and the presence of cysteine when compared to the alpha, gamma and delta lysins. Methionine is also absent. It appears that this analysis is the only one as yet provided for the beta hemolysin.

Several investigators have separated a major (cationic) and a minor (anionic) component from preparations of the beta hemolysin (Haque, 1963; Maheswaran et al., 1967; Mollby and Wadstrom, 1970). The latter authors found that 95% of their crude lysin subjected to isoelectric focusing was the cationic form with pI = 9.5 ± 0.1. About 5% of the crude lysin was in the anionic form (pI = 3 - 4). They also showed that occurrence of the anionic lysin was probably due
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Alpha</th>
<th>Beta</th>
<th>Gamma</th>
<th>Delta</th>
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</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>High</td>
<td>441.6</td>
<td>141.4</td>
<td>218.5</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>121.2</td>
<td>135.1</td>
<td>142.0</td>
<td>164.1</td>
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<tr>
<td>Threonine</td>
<td>56.9</td>
<td>53.8</td>
<td>68.0</td>
<td>119.9</td>
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<tr>
<td>Serine</td>
<td>65.1</td>
<td>98.7</td>
<td>77.4</td>
<td>96.3</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>93.0</td>
<td>116.9</td>
<td>102.4</td>
<td>66.4</td>
</tr>
<tr>
<td>Proline</td>
<td>81.2</td>
<td>-</td>
<td>50.2</td>
<td>14.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>170.1</td>
<td>121.2</td>
<td>125.0</td>
<td>112.4</td>
</tr>
<tr>
<td>Alanine</td>
<td>75.8</td>
<td>78.2</td>
<td>63.2</td>
<td>75.2</td>
</tr>
<tr>
<td>Valine</td>
<td>50.1</td>
<td>57.8</td>
<td>51.0</td>
<td>76.0</td>
</tr>
<tr>
<td>Cysteine</td>
<td>-</td>
<td>12.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methionine</td>
<td>12.1</td>
<td>-</td>
<td>3.5</td>
<td>-</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>40.4</td>
<td>52.5</td>
<td>44.4</td>
<td>102.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>46.0</td>
<td>58.8</td>
<td>57.8</td>
<td>61.2</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>33.5</td>
<td>6.3</td>
<td>30.6</td>
<td>9.3</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>28.3</td>
<td>41.4</td>
<td>35.8</td>
<td>53.5</td>
</tr>
<tr>
<td>Lysine</td>
<td>70.9</td>
<td>98.0</td>
<td>78.4</td>
<td>24.3</td>
</tr>
<tr>
<td>Histidine</td>
<td>20.0</td>
<td>24.8</td>
<td>27.2</td>
<td>3.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>34.5</td>
<td>39.6</td>
<td>38.6</td>
<td>21.4</td>
</tr>
</tbody>
</table>

*Residues/1,000 amino acid residues (after Packrell and Wiseman, 1974c).
to aggregation (of some of the cationic form, presumably) and that passage of crude lysin through ion exchange columns which contained 6 M urea resulted in the appearance of only the cationic form. In a later publication, Wadstrom and Mollby (1971a) revised the pI to 9.4 ± 0.1.

Table X summarizes information on the physical characteristics of the beta hemolysin.

Mode of Action: The mode of action of the beta hemolysin has been discussed by Wiseman (1970). Doery et al. (1963, 1965) first noted that beta hemolysin released acid-soluble phosphorus from red cells, which originated from the hydrolysis of sphingomyelin, a common constituent of membranes. This observation has been confirmed by several other investigators (Maheswaran and Lindorfer, 1967; Wiseman and Caird, 1967; Wadstrom and Mollby, 1971a, 1971b). Chesbro et al. (1965) had reported earlier that beta hemolysin degraded cell walls of staphylococci and streptococci, as well as rabbit red cell stromata with the liberation of polysaccharides and breakdown products of these. Their purified hemolysin was undoubtedly contaminated with other enzymes; nor is the substrate, sphingomyelin, found in the cell walls.

Relatively little is known of the substrate specificity of the beta lysin. Of several phospholipids tested, Wiseman and Caird (1967) found that the rate of hydrolysis was most rapid if sphingomyelin were used as substrate. Little or no hydrolysis of phosphatidylethanolamine or

1Publication in Appendix.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Strain</th>
<th>Sed. Coeff. $(S_{20w})$</th>
<th>Mol. Wt.</th>
<th>pI</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mollby and Wadstrom (1970)</td>
<td>R-1</td>
<td>-</td>
<td>-</td>
<td>9.5±0.1</td>
<td>?</td>
</tr>
<tr>
<td>Gow and Robinson (1969)</td>
<td>MB534</td>
<td>1.7</td>
<td>-</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>Chesbro et al. (1965)</td>
<td>UNH-Donita</td>
<td>-</td>
<td>59,000$^2$</td>
<td>8.6-8.9</td>
<td>carbohydrate</td>
</tr>
<tr>
<td>Wadstrom and Mollby (1971a)</td>
<td>R-1</td>
<td>-</td>
<td>38,000$^3$</td>
<td>9.4±0.1</td>
<td>phospho-lipase</td>
</tr>
<tr>
<td>Fackrell and Wiseman (1974c)</td>
<td>R-1</td>
<td>1.8</td>
<td>26,000$^3$</td>
<td>9.5</td>
<td>phospho-lipase</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16,110$^4$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^1$Calculated from ultracentrifugation data and identical with the weight obtained from gel filtration.

$^2$Density gradient centrifugation.

$^3$Gel filtration.

$^4$Amino acid analysis (minimal molecular weight).
phosphatidylcholine was observed. Other phosphate bonds of RNA, beta-glycerophosphate and phenylphosphate were not attacked. Doery et al. (1965) observed that beta lysin also attacked lysophosphatidylcholine. Wiseman and Caird obtained good agreement between the sphingomyelin content of eight species of red cells and their hemolytic sensitivity to beta lysin.

Requirement of the beta hemolysin for Mg\(^{++}\) ions has been documented by various workers (Haque and Baldwin, 1964; Wiseman, 1965a; Doery et al., 1965; Wiseman and Caird, 1967; Maheswaran and Lindorfer, 1967; Gow and Robinson, 1969). Other metal cations such as Co\(^{++}\) and Mn\(^{++}\) have also been observed to enhance hemolysis of red cells in the presence of the hemolysin (Wiseman, 1965a).

**Reaction kinetics:** Though a thorough investigation of reaction kinetics of beta hemolysin has not been undertaken, some data are available. Wiseman (1965a,b\(^1\)), working with partially purified hemolysin, noted that a plot of reaction time against hemolysis in the presence of the lysin resulted in a straight line. However, this observation was not true for early or late periods of erythrocyte-hemolysin interaction. Plots of reaction velocity versus lysin concentrations were linear. Wiseman (1965b) noted that the reaction rate of the hemolysin with red cells as substrate was maximal at a pH of about 7.5, which was observed by Doery et al. (1965) in connection with lysophospholipase

\(^1\)Publications in Appendix.
and sphingomyelinase activities of beta lysin on purified substrates. With regard to temperature, Wiseman found that the reaction rate increased linearly between 20-45°C. The activation energy value derived from an Arrhenius plot was calculated to be 14,100 cal. Wiseman's data no doubt reflect the enzymatic nature of the hemolysin-red cell interaction, but it must be pointed out that they were obtained with red cells as substrate.

Maheswaran and Lindorfer (1967) have also investigated the kinetics of hydrolysis of sphingomyelin by purified beta lysin. They found that release of acid-soluble phosphorus from red cell ghosts by the hemolysin is linear between 37-45°C, although release was maximal at 41°C when purified sphingomyelin was the substrate. When hemolysin concentration was plotted against phosphorus released, a straight line was obtained which indicated a first order reaction. At the present time $K_m$ and $V_{max}$ have not been determined for the beta hemolysin nor has a detailed study of its specificity been performed.

"Hot-Cold" reaction: As noted previously, the beta hemolysin, along with several other similar agents (e.g., the alpha toxin of Clostridium perfringens), is a "hot-cold" hemolysin; that is, hemolysis is significantly increased if incubation at 37°C is followed by a period of holding at a lower temperature. Much has been written about this reaction, but it is probably true to say that it is still imperfectly
understood. Wiseman (1965b) has discussed the phenomenon, finding that rapid alteration of pH or NaCl concentration in suspensions of red cells treated with hemolysin would cause intensified hemolysis at 37°C. Reduction of temperature may act in a manner analogous to these observations. Sudden contraction of the red cell membrane as the result of lowered temperature may weaken the structure to the point of its giving way.

Recently, Meduski and Hochstein (1972) have advanced the hypothesis that the "hot" phase results from changes in the choline residues of membrane sphingomyelin. They found that the lytic effects of $I^-_3$ ions on red cells are similar to those observed in "hot-cold" hemolysis. The addition of dipalmitoyl lecithin (phosphatidylcholine) to the system prevented $I^-_3$ hemolysis of red cells, provided it was added before initiation of the "hot" phase. $I^-_3$ also bound to the lecithin as a stable complex, a triiodide, which could be identified by chromatography. Apparently $I^-_3$ also can interact with $-N^+ (CH_3)_3$ groups of other phospholipids at pH 7.0 which also leads to "hot-cold" hemolysis. They point out that the sphingomyelins may be the most actively bound with $I^-_3$ because of their physicochemical properties. The red cells of sheep, man and rat are, in that order, decreasingly sensitive to the action of beta hemolysin as they are to $I^-_3$ hemolysis. Meduski and Hochstein thus argue that the fixed positive charge of the phospholipids
mediates the leakage of hemoglobin through the membrane and that this leakage reaches an equilibrium during the "hot" phase. If the temperature is reduced, the equilibrium is shifted to increasing the leakage. Their view is that "hot-cold" hemolysis is a feature of the response of red cells to any agent which affects -N⁺(CH₃)₃ groups of membrane phospholipids.

**Toxic properties of the beta hemolysin**: Most present day controversy focuses on the toxic properties of the hemolysin. Since Wiseman's (1970) review, the situation has been further complicated by contradictory findings and it is therefore impossible to come to definite conclusions about the status of the beta hemolysin as a toxin. On one hand, a number of observations have been made of the action of the beta lysin on mammalian cell cultures and suspensions. As pointed out by Wiseman, several investigators, working with preparations of variable purity, have observed toxic effects (Chesbro et al., 1965; Wiseman, 1968¹; Korbecki and Jeljaszewicz, 1965; Jeljaszewicz et al., 1965). More recently, Wadstrom and Mollby (1972) have reported that beta hemolysin is cytotoxic for HeLa cells, human fibroblasts and human thrombocytes, although Thelestam et al. (1973) did not confirm this finding for lysin acting on human fibroblasts. Contradictory results have also been obtained by Gladstone and Yoshida (1967). These authors found that crude beta hemolysin had no effect on a variety

¹Publication in Appendix.
of cultured cells including HeLa, L, HL, FL, HeP, chick fibroblasts and rat heart connective tissue cells. Hallander and Bengtsson (1967) also did not observe toxicity when beta lysin was incubated with human, bovine and monkey kidney cells.

On the other hand, conflicting observations have been made of the effects of beta lysin injected into various animals. Heydrick and Chesbro (1962) claimed that intraperitoneal injection of the beta lysin into guinea pigs was only lethal if given with Mg\(^{++}\) ions. Wiseman (1965a) found that the partially-purified preparations from two strains of *S. aureus* were not lethal for mice or rabbits whether or not Mg\(^{++}\) ions were included in the inoculum.

Intradermal injection of the hemolysin into rabbits caused slight swelling with an erythematous flush, but no necrosis. Maheswaran *et al.* (1967) could not demonstrate dermonecrosis in rabbits after intradermal injection of rabbits with or without added Mg\(^{++}\). Gow and Robinson (1969) reported that the lysin killed rabbits when injected in doses of 40-160 \(\mu\)g. Wadstrom and Mollby (1971b, 1972) reported that the LD\(_{50}\) dose of beta lysin was in the range of 10-100 \(\mu\)g for mice, rabbits and guinea pigs, and in chicken embryos, the dose was 0.25-10 \(\mu\)g. Their view is that the toxin on a weight basis is as toxic as alpha toxin. However, their results were not precisely reported as \(\mu\)g/kg body weight. Arbuthnott (1970) and Fackrell and Wiseman
(1974c) have reported that the LD$_{50}$ of alpha toxin is 0.68-1 LD$_{50}$/µg protein in mice. Thus for rabbits, mice and guinea pigs, if we are to accept the findings of Wadstrom and Mollby, Gow and Robinson, beta hemolysin is 10-160 times less toxic than alpha toxin.

It is indeed difficult to understand the discrepancies in these reports of beta lysin toxicity. Perhaps an inhibitor was present in the beta lysin preparations of Wiseman (1965a) and of Maheswaran et al. (1967). It is remotely possible that such an inhibitor was co-purified with the hemolysin. If this were so, one would expect enzymatic activity in vitro also to be inhibited. It is conceivable that the preparations of Gow and Wadstrom were contaminated with gamma hemolysin, although they were of a high degree of purity. At any rate one can only speculate at this time as to the basis of these conflicting reports.

**Role of beta hemolysin in the metabolism of S. aureus:** Little has been accomplished in this area, but Fritsche (1970) was unable to link beta hemolysin production to lipid metabolism in strains which produced it. He could not extract sphingomyelin from the organisms and consequently felt that beta hemolysin had no function in their internal metabolism, but rather liberated metabolites from membranes of the host cells.

The beta hemolysin is not produced by all or even
the majority of strains of *S. aureus*. It has long been known that many strains isolated from animals other than man produce the hemolysin (Elek, 1959) and that the hemolysin is less commonly found in strains isolated from man. The surveys that produced these results, however, were all performed many years ago. In Australia, Christie and North (1941) reported that a larger number of beta lysin-producing strains was isolated from man. Whether these surveys are still valid today and whether the findings apply to all countries is questionable.
The Delta Hemolysin
The Delta Hemolysin

The delta hemolysin was discovered by Williams and Harper (1947) in strains of *S. aureus* which were grown on sheep blood agar in the presence of alpha and beta antihemolysins.

**Production**

Production of delta hemolysin on various media and the effect of CO$_2$ on titres have been reviewed by Wiseman (1970). Two methods have largely been favoured; cultivation on solid media overlain with cellophane and cultivation in casein hydrolysate liquid media supplemented with yeast diffusate. The cellophane-on-agar technique (Birch-Hirschfeld, 1934) has been used with some variation in the composition of the medium, by Marks and Vaughan (1950), Jackson and Little (1958a), Hoffmann and Streitfeld (1965), Murphy and Haque (1967), and Wiseman and Caird (1968$^1$). The semi-defined medium of Bramann and Norlin (1951) has been used with some variations by its advocates; Yoshida (1963), Heatley (1971), Kreger *et al.* (1971), Kapral and Miller (1971) and Kantor *et al.* (1972). Since Williams and Harper had observed that delta hemolysin was not produced in a liquid medium and that its production did not require CO$_2$, investigators who developed liquid media generally aerate their cultures with oxygen or air at a controlled rate with or without added CO$_2$. Satisfactory titres have thus been

$^1$Publication in Appendix.
obtained.

Purification

Purification methods developed by different investigators are shown in Table XI. It is interesting to note that Yoshida's (1963) extensive procedure yielded delta hemolysin contaminated with beta hemolysin and RNAse (Gladstone and Yoshida, 1967) while the one-step procedures of Kreger et al. (1971) and Kantor et al. (1972) are claimed to yield highly purified, homogeneous hemolysin. It is indeed remarkable that simple adsorption of hemolysin to aluminum hydroxide or to hydroxylapatite, as given by these authors, is sufficient to provide a homogeneous product.

Properties

Physicochemical characteristics: The activity of delta hemolysin is not affected by EDTA or citrate, or any metal cation (Jackson and Little, 1958a; Wiseman, 1970). A property of the hemolysin which sets it apart from other hemolytic proteins of S. aureus is its inhibition by serum and serum components as shown by Jackson and Little, Gladstone and Yoshida, Maniar et al. (1967), Wiseman and Caird (1968), and Kreger et al. According to Kapral (1972), the phospholipid content of sera or crude protein fractions might account for the inhibitory activity. In this connection, Kapral (1967) had earlier shown that the hemolytic activity of the delta hemolysin was inhibited by several
# Table XI

## Purification of Staphylococcal Delta Hemolysin

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Jackson and Little</td>
<td>1363, 2426,2428, 2429</td>
<td>H, D, G</td>
<td>3200</td>
</tr>
<tr>
<td>Caird and Wiseman</td>
<td>E-delta</td>
<td>D, D, B, B</td>
<td>12,000</td>
</tr>
<tr>
<td>Kantor et al. (1972)</td>
<td>Wood 46M</td>
<td>B</td>
<td>75</td>
</tr>
</tbody>
</table>


[^3],[^5] Soluble delta lysin.


phospholipids, including sphingomyelin, phosphatidylcholine, phosphatidylserine, and phosphatidylinositol.

Maheswaran and Lindorfer (1970) found that delta hemolysin from strain 146P could be fractionated into three parts of pI values 3.32 (delta I), 3.75 (delta II), and 8.45 (delta III) on an electrofocusing column, as shown in Table XII. The results of Kreger et al. (1971) differed from those of Maheswaran and Lindorfer in that only two peaks of pI 9.5 ± 0.3 and 5.0 ± 0.2 were obtained. The 9.5 peak represented about 70% of the hemolytic activity. In Maheswaran's case, the 3.75 peak appeared to represent the major portion. Kantor et al. (1972) found only one peak of pI 5.2 in the presence of 0.1% Tween 80. Without detergent, three peaks were obtained of pI 4.65, 6.7 and 9.0. About 30% of the total hemolytic activity was contained in each peak. Mollby and Wadstrom (1970) found the pI of their delta lysin preparation to be 9.6 ± 0.2. It would seem that slight alterations of experimental conditions and the use of preparations from different strains provide results that are somewhat different. Fackrell and Wiseman (1974c) have found their preparation to have a value of pI = 9.6. There is some agreement that a major proportion of delta hemolytic activity has a pI in the range of 9-10.

Various molecular weights have been reported (see Table XII). It is not surprising that there is no agreement, since different methods have been used in the determination
<table>
<thead>
<tr>
<th>Reference</th>
<th>Delta lysin conc.</th>
<th>pI</th>
<th>Mol. Wt.</th>
<th>Sed. Coeff. (S_{20w})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yoshida (1963)</td>
<td>0.4%</td>
<td>-</td>
<td>68,320(^1)</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>74,000(^2)</td>
<td></td>
</tr>
<tr>
<td>Hallander (1963)</td>
<td>?</td>
<td>-</td>
<td>&gt;200,000(^3)</td>
<td></td>
</tr>
<tr>
<td>Mollby and Wadstrom (1970)</td>
<td>?</td>
<td>9.6(\pm)0.2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Maheswaran and Lindorfer (1970)</td>
<td>?</td>
<td>(I) 3.32</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(II) 3.75</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(III) 8.45</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Caird and Wiseman (1970)</td>
<td>6.0 mg/ml</td>
<td>-</td>
<td>&gt;200,000(^4)</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.8</td>
</tr>
<tr>
<td>Kreger et al. (1971)</td>
<td>0.6%(^7)</td>
<td>(I) 9.5(\pm)0.3</td>
<td>-</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(II) 5.0(\pm)0.2</td>
<td>-</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11.9</td>
</tr>
<tr>
<td>Kantor et al. (1972)</td>
<td>?</td>
<td>(I) 4.65</td>
<td>103,000(^5)</td>
<td>6.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(II) 6.70</td>
<td>195,000(^6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(III) 9.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heatley (1971)</td>
<td>6.0 mg/ml</td>
<td>-</td>
<td>-</td>
<td>4.9</td>
</tr>
</tbody>
</table>

\(^1\) Calculated from amino acid analysis.
\(^2\) Derived from the sed. coeff.
\(^3\),\(^4\) Sephadex gel filtration (G-200).
\(^5\) From sucrose density gradient data.
\(^6\) Biogel A5m filtration.
\(^7\) Concentration applies to ultracentrifuge experiments. Concentration in electrophoresis focusing column was a total of 20 mg.
of the molecular weight. However, there is some agreement in molecular weights determined by gel filtration. Kantor et al. (1972) obtained a weight of 195,000, Caird and Wiseman (1970) a weight of > 200,000, and Hallander (1963, 1968) > 200,000 daltons. This similarity is probably more apparent than real. With regard to the pI and molecular weight-determinations of Kantor et al., it is not clear which of the pI values related to the two molecular weight values. Furthermore, the apparent agreement between the sedimentation constant and molecular weight determination of Kantor et al. and Yoshida are all the more remarkable in the light of demonstrated impurities in Yoshida's preparation (Gladstone and Yoshida, 1967).

Some views have been put forward concerning the structure of the delta hemolysin molecule. Kantor et al. claim that delta lysin of molecular weight 103,000 (as determined by sucrose density gradient), consists of at least five subunits of molecular weight 21,000 daltons as shown by dissociation in the presence of 0.1% Tween 80. Even smaller polypeptide units may be obtained, they state, by further treatment with detergents. Kantor et al. felt that the 21,000 molecular weight fragments each represent a tetramer of identical polypeptide chains and that the so-called "native" form of delta hemolysin is a structure which contains five of these tetramers oriented in a planar-radial configuration.

1Paper in Appendix.
Apart from the use of different strains grown on various media, and the application of widely differing techniques of purification, one of the greatest difficulties in the author's view is the question of lysin concentration in relation to pronouncements of purity. Kantor et al. did not state the concentration of delta lysin (i.e., as mg protein or N/ml) which they used in their characterization studies, except in the case of amino acid analyses where 1 mg (dry weight) samples were used.

Kreger et al. (1971) used several concentrations of delta lysin in their work, ranging from 1 mg dry weights, 1 ml samples, to 1% solutions (w/v). Heatley (1971) did not generally state the concentration he employed except that ultracentrifuge studies were based on a solution which contained 6.0 mg/ml. Heatley also stated that the lysin was adsorbed to glass, which he claimed made accurate assay difficult. Caird and Wiseman (1970) used a concentration of 6 mg/ml in assessing homogeneity by various techniques.

Hemolytic activity: It has been noted (Wiseman, 1970) that erythrocytes of various species are more uniformly sensitive to delta lysin than is true of alpha, beta or gamma lysins. That is, interspecies differences in susceptibility to delta lysin are considerably less (see also Fackrell and Wiseman, 1974c).

Reaction kinetics: Jackson and Little (1958a) observed that there was a linear relationship between degree
of hemolysis of human red cells and delta lysin concentration within 30-65% limits of hemolysis. This finding is in fair agreement with that of Yoshida (1963) who reported that hemolysis was related linearly to lysin concentration, over the range of 35-70% lysis. Heatley (1971) found that the lysin concentration-hemolysis relationship was linear within an approximate range of 10-60% lysis.

Other than those limited studies with erythrocytes as substrate, little work has been reported because of the inability of investigators to agree on the mode of action of the hemolysin. However, Wiseman and Caird have studied the reaction between delta lysin and phosphatidylinositol as shown in Fig. 2, in which it is observed that P released versus time is approximately linear over the range 0-10 min. In Fig. 3, it is shown that the rate of the reaction is linear over a concentration range of 50-500 HU. With regard to the effect of temperature on this reaction, the reaction rate is directly proportional to temperature between 30-56°C, as shown in Fig. 4. An Arrhenius plot of the reaction is given in Fig. 5 from which it can be calculated that $\mu = 18,750$ cal.

Mode of action: Some controversy surrounds the mode of action of the delta hemolysin. On one hand there are those who believe that the lysin is a surface active agent acting in some non-enzymatic way on the phospholipids of membranes resulting in their disruption. However, it is
FIG. 2 DEGRADATION OF PHOSPHATIDYLINOSITOL BY DELTA LYSIN.
FIG. 3  RATE OF DEGRADATION OF PHOSPHATIDYLINOSITOL BY DELTA LYSIN.
FIG. 4 EFFECT OF TEMPERATURE ON RATE OF DEGRADATION OF PHOSPHATIDYLINOSITOL BY DELTA LYSIN.
FIG. 5. ARRHENIUS PLOT OF RATE OF DEGRADATION OF PHOSPHATIDYLINOSITOL BY DELTA LYSIN.
useful to remember that interaction of proteins and phospholipids is a common phenomenon. On the other hand, the view has been put forward that the delta lysin is a phospholipase which hydrolyzes phosphatidylinositol, a phospholipid which is widely distributed in erythrocyte species in small amounts.

The concept of delta lysin as a surface active agent has been supported by Heatley (1971) who found that the lysin was surface-active as shown by a change in contact angle when it was added to a drop of water on a water-repellent surface. No values for surface tension were supplied by Heatley, however. Heatley's preparation did not hydrolyze phosphatidylinositol. Kreger et al. (1971) confirmed Kapral's (1967, 1972) observation that hemolytic activity of delta hemolysin was inhibited by several phospholipids and were also unable to detect phospholipase activity. Their preparation was also surface-active. Kreger et al. reason that in view of the observations of Marks and Vaughan (1950), Jackson and Little (1958a), and Bernheimer (1970) to the effect that incubation of constant amounts of the lysin with increasing concentrations on red cells results in decreasing hemolytic titres, the level of hemolysis should remain constant if the delta lysin were acting as an enzyme. In our view, this reasoning, as applied to lysin-erythrocyte mixtures, is faulty, because the presence of inhibitors (even the reaction products
themselves) could be responsible for the observed reduction in titres. One must remember that red cells used as a substrate are a complex system, and if he were to use a purified substrate the kinetics would very likely be first order as shown by us (above). Kapral (1972) was also unable to detect phospholipase activity in his delta lysin preparations. Rahal (1972) has reported the action of Kreger's lysin on mitochondria, claiming that it was similar to Triton X-100 and desoxycholate in this respect. He could detect no phospholipase activity in Kreger's preparation. Mitochondrial metabolism was affected with apparent uncoupling of oxidative phosphorylation, but this was probably due to lysis of the mitochondria by the delta hemolysin.

The view that delta lysin is a phospholipase has been advanced by Wiseman and Caird (1968\(^1\)), Caird and Wiseman (1970) and evidence has been discussed in the review by Wiseman (1970). Perhaps the most important observation has been the discovery of a relationship between hemolytic sensitivity of erythrocyte species to delta lysin and its ability to liberate organic phosphorus from intact cells or phospholipid extracts. This relationship is similar to that detected with beta lysin (Wiseman and Caird, 1967) and with alpha toxin (Wiseman and Caird, 1970). Of several phospholipids tested as substrates, phosphatidylinositol was hydrolyzed to a much greater extent than phosphatidylserine or phosphatidylcholine. Sphingomyelin was not attacked (see \(^1\)Publication in Appendix.)
Wiseman and Caird, 1968). Red cells of the type used in that study have been shown by Nelson (1967) to contain these phospholipids.

The major difficulty is that we have not shown that phosphatidylinositol levels in red cells are altered in the presence of lysin. Unlike sphingomyelin, the substrate of beta lysin, phosphatidylinositol levels in red cells comprise only a small percentage of the total phospholipid as reported by Nelson. In ten types of red cell studied, he found that phosphatidylinositol concentration ranged from < 0.3 - 7.4% of total phospholipid. Thus a correlation of phosphatidylinositol content of the cells with their hemolytic sensitivity to delta lysin (Table XIII) is not as clear as it is with their sphingomyelin content and sensitivity to beta lysin (Table XIV). It will be remembered that the range of sphingomyelin concentrations in various erythrocyte species is far greater than their phosphatidylinositol content.

At present it is not possible to reconcile the two points of view concerning the mode of action of the delta lysin. However, attention should be drawn to the fact that of the preparations of Kreger et al., Kantor et al., Heatley and Kapral, all had low specific activities. In fact, as pointed out by Kreger, his preparation was 45-fold lower in specific activity than our delta lysin material. It may be difficult to detect enzymatic activity in preparations with
Table XIII

Erythrocyte Sensitivity to Delta Lysin in Relation to their Phospholipid Content

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>HU/mg protein(^1)</th>
<th>% total phospholipid(^2)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>phosphatidyl-ethanolamine</td>
<td>phosphatidyl-serine</td>
<td>phosphatidyl-inositol</td>
<td></td>
</tr>
<tr>
<td>sheep</td>
<td>2929</td>
<td>26.2</td>
<td>14.1</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>rat</td>
<td>1700</td>
<td>21.5</td>
<td>10.8</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>rabbit</td>
<td>1644</td>
<td>31.9</td>
<td>12.2</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>cat</td>
<td>1500</td>
<td>22.2</td>
<td>13.2</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>dog</td>
<td>882</td>
<td>22.4</td>
<td>15.4</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>guinea pig</td>
<td>803</td>
<td>24.6</td>
<td>16.8</td>
<td>2.4</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)From Fackrell and Wiseman (1974c).
\(^2\)After Nelson (1967).
Table XIV
Correlation of Hemolytic Sensitivity of Red Cells to Beta Lysin with their Sphingomyelin Content

<table>
<thead>
<tr>
<th>Species</th>
<th>Beta lysin titre (HU/ml)</th>
<th>% Sphingomyelin¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wiseman and Caird</td>
</tr>
<tr>
<td>Sheep</td>
<td>1810</td>
<td>50.5</td>
</tr>
<tr>
<td>Ox</td>
<td>1280</td>
<td>45.0</td>
</tr>
<tr>
<td>Man</td>
<td>256</td>
<td>21.6</td>
</tr>
<tr>
<td>Rabbit</td>
<td>160</td>
<td>19.7</td>
</tr>
<tr>
<td>Goat</td>
<td>113</td>
<td>38.0</td>
</tr>
<tr>
<td>Horse</td>
<td>&lt; 20</td>
<td>15.0</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>&lt; 20</td>
<td>16.6</td>
</tr>
<tr>
<td>Monkey</td>
<td>&lt; 20</td>
<td>11.4</td>
</tr>
</tbody>
</table>

¹From Wiseman and Caird (1967), supplemented by the analyses of Nelson (1967).
such low activity. Purified delta lysin sent to this laboratory by Dr. Kreger was contaminated with alpha and gamma lysins (Fackrell and Wiseman, 1974a). Furthermore, his delta lysin was produced by a coagulase-negative mutant of strain Wood 46 obtained by UV irradiation. This was also the strain which Kantor's group used.

Kreger et al. (1971) and Kayser (1968) claimed that delta lysin clarified egg yolk agar. It is not quite clear what is responsible for the reduction in turbidity and it is unlikely that the mechanism is similar to that responsible for clearing of egg yolk suspensions by alpha toxin of Clostridium perfringens (MacFarlane and Knight, 1941). Egg yolk clearing by delta lysin is suggestive of enzymatic activity, and while phosphatidylcholine inhibits the lysin's hemolytic activity (Kapral, 1972; Kreger et al., 1971), the phospholipid itself is not degraded (Wiseman and Caird, 1968). The most logical explanation of the egg yolk reaction at present is that the preparations which have been tested are contaminated with a lecithinase. When one reflects upon this phenomenon, it is remarkable that the delta lysin could clear the yolk in the presence of phosphatidylcholine, a constituent of the yolk.

Toxicity of the delta lysin: Wiseman (1970) has reviewed the delta lysin's properties. Most investigators agree that it has an injurious effect on a wide variety of cells in culture and on leucocyte suspensions (Jackson and

1Paper in Appendix.
Little, 1956, 1957; Gladstone and Van Heyningen, 1957; Gladstone, 1966; Gladstone and Yoshida, 1967). According to Kreger et al. (1971) and Kreger and Bernheimer (1971), delta lysin disrupts bacterial protoplasts, spheroplasts, lysosomes, and inhibits the growth of several strains of bacteria. This group also claimed that delta lysin killed mice, rabbits and guinea pigs (Table XV) and caused dermonecrosis in rabbits if injected in amounts of 1 mg or more. Regrettably, their findings were expressed as minimum lethal dose (MLD), a rather imprecise method. Fackrell and Wiseman (1974c) have calculated the LD<sub>50</sub> dose of Kreger's delta lysin from the data supplied in his paper, and it is of the order of 2 mg for mice, or 7.17 mg for guinea pigs. Wadstrom and Mollby (1972) reported an LD<sub>50</sub> dose of 125 mg for delta lysin in mice, very large indeed. A dose this size is of little significance, since it could be argued that a contaminant was present in concentrations sufficient to kill the animals. As shown in the Table, Fackrell and Wiseman have determined that the LD<sub>50</sub> of delta lysin in mice and guinea pigs is $>0.1$ mg or $>4$ mg/kg.

Gladstone (1966) has also reported some properties of Yoshida's crystalline delta lysin preparation, although it must be remembered that this material was contaminated with beta lysin and RNAse as mentioned elsewhere. The LD<sub>50</sub> or MLD was greater than 0.5 mg when a 55% pure preparation was used, but the number of mice was small. However, he did
Table XV
Lethal Effect of Delta Hemolysin in Animals

<table>
<thead>
<tr>
<th>Reference</th>
<th>Animal</th>
<th>Dose (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lethal</td>
</tr>
<tr>
<td>Kreger et al. (1971)</td>
<td>mouse</td>
<td>110 (MLD)</td>
</tr>
<tr>
<td></td>
<td>rabbit</td>
<td>30 (MLD)</td>
</tr>
<tr>
<td></td>
<td>guinea pig</td>
<td>-</td>
</tr>
<tr>
<td>Kreger et al. (1971)¹</td>
<td>rabbit</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>guinea pig</td>
<td>-</td>
</tr>
<tr>
<td>Gladstone (1966)²</td>
<td>mouse</td>
<td>&gt;10 (LD50)</td>
</tr>
<tr>
<td></td>
<td>rabbit</td>
<td>-</td>
</tr>
<tr>
<td>Wadstrom and Mollby (1972)</td>
<td>rabbit</td>
<td>5000 (LD50)</td>
</tr>
<tr>
<td>Fackrell and Wiseman (1974c)</td>
<td>mouse</td>
<td>4 (LD50)</td>
</tr>
<tr>
<td></td>
<td>guinea pig</td>
<td>4 (LD50)</td>
</tr>
</tbody>
</table>

¹Delta lysin supplied by Dr. Kapral (Infect. Immun. 4, 541, 1971).
³Calculated on the basis of 25 g. mouse, 1 kg. rabbit, average weights.
⁴These doses cause dermonecrosis, but information regarding the minimum or 50% necrotic dose was not available.
observe that 0.5 mg doses caused necrosis in rabbits. Lower doses were erythematous, but no necrosis took place.

The fact which emerges is that the delta lysin is certainly not very toxic when compared to other bacterial toxins, in spite of the evident discrepancies in technique, dose and numbers of mice used by various investigators.

**Immunogenicity of the delta lysin:** Several investigators (Gladstone and Yoshida, 1967; Hallander, 1968; Kantor *et al.*, 1972, and others) have not been able to produce antibody against the delta lysin. Gladstone and Yoshida claimed that the lysin was neutralized by alpha and beta globulin fractions of serum but only to a limited extent by gamma globulin. Kapral (1972) felt that this neutralizing activity was due to the phospholipid content of the serum.

Fackrell and Wiseman (1974a) have prepared antibody to highly purified delta lysin in rabbits. Non-specific inhibitors were removed from the serum by ammonium sulphate precipitation and ion exchange chromatography. Purified antibody to delta lysin showed precipitin lines in the presence of the lysin when subjected to agar gel diffusion and immunoelectrophoresis, in contrast with the behaviour of pre-immune gamma globulin. It was also shown that the delta lysin antibody neutralized the hemolytic activity of delta lysin.
The Gamma Hemolysin
The Gamma Lysin

Although gamma lysin was described by Smith and Price (1930) and its existence confirmed by Marks (1951), most investigators, until very recently, accepted the view of Elek and Levy (1950) that the alpha, gamma and delta lysins were identical. It now seems clear that Elek and Levy could not possibly have detected gamma lysin on their blood agar plates since its activity is inhibited by agar. Reports by Jackson (1962), Guyonnet et al. (1968), Guyonnet and Plommet (1970) and by Mollby and Wadstrom (1971) have amply demonstrated the existence of a hemolysin separate from the alpha, beta and delta lysins.

Production

Gamma lysin has been produced by essentially the same techniques which have been applied to production of the other hemolysins. The method of Birch-Hirschfeld (1934), in which cells are grown on cellophane overlaying agar media, has been used by Jackson (1962) and by Fackrell and Wiseman (1974b). The French and Swedish groups (Guyonnet et al., 1968; Guyonnet and Plommet, 1970; and Mollby and Wadstrom 1971) have used the liquid CCY medium of Gladstone and Van Heyningen (1957) with production of acceptable yields. Guyonnet and Plommet aerated their liquid cultures with a mixture of 25% CO₂ and oxygen. Mollby and Wadstrom did not aerate the liquid cultures apart from that obtained

¹Paper in Appendix.
by shaking as cultivation proceeded. In fact, they reported that aeration resulted in low yields (< 10 HU/ml). Fackrell and Wiseman investigated production of the lysin under a variety of conditions, finding that highest titres were obtained when trays were incubated in a mixture of 10% CO₂ in air, at pH 7.0 for 24 hours at 37°C. Hemolysin production was minimal beyond the pH range 6.0-8.0.

Fackrell and Wiseman also found that the gamma lysin is produced by the 5R strain during the late log phase of growth. When intracellular levels of gamma lysin were compared to that found in the supernatant in the late log phase, it was evident that the hemolysin was not cell bound to any extent.

Purification

As shown in Table XVI, only three attempts at purification of the gamma lysin have been made, reflecting the relatively recent resurgence of interest in this lysin. Guyonnet et al. (1968) and Guyonnet and Plommet (1970) used hydroxylapatite in its purification, and found that the lysin was retained on the column. If a molar gradient of buffer were established between 0.1 - 0.6 M, two inactive peaks were eluted, but recombination restored hemolytic activity. Mollby and Wadstrom (1971) combined ion exchange chromatography on DEAE Sephadex with electrofocusing, but did not state the degree of purity achieved. Fackrell and
Table XVI

Purification of Staphylococcal Gamma Hemolysin

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<td>Guyonnet et al. (1968)</td>
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<td>Mollby and Wadstrom (1971)</td>
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<td>Fackrell and Wiseman (1974b)</td>
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1B=ion exchange chromatography, A=electrofocusing, I=ultrafiltration, C=gel filtration, D=precipitation, F=other (NaCl extraction).
Wiseman have achieved a 2736-fold purification by concentration of the lysin on an XM-50 membrane, gel filtration on Sephadex G-75 and ammonium sulphate precipitation. The specific activity of the highly purified hemolysin was $1.08 \times 10^5$ HU/mg protein.

**Properties**

**Physicochemical characteristics:** Guyonnet et al. and Guyonnet and Plommet found that gamma lysin from strain 5R could be separated into two inactive peaks and, as indicated above, hemolytic activity was restored when the two components were mixed. Neither Mollby and Wadstrom nor Fackrell and Wiseman were able to confirm this finding with the 5R strain.

Jackson (1962) observed that gamma lysin was inactivated by heating at 55°C for 10 min or by addition of cysteine or ascorbic acid. He also noted that agar inhibited the lysin's hemolytic activity. In these ways, Jackson claimed to be able to separate gamma from delta lysin. However, Guyonnet and Plommet did not confirm Jackson's findings with cysteine and ascorbic acid, but agar in their hands inhibited activity. It is not known what strain of *Staphylococcus* Jackson used. Perhaps the most important difference between the work of Jackson on the one hand and Guyonnet's group on the other, is the latter's observation that the activity of one of their synergistic fractions was
inactivated by heating at 60°C for 10 min while the other was refractory. However, it is conceivable that a difference of 5°C in temperature while heating gamma lysin could destroy lysin which was stable at 55°C. Mollby and Wadstrom confirmed inactivation of the hemolysin by heating at 60°C for 10 min. They also noted that 80% of the lytic activity was lost in the presence of 0.1% agar. Noble Agar (Difco) was less effective as an inhibitor while hemolytic activity in the presence of 0.1% agarose was unchanged. Isoelectric focusing experiments indicated that the pI of gamma lysin was 9.5. Oddly enough, DEAE Sephadex at pH 8.5 adsorbed the lysin in contrast with its behaviour towards alpha, beta and delta lysins, all of which are reputedly basic proteins.

Fackrell and Wiseman (1974c) have obtained some data on the properties of the gamma lysin. In our hands, gamma lysin when compared to alpha, beta and delta lysins can be distinguished from them on the basis of $S_{20w}$, pI, extinction coefficient, amino acid N-terminus and molecular weight. However, comparison of our gamma lysin preparation to preparations of alpha, beta and delta lysins obtained by other investigators is probably meaningless when one considers the wide variation in data found in the literature.

**Hemolytic activity:** Guyonnet and Plommet (1970) stated that gamma lysin acted on human, rabbit and sheep but not horse red cells provided that the two fractions they had isolated were present. Mollby and Wadstrom (1971) found
that rabbit erythrocytes were most sensitive to gamma lysin while those of the chicken were least sensitive. Other species, sheep, goat, human and dog were of intermediate sensitivity, while horse cells were slightly lysed. Perhaps the most reliable hemolytic assays of the 5R gamma lysin are those given by Fackrell and Wiseman (1974c) in which the gamma lysin is compared with other lysins on the basis of specific activity/mg protein. The erythrocytes of rabbit and sheep are the most sensitive, while those of chicken and pigeon are the least. There is some agreement with the findings of Mollby and Wadstrom with the exception of human red cells, a surprising fact in view of the variability of the cells and the difficulties of cross-comparison of results. As shown by us, the hemolytic spectrum of gamma lysin does not resemble that of the alpha, beta or delta lysins.

**Toxicity:** When injected subcutaneously with 100 μg of gamma lysin, guinea pigs and rabbits showed no effect. The same dose injected into mice intraperitoneally or by the intravenous route did not affect the mice, but guinea pigs were killed in a few minutes if 50 μg quantities were injected intracardially. Autopsy findings indicated massive hemorrhage of the kidney and serosal surfaces of the intestines accompanied by lysis of red cells in major veins and arteries. Incubation of gamma lysin with C-6 cells (Fackrell and Wiseman) increased the rate at which the cells took up
trypan blue. Both rabbit and human platelet suspensions were lysed by gamma lysin as indicated by a decrease in optical density. A reduction in optical density was also observed when human leucocytes were incubated with the lysin.

**Reaction Kinetics**

Features of the interaction between gamma lysin and erythrocytes are not unlike those of the alpha, beta and delta lysins. When hemolysis is plotted against time, at various concentrations of gamma lysin, a direct correlation is obtained except for early and late phases of the reaction (Fig. 6). When reaction velocity is plotted against lysin concentration, a straight-line relationship is observed (Fig. 7) in the range of 0-10 HU. Such a relationship is not incompatible with first order kinetics, although it must be remembered that the substrate present in the erythrocyte has not been identified. The plateau observed in Fig. 7 probably reflects limitations of experimental conditions which measure velocity at high concentrations of lysin, as pointed out by Fackrell (1973). Fackrell also investigated the effect of temperature and pH on reaction kinetics. The reaction rate of the lysin-red cell interaction is linear between 20-37°C (Fig. 8), but beyond this we cannot be sure that temperature is not affecting the erythrocytes. An Arrhenius plot of the data indicated that for gamma lysin, the curve is biphasic, with $E (\mu)$ values of 1600 and 4800
FIG. 6. HEMOLYSIS OF RABBIT ERYTHROCYTES IN THE PRESENCE OF GAMMA LYSIN. LYSIN CONCENTRATION (HU/ml) VERSUS TIME.
FIG. 7. HEMOLYSIS OF RABBIT ERYTHROCYTES IN THE PRESENCE OF GAMMA LYSIN. REACTION VELOCITY VERSUS LYSIN CONCENTRATION.
FIG. 8. EFFECT OF TEMPERATURE ON GAMMA LYSIN-RED CELL INTERACTION. ARRHENIUS PLOT IN UPPER RIGHT CORNER.
cal. respectively. This contrasts with a value of 14,100 cal. for beta lysin and 18,750 cal. for delta lysin. While these data probably represent genuine differences, it has to be remembered that with the exception of the delta lysin, values for beta and gamma lysins were obtained with red cells and not with the isolated substrate. The values obtained for gamma lysin are still within the range of 1000-25,000 cal. observed by Sizer (1943) for most enzymes. In Fig. 9, the reaction rate at various pH levels has been investigated. The rate is maximal at pH 7.0 and falls off sharply on either side of neutrality.

Gamma lysin from the 5R strain has also been shown by Fackrell and Wiseman (1974d) to be inhibited by EDTA and citrate. Sodium ions appear to be required for lysis.

Mode of action: At the present time, little is known of the mode of action of the gamma lysin. Nevertheless, there is no doubt that the lysin is a protein and that its reaction kinetics with red cells are compatible with an enzymatic mechanism. Evidence of a cation requirement (Na⁺) supports this contention. Fackrell and Wiseman (1974d) have obtained some data which indicate that gamma lysin liberates nitrogen and acid-soluble phosphorus from erythrocyte ghosts. Phospholipids extracted from human red cells by the method of Rose and Oklander (1965) did not release nitrogen and phosphorus in the presence of gamma lysin in contrast with the ghosts as substrate. Whether

¹Paper in Appendix.
FIG. 9. EFFECT OF pH ON GAMMA LYSIN-RED CELL INTERACTION.
or not phospholipids are in fact attacked by gamma lysin, it certainly had no effect on phosphatidylserine, phosphatidylethanolamine, sphingomyelin or phosphatidylinositol. These observations rule out contamination of the gamma lysin with beta and delta lysins. Furthermore, neither TAME nor Azocoll were attacked by the lysin whether or not it was "activated", thus ruling out contamination with alpha lysin. Further evidence has nevertheless been obtained to the effect that gamma lysin degrades a phospholipid present in the intact red cell and in ghosts, as alluded to by Fackrell and Wiseman in their paper.

In summary, evidence available at present favours the enzymatic degradation of a phospholipid in the red cell membrane by gamma lysin. However, it has not been possible to detect changes in phospholipid composition. Perhaps the extraction procedure may have denatured the substrate, or it could be that conformation of the phospholipid substrate changes during extraction, rendering it refractory to the lysin's action.
References
References


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Appendix
IMMUNOGENICITY OF THE DELTA HEMOLYSIN OF

STAPHYLOCOCCUS AUREUS

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INTRODUCTION

Attempts by several investigators have failed to demonstrate the immunogenicity of the delta hemolysin of Staphylococcus aureus. Gladstone and Yoshida (1967) did not convincingly demonstrate that delta lysin was immunogenic and showed that inhibitors of its hemolytic activity were present in serum. Recent evidence suggests that these serum inhibitors are lipoproteins (Donahue, 1969; Kantor, Temples and Shaw, 1972).

In this study, the immunogenicity of delta lysin has been demonstrated by the use of purified antibody preparations.

MATERIALS AND METHODS

The strain employed, methods of production and purification of delta lysin were those of Caird and Wiseman (1970). Hemolysin titrations were performed as described by Wiseman and Caird (1972). Buffers were prepared according to Gomori (1955) and where required, 0.01 M phosphate buffer at pH 7.0 was supplemented with 0.85 per cent. NaCl. Nitrogen content was assayed by the micro-Kjeldahl technique as described by Markham (1942) and where an estimate of the protein content was required, its value was multiplied by 6.25.

Alpha and gamma hemolysins were prepared and purified as described elsewhere (Wiseman, Caird and Pack-
reill, 1974; Fackrell and Wiseman, 1974). Purified beta lysin was used in one immunodiffusion study and was prepared by a modification of the method of Wiseman and Caird (1967) in which the hemolysin was passed through Sephadex G-75 equilibrated with Hallander's (1963) buffer. The active lysin was pooled when removed from the column, dialyzed against distilled water for 48 hr, centrifuged and the supernatant lyophilized.

Immunodiffusion, immunoelectrophoresis and quantitative precipitin methods were those of Campbell et al. (1970). The blood agarose required in some immunodiffusion tests was prepared by the addition of 1.0 ml of packed, washed human group O erythrocytes to 100 ml of a 1 per cent. (w/v) solution of Difco agarose in phosphate buffered saline held at 50°C.

Antibodies to the hemolysins were prepared in two month old New Zealand white rabbits purchased from the Canadian Research Animal Farm, Bradford, Ontario. A solution of purified hemolysin (2 mg/ml) was prepared in phosphate buffered saline and injected subcutaneously into the animals at three-day intervals for three weeks. After a two week period during which no injections were given, the schedule was repeated once. The first three injections of the schedules consisted of 0.10, 0.25 and 0.5 ml of hemolysin followed by 1.0 ml volumes thereafter. Two days after the last injection, the animals
were bled from the heart and the serum was stored at 
-20°C.

Immune and control sera were twice precipitated 
with ammonium sulphate and fractionated on diethylamino-
ethyl cellulose (Campbell et al.). The concentration 
of these purified sera was adjusted to 5 mg protein/ml. 
A single line of precipitation was observed with goat 
anti-rabbit serum (Hyland Laboratories, Costa Mesa, 
Calif.) when the purified antibody was subjected to 
immunoelectrophoresis.

Purified delta lysin was also kindly sent to us 
by Dr. Arnold Kreger.

RESULTS

A single line of precipitation was observed 
when purified delta lysin was electrophoresed against 
purified (IgG) anti-delta lysin as shown in Fig. 1. 
Ouchterlony agar gel diffusion studies of the delta 
lysin also indicated the presence of one line of 
precipitation (Fig. 2).

The quantitative precipitin test shown in Fig. 
3 gave a characteristic curve when increasing amounts 
of purified delta lysin were mixed with a constant 
volume of antibody, yet no precipitate was detected 
with pre-immune gamma globulin prepared from normal 
serum. Gamma lysin gave no precipitate with anti-
delta lysin, proving that the two hemolysins are 
distinct from each other.
In another experiment, an immunodiffusion test was performed in which the agarose contained human erythrocytes. The slides were developed at 4°C for two days and then incubated at 37°C for 1 hr. As shown in Fig. 4, the edge of the zone of hemolysis coincided with the precipitin line formed between delta lysin and its antibody. Pre-immune serum showed no inhibition of hemolysis.

A sample of purified delta lysin which was obtained from Dr. Kreger (Kreger et al., 1971) gave a line of identity with our delta lysin when diffused against the antibody (Figs. 2, 4). The Kreger lysin was injected into rabbits and the antiserum obtained was fractionated in the usual manner. It was interesting to note (Fig. 5) that this purified anti-delta lysin gave a strong and weak line of precipitation when incubated in an agar gel diffusion test with Kreger's purified antigen. As shown in the same Figure, alpha and gamma lysins were also detected in Kreger's preparation.

DISCUSSION

In contrast with the earlier observation of Gladstone and Yoshida, we found that in blood agarose gel diffusion tests, the line of precipitation resulting from the interaction of delta lysin with IgG coincided with the zone of hemolysis. Gladstone's inability to observe coincident hemolysin and precipitation zones
may have been due to the fact that he was not using a purified antibody. It would appear that most of the difficulty in detection of antibody to delta lysin is due to inhibition of hemolysis by serum proteins other than IgG as shown by Gladstone and Yoshida and others (Donahue, 1969). Kantor et al. have been unable to produce antibody to their delta lysin preparation and found that lines of precipitation occurred when sera from unimmunized animals were subjected to agar gel diffusion against the antigen. Whether these animals were unimmunized is open to question and the lines of precipitation they obtained may or may not be regarded as nonspecific. It is obvious that reliable results can only be obtained in the presence of purified antibody.

Comparison of Kreger's delta lysin preparation with our own purified material indicated that the two are identical antigenically but it was observed that his lysin also contained alpha and gamma lysins. It would thus seem that Kreger's method of purification was no more satisfactory than that of Yoshida (1963), whose delta lysin was subsequently shown to contain beta lysin and ribonuclease. The contamination of Kreger's preparation with alpha and gamma lysins might account for the differences between his preparation and our own.

The nature of the curve obtained for delta lysin in the quantitative precipitin test proves that
antibody to the lysin was produced. Pre-immune gamma globulin and gamma lysin IgG were unreactive with the delta lysin antigen.

**SUMMARY**

Antibody to delta lysin of *Staphylococcus aureus* has been prepared in rabbits injected with purified antigen. Removal of normal serum inhibitors was effected by ammonium sulphate fractionation and ion exchange chromatography. The purified anti-delta lysin showed a single line of precipitation against purified antigen in Ouchterlony agar gel diffusion tests and when subjected to immunoelectrophoresis. The line of precipitation also coincided with the zone of hemolysis caused by delta lysin when the agar gel diffusion test was performed in blood agarose plates.

A quantitative precipitin test was performed with purified antibody and a characteristic curve of delta lysin precipitation was observed in contrast with non-immune sera in which no precipitation was evident.

**ACKNOWLEDGMENTS**

This work was supported by the Medical Research Council of Canada.
REFERENCES


CAPTIONS OF FIGURES

Figure 1. Immunoelectrophoresis of purified delta hemolysin. The well contains the authors' delta lysin antigen. The trough contains anti-delta IgG. The anode is to the left.

Figure 2. Ouchterlony double diffusion of the authors' purified delta lysin. Centre well contains authors' delta lysin. Well (1) contains authors' anti-delta IgG. Well (2) contains IgG prepared from an animal injected with Kreger's purified delta lysin.

Figure 3. Quantitative precipitation of purified gamma and delta lysins by anti-gamma and anti-delta IgG.

Figure 4. Immunodiffusion of authors' purified delta lysin in human blood agarose. Centre well contains delta lysin. Well (1) contains authors' anti-delta IgG. Well (2) contains authors' pre-immune gamma globulin. Well (3) contains pre-immune gamma-globulin from an animal which received Kreger's delta lysin. Well (4) contains IgG to Kreger's delta lysin.
Figure 5. Ouchterlony double diffusion of Kreger's purified delta lysin. The centre well contains Kreger's delta lysin. The outer wells contain authors' IgG prepared from animals injected with (1) alpha lysin, (2) Kreger's delta lysin, (3) gamma lysin, and (4) beta lysin.
Fig. 1
Fig. 2
Fig. 3

Graph showing the effect of anti-gamma lytin and anti-delta lytin on the precipitation of gamma lysin and delta lysin. The x-axis represents the concentration of gamma lysin and delta lysin in micrograms nitrogen (μg N), while the y-axis represents the precipitation in units of 10² μg N.
Fig. 5
TRYPSIN-MEDIATED ACTIVATION OF THE ALPHA HEMOLYSIN OF STAPHYLOCOCCUS AUREUS "WOOD 46"

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INTRODUCTION

Recent work in this laboratory indicated that the alpha toxin of *Staphylococcus aureus* "Wood 46" is produced by the organisms as an inactive proteolytic enzyme (Wiseman and Caird, 1970). Both proteolytic and hemolytic activities of activated toxin have been shown to be neutralized by alpha antitoxin (Wiseman and Caird, 1972). It has also been suggested that this alpha "protoxin" is activated by erythrocyte proteases and that the level of protease activity in various red cell species explains their differential sensitivity to the toxin. In our study of this phenomenon, we were interested to know whether other proteolytic enzymes could serve as activators and our attention was thus directed toward trypsin.

MATERIALS AND METHODS

Crude protoxin was prepared from the Wood 46 strain of *S. aureus* by a method similar to that given by Gow and Robinson (1969) except that in cultivation of the organisms, air rather than pure oxygen was used in conjunction with carbon dioxide to provide the gaseous environment.
Purification Procedure

Gel filtration and ion exchange chromatographic procedures are given as follows. A K50x100 cm column of Sephadex G-75 (Pharmacia, Montreal, Que.) was set up according to the manufacturer's instructions and equilibrated with Hallander's buffer (Hallander, 1963). Five ml volumes of hemolysin were applied to the column and 15 ml fractions were collected by upward elution.

Ion exchange chromatography was performed on a column of carboxymethylcellulose (CMC) according to the method of Robinson, Thatcher and Montford (1960). Hemolysin samples were dialyzed against distilled water for 24 hr, centrifuged and then dialyzed against 0.056 M phosphate buffer, pH 6.0. Five ml volumes of hemolysin were applied to a column equilibrated with the same buffer. The hemolysin was eluted in 15 ml fractions from the column by the gradient described by Robinson et al.

Ammonium sulphate fractionation of partially purified lysin was performed in which lysin was divided into eight fractions, to each of which the salt was added in concentrations ranging from 0-100 per cent. saturation. The precipitates, held overnight at 4°C, were centrifuged, resuspended and dialyzed against phosphate buffered saline.
The purification procedure was the method developed in this laboratory (Packrell, 1973). The technique of methanol precipitation of alpha hemolysin, described by Wittler and Pillemer (1948), was re-examined. Crude hemolysin at pH 4.0 was mixed with methanol at $-20^\circ$C to a final concentration of 35 per cent. solvent and the collected precipitate was dialyzed against phosphate buffered saline. As a second step, methanol-precipitated lysin was further purified by precipitation in the range of 50-60 per cent. ammonium sulphate saturation. About 70 per cent. of the hemolysin was precipitated at this concentration of the salt and was accompanied by a two-fold increase in specific activity.

Fractionation of the alpha lysin on Sephadex G-75 was a third step which gave a further six-fold increase in specific activity. The relative elution volume of the lysin was 1.45, the hemolytic activity appearing between two major peaks of protein. A second ammonium sulphated precipitation was performed at this stage primarily to concentrate and stabilize the activity in the Sephadex effluent. The precipitate obtained was prepared for chromatography on carboxymethylcellulose (CMC). The alpha lysin was eluted from the CMC column with the third peak of protein, as shown earlier by Gow (1968). Most of the hemolytic activity was recovered in this step and the purified
product was stored under saturated ammonium sulphate at 4°C.

A summary of the procedure is given in Table I, where it is observed that a 308-fold purification of the alpha toxin has been achieved with 39 per cent. recovery. Specific activity of the final product is 125,000 Hemolytic Units/mg protein, rather higher than activities reported for earlier procedures by Arbuthnott (1970).

Purified toxin was dermonecrotic and lethal for rabbits and mice, possessed a hemolytic spectrum similar to that given by Bernheimer and Schwartz (1963) and by Wiseman and Caird (1970, 1972), and its activity was neutralized by Wellcome CPP97/63 antitoxin. The $S_{20\, w}$ value of the toxin was 1.4 but became 3.0 after standing for several days (Fackrell and Wiseman, 1974). Furthermore, the purified alpha toxin was not immuno-logically reactive with immune gamma globulins prepared from rabbits injected with purified beta, gamma and delta hemolysins. Evidence of homogeneity of the toxin preparation used in the present study has been presented elsewhere (Fackrell and Wiseman).

**Assay Methods**

Hemolytic activity was measured according to the method outlined by Wiseman and Caird (1972). Proteolytic activity was assayed according to the technique
described by Hummel (1959) in which p-toluenesulphonyl-
L-arginine methylester (TAME) was used as a substrate. 
The method of Fraenkel-Conrat, Harris and Levy (1955) 
was used in the preparation of N-terminal amino acids 
from the protein. Standard dinitrophenyl (DNP) amino 
acids were purchased from Schwarz-Mann, New York. The 
N-terminal groups were separated and identified by 
thin layer chromatography as described elsewhere in 
Materials and Methods.

Preparation of CMC-trypsin Complex

Trypsin (Worthington, crystallized) was coupled 
to carboxymethylcellulose (CMC) in the presence of 
N,N'-dicyclohexylcarbodiimide (Sigma) by the method 
given in Campbell et al. (1970). In the product 
obtained, proteolytic activity was associated with the 
CMC particles but was absent from the supernatant 
fluid.

Spectrophotometry

A Unicam SP800 B double beam recording spectro-
photometer equipped with a SP825 series B program 
controller was used. In some experiments, it was 
advantageous to use a split spectrophotometer cell 
(Pyrocell Mfg., Westwood, N.J.) in which the substrate 
(TAME) and enzyme (trypsin or activated toxin) were 
placed respectively in the two compartments in tandem 
positions. The contents of this cell were then
compared to those of a regular cell which contained both enzyme and substrate mixed together, similar to the techniques used in obtaining difference spectra as described by Herskovits (1967).

**Thin-layer Chromatography (TLC)**

Glass plates measuring 20 x 20 cm were coated with an 0.25 mm thickness of Silica Gel G (Merck and Co., Darmstadt) in a Fisher "Quick Fit" TLC apparatus equipped with automatic plate levelling. The silica gel was prepared as a slurry in water, one part gel to two parts water, before being applied to the plates. Prepared plates were dried overnight at room temperature.

Samples of DNP-amino acids were applied 1 cm from the edge of the plate and were developed until the solvent had travelled 10-15 cm upward. Plates were removed from the tanks, dried, and the positions of the spots recorded by drawing or photography.

For single dimensional TLC, several solvent systems were used as described by Pataki (1966). These were chloroform/benzyl alcohol/acetic acid (70:30:3) v/v and benzene/pyridine/acetic acid (80:20:2). For the separation of leucine and isoleucine, two-dimensional TLC was attempted. These chromatograms were developed initially in toluene/pyridine/2-chloroethanol/25% NH₄OH (50:15:35:7), and secondary
development was effected at right angles to the first in chloroform/methanol/acetic acid (95:5:1).

RESULTS

Degradation of Protoxin Protein by Trypsin

The action of trypsin on protoxin was investigated in the absence of CMC before activation studies were initiated. Protoxin was mixed with an equal volume of trypsin dissolved in Tris buffer at pH 7.0. This mixture was incubated at 37°C in a water bath for a period ranging from 30 s to 60 min. At various times, soybean trypsin inhibitor was added to stop the reaction. After this the tubes were plunged into ice water. Results shown in Table II indicate that protoxin is almost immediately degraded, but that the maximal rate of degradation is not reached before 5 min. exposure. It will also be noted that proteolytic activity is degraded hand-in-hand with hemolytic activity. In subsequent experiments, protoxin was exposed to CMC-trypsin for not more than 30 s before centrifugation in the cold.

Activation of Protoxin by Trypsin

The action of CMC-trypsin complex on alpha protoxin was investigated according to the experimental design given in Table III. Tubes which contained the
reagents given in the Table were incubated at 37°C for 30 s with gentle shaking after which they were immersed in ice water. No soybean trypsin inhibitor was added. The contents were centrifuged at 2000 g for 15 min. and passed through Swinney-type filters equipped with cellulose acetate membranes of pore size 0.45 μm (RB Filters, Toronto, Ontario). This was necessary because CMC-trypsin supernatants contained finely suspended CMC particles with consequent proteolytic activity. Filtration removed this particulate material and its associated activity. Supernatant fluids were then assayed for hydrolysis of TAME.

Results are given in Fig. 1 in which it is observed that hydrolysis of TAME in the presence of toxin activated by CMC-trypsin complex proceeds approximately in a linear manner over a period of 13 min. Separate controls of protoxin and supernatant fluids of CMC, CMC and protoxin, and CMC-trypsin complex show no hydrolytic activity.

**Effect of Filtration on Protoxin and Activated Toxin**

Passage of small volumes of protoxin through the Swinney filter resulted in recovery of only 5 per cent. of the material, as shown in Table IV. It should be understood that protoxin by definition is not hemolytic, but if it is to be detected, the protoxin must be titrated against erythrocytes which contain the activating
protease. In contrast with the recovery of 5 per cent. of protoxin, 65 per cent. of the trypsin-activated toxin was recovered as shown in the Table. Some of the hemolytic activity has been removed by CMC.

N-Termini of Filtered Protoxin and Activated Toxin

As shown in Fig. 2, single dimensional TLC of DNP-amino acids of protoxin in benzene/pyridine/acetic acid confirmed that histidine is the N-terminus. This determination was based on unfiltered protoxin, since filtration removed much of the hemolytic activity. Chromatograms of filtered activated toxin showed two spots, one of which was histidine. The other spot migrated in the solvent close to leucine and isoleucine, the Rf of which were nearly identical. Methionine also moved closely to the unknown, but was not identical with it, as shown in the Figure. This was confirmed with the use of chloroform/benzyl alcohol/acetic acid in which methionine was well-separated from the unknown (data not shown). The only amino acids which moved in benzene/pyridine/acetic acid with Rf values close to that of the unknown were in fact methionine, leucine and isoleucine. The Rf values of 20 other amino acids tested were quite different when compared to the unknown. Filtration had no effect on resolution of DNP-amino acids of activated toxin.
Comparative Kinetics of TAME Hydrolysis by Activated Toxin and Trypsin

In this experiment, TAME and activated toxin dissolved in 0.01 M phosphate buffer at pH 7.0 were separately added to a series of tubes such that the final concentration of TAME ranged from 40-2000 µg. The amount of toxin contained in each tube was constant at 37 HU/ml. In the trypsin experiment, the range of concentration of TAME was the same but that of trypsin was 20 µg per tube. These solutions were incubated at 37°C in regular cells in the spectrophotometer and readings at 247 nm were taken automatically at 3 s intervals over a period of seven min. A Lineweaver-Burk plot was constructed from the data obtained and is shown in Fig. 3. Plots of 1/v versus 1/s for toxin and trypsin are significantly different as shown by 95% confidence limits placed about the lines. From the graph it can be determined that $V_{\text{max}}$ for toxin is 0.26 min$^{-1}$ while that for trypsin is nearly identical at 0.27. However, the value of $K_m$ for toxin as obtained by extrapolation is 0.040 µM in contrast with a value of 0.072 µM for trypsin. Although dry weight concentrations of toxin and trypsin in this experiment were not strictly comparable, advantage was taken of the fact that under specified conditions and with the same substrate, $K_m$ is independent of enzyme concentration.
DISCUSSION

We feel that these observations contribute further to the theory that the alpha toxin of \textit{S. aureus} is a zymogen. Conversion of the protoxin to the active form can be effected by activating enzymes derived from suitable erythrocyte membranes or by trypsin. The time of exposure of protoxin to CMC-trypsin complex is important, since activation is quickly followed by degradation of the toxin molecule. It is impossible to prevent some loss of toxin activity but this can be minimized to an acceptable level provided that the 30 s period of incubation followed by a 15 min. "cold" centrifugation is not exceeded.

It might be argued that alpha toxin in contact with CMC-trypsin complex removed the trypsin from the CMC and that the liberated trypsin is responsible for hydrolysis of TAME. There would appear to be no force to this argument since statistical analysis of the data (Fig. 3) showed that plots of $1/v$ versus $1/s$ for trypsin and toxin are significantly different at the 1 per cent. level, indicating that the toxin has a greater affinity for TAME than trypsin. Apart from this, activated toxin and trypsin are similar in several respects. Both are produced as inactive precursors and will attack TAME. Furthermore, the N-terminus of bovine
trypsin is isoleucine as shown by Rovery, Fabre and Desnuelle (1952, cited by Dixon and Webb, 1964). The N-terminus of activated alpha toxin is either leucine or isoleucine. Trypsin and erythrocyte membrane proteases may activate alpha protoxin by removing a short peptide (with histidine as the N-terminus) from the N-terminal end of the molecule which exposes the active centre, analogous to the manner in which trypsinogen is activated.

Little or no protoxin was recovered after passage through a Swinney filter in contrast with 65 per cent. recovery of active toxin. It is difficult to explain why partial rather than full recovery of the toxin was obtained. It may be the result of incomplete activation of protoxin by trypsin with 35 per cent. of the toxin unfiltrable because it was not activated. At any rate, the differential recovery of the two toxins must reflect a change in size, conformation or charge when the molecule is activated.

It has come to our attention that Freer, Arbuthnott and Billcliffe (1973) have been unable to associate proteolytic activity with their alpha toxin preparations derived from the Wood 46 strain. However, they failed to look for protease activity in their membrane suspensions. It is quite possible that activator was lost in the preparation of their ghosts or that under the conditions of their experiments the protease acti-
vator was inactive. In this connection it is well to take note of the work of Dodge, Mitchell and Hanahan (1963), who showed that the method of preparation greatly affected the quality of the ghosts.

Alpha toxin has also been reported to have surface activity. Weissmann, Sessa and Bernheimer (1966) and Freer, Arbuthnott and Bernheimer (1968) have both observed that alpha toxin disrupted artificial lipid membranes. Buckelew and Colacicco (1971) have demonstrated that the toxin readily spreads as a film on aqueous media and that penetration of toxin into lipid monolayers is related to the structure of the lipid. Film penetration was greatest with cholesterol and least with ganglioside. Arbuthnott, Freer and Billcliffe (1973) have noted that lipids differed in their ability to induce polymerization of 3 S toxin, diglyceride being the most effective and lyssolecithin the least.

However, as pointed out by Camejo, Colacicco and Rapport (1968), most lipids and proteins display some degree of association when brought together. There is a difficulty in connection with the view that surface activity of alpha toxin and its interaction with membrane lipids is responsible for hemolysis of sensitive erythrocytes and other biological properties attributed to the toxin. This is that it has not been possible to distinguish whether the surface activity
of toxin is specific or nonspecific.

At present it is not entirely possible to reconcile the alpha toxin's surface activity with our view that the toxin is an enzyme which degrades membrane protein when activated by membrane protease. Perhaps the interaction of toxin with membrane lipids might serve to anchor it in a particular conformation which is susceptible to the action of the activating protease.

SUMMARY

Alpha protoxin of \textit{Staphylococcus aureus} "Wood 46" was activated by trypsin which had been coupled to carboxymethylcellulose, as indicated by the toxin's ability to hydrolyse tosyl-arginine methylester (TAME). A Lineweaver-Burk plot of the degradation of TAME by toxin and trypsin showed that toxin had a greater affinity for the substrate than trypsin. N-terminal amino acid analyses of activated toxin suggested that leucine or isoleucine is the N-terminus in contrast with protoxin, the N-terminus of which is histidine.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of Canada.
REFERENCES


### TABLE I

**SUMMARY OF DATA FOR PURIFICATION OF ALPHA HEMOLYSIN**

<table>
<thead>
<tr>
<th>STEP</th>
<th>TOTAL HEMOLYSIN (HU)*</th>
<th>RECOVERY (%)</th>
<th>TOTAL PROTEIN (mg)</th>
<th>SPECIFIC ACTIVITY (HU/mg)</th>
<th>PURIFICATION (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude lysin</td>
<td>20,235,000</td>
<td>100</td>
<td>49,962</td>
<td>405</td>
<td>1</td>
</tr>
<tr>
<td>Methanol precipitate</td>
<td>11,347,200</td>
<td>56</td>
<td>6,304</td>
<td>1,800</td>
<td>4</td>
</tr>
<tr>
<td>Ammonium sulphate precip.</td>
<td>7,375,500</td>
<td>36</td>
<td>1,755</td>
<td>4,200</td>
<td>10</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>8,118,000</td>
<td>40</td>
<td>335</td>
<td>24,200</td>
<td>60</td>
</tr>
<tr>
<td>Ammonium sulphate precip.</td>
<td>8,200,000</td>
<td>41</td>
<td>185</td>
<td>44,200</td>
<td>109</td>
</tr>
<tr>
<td>CM cellulose</td>
<td>7,790,000</td>
<td>39</td>
<td>62.3</td>
<td>125,000</td>
<td>308</td>
</tr>
</tbody>
</table>

*Hemolytic units.*
TABLE II
EFFECT OF TRYPsin ON ALPHA PROTOXIN OF STAPHYLOCOCCUS AUREUS

<table>
<thead>
<tr>
<th>Test</th>
<th>Hemolytic activity (HU/ml)</th>
<th>Proteolytic activity (O.D. 520 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin control¹</td>
<td>&lt;10</td>
<td>1.64</td>
</tr>
<tr>
<td>Trypsin + STI²</td>
<td>&lt;10</td>
<td>0.06</td>
</tr>
<tr>
<td>STI control</td>
<td>&lt;10</td>
<td>0.04</td>
</tr>
<tr>
<td>Toxin control³</td>
<td>747</td>
<td>0.02</td>
</tr>
<tr>
<td>Toxin + trypsin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 s exposure</td>
<td>730</td>
<td>0.88</td>
</tr>
<tr>
<td>1 min. &quot;</td>
<td>678</td>
<td>0.88</td>
</tr>
<tr>
<td>5 min. &quot;</td>
<td>526</td>
<td>0.57</td>
</tr>
<tr>
<td>15 min. &quot;</td>
<td>170</td>
<td>0.26</td>
</tr>
<tr>
<td>30 min. &quot;</td>
<td>53</td>
<td>0.20</td>
</tr>
<tr>
<td>60 min. &quot;</td>
<td>21</td>
<td>0.21</td>
</tr>
</tbody>
</table>

¹Trypsin concentration = 200 µg/ml.

²Soybean trypsin inhibitor. Action of trypsin was stopped by addition of 200 µg/ml STI followed by immersion in ice water.

³Protoxin is by definition nonhemolytic, but becomes hemolytic in the presence of red cells because of their content of activating protease.
### TABLE III
EXPERIMENTAL DESIGN

<table>
<thead>
<tr>
<th>Additions</th>
<th>Volume (ml) of reagents added to tube numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Alpha protoxin*</td>
<td>2.0</td>
</tr>
<tr>
<td>CMC slurry**</td>
<td>0</td>
</tr>
<tr>
<td>CMC-trypsin complex***</td>
<td>0</td>
</tr>
<tr>
<td>Buffer****</td>
<td>3.0</td>
</tr>
</tbody>
</table>

*Hemolytic activity was 901 HU/ml.
**CMC concentration was 32 mg/ml dry weight.
***Trypsin activity was 22 units/mg dry weight CMC.
****Buffer was 0.01 M phosphate, pH 7.0, made 0.15 M with respect to NaCl.
### TABLE IV

EFFECT OF FILTRATION UPON RECOVERY OF TOXIN

<table>
<thead>
<tr>
<th>Samples from tube</th>
<th>Hemolytic titres (HU/ml) of Unfiltered toxin</th>
<th>Filtered toxin</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>901**</td>
<td>45</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>671</td>
<td>34</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>531</td>
<td>341</td>
<td>65</td>
</tr>
<tr>
<td>4</td>
<td>&lt;16</td>
<td>&lt;16</td>
<td>-</td>
</tr>
</tbody>
</table>

*Tube contents are given in Table I.

**Each titre is the mean of four determinations.
CAPTIONS FOR FIGURES

Figure 1. Hydrolysis of TAME by alpha toxin activated by CMC-trypsin complex.

- Tube 1 contained protoxin alone.
- Tube 2 contained supernatant of CMC-trypsin complex.
- Tube 3 contained supernatant of a mixture of protoxin and CMC.
- Tube 4 contained supernatant of a mixture of protoxin and CMC-trypsin complex.

Figure 2. TLC of dinitrophenylated N-terminal amino acids of alpha protoxin and activated toxin. DNP-amino acid spots corresponding to positions 1 and 8 are unfiltered protoxin. Position 9 is filtered activated toxin. Position 2 is histidine and 5 is methionine. Position 6 is leucine and 7 and 10 are isoleucine. Positions 3 and 4 are equivalent to the material applied to position 9 except that histidine and the unknown were removed from the glass plate and separately rechromatographed.

Figure 3. Plot of $1/v$ against $1/s$ (Lineweaver-Burk) for activated alpha toxin and trypsin. $V_{\text{max}}$ (O.D. change min.$^{-1}$ at 247 nm) is the
Figure 3. (cont'd.) reciprocal of the y-intercept and $K_m$ was calculated from an extrapolation of the two lines to the x-axis. $K_m$ is independent of enzyme concentration.
Fig. 1

Time (min.)

OD 247 nm
PRODUCTION AND PURIFICATION OF THE GAMMA HEMOLYSIN OF STAPHYLOCOCCUS AUREUS "SMITH 5R"

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Running Head: STAPHYLOCOCCAL GAMMA LYSIN

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SUMMARY

The gamma hemolysin of *S. aureus* Smith 5R was produced on Dolman-Wilson agar overlain with cellophane. Maximal yields of crude lysisin with titres of 2000-4000 hemolytic units/ml were obtained after 24 h incubation at 37°C in 10% carbon dioxide on medium adjusted to pH 7.0.

The crude lysisin was purified 2700-fold with 75% recovery by ultrafiltration, gel filtration and ammonium sulphate fractionation. The specific activity of the lysisin was calculated to be $10^5$ hemolytic units/mg protein after the dialyzed active precipitate was extracted with NaCl and reprecipitated with ammonium sulphate.

Purified gamma lysisin was shown to be homogeneous by disc electrophoresis and immunoelectrophoresis.
INTRODUCTION

The existence of gamma hemolysin was first reported by Smith & Price (1938) in their investigation of some strains of Staphylococcus aureus. These observations were confirmed by Marks (1951) who agreed that the gamma lysin was distinct from the alpha, beta and delta lysins. Elek (1959) was of the opinion that the existence of gamma lysin had not been proved since its presence was not demonstrated in his antitoxin-blood agar plate test. However, it is now known that agar inhibits activity of the gamma lysin (Mollby & Wadstrom, 1971; Guyonnet & Plommet, 1970; Jackson, 1962; Packrell, 1973).

Production and purification of the gamma lysin have been described by Plommet & Bouillanne (1966) and by Mollby & Wadstrom (1971). We have not been able to produce gamma lysin by the method of Plommet and Bouillanne and have found that the Mollby-Wadstrom procedure yields a preparation of very low titre.

The present paper presents a method of production and purification of gamma lysin which results in higher yields of material which have a high specific activity.

MATERIALS AND METHODS

The Smith 5R strain of S. aureus used for the production of gamma lysin was obtained from Dr. F. Guyonnet.
The organism was subcultured once monthly on Brain Heart Infusion agar slants incubated at 37°C for 24 h and stored at 4°C.

Growth was determined by a procedure in which cell cultures were diluted 1:20 with phosphate buffered saline, after which the optical density of the diluted suspension was determined at 650 nm in a Pye Unicam SP 800B double beam recording spectrophotometer. Viable counts were determined by the standard pour plate method on Dolman-Wilson agar (1940).

Hemolytic titrations of gamma lysin were performed with human red cells as described by Wiseman & Caird (1972). Human blood (type O Rh+) obtained from the Winnipeg Red Cross was stored in acid-citrate-dextrose solution as supplied.

Proteolytic activity of cultures in some experiments was determined as described by Wiseman & Caird (1972).

Gel filtration of gamma lysin was performed in K50 x 100 cm columns of Sephadex suspended in phosphate buffer, pH 7.0, which contained 0.5M NaCl. The column packing procedure was that described by the manufacturer, Pharmacia Canada Ltd., Montreal, Que. Volumes of 10-15 ml were collected by upward elution.

The technique of Birch-Hirschfeld (1934) was applied to the production of crude gamma lysin by the Smith 5R strain. Difco agar (1.5% w/v) was added to 500 ml quantities of Dolman-Wilson broth and the autoclaved medium was poured
into sterile stainless steel trays 1800 cm² in area. After cooling, the solidified medium was overlaid with cellophane (Dennison Mfg., Drummondville, Que., number 57001) which had been sterilized by immersion in water and exposure to steam for 1 h. The inoculum was prepared from slants of 16 h cultures to which were added 5 ml of sterile phosphate buffered saline, and the contents distributed over the surface of the cellophane with a glass spreader. The trays were covered, placed in Perspex boxes and flushed with 10% carbon dioxide in air at a rate of 20 L/min for 10 min before being incubated at 37°C for 24 h. At the end of the period, the trays were removed and the cellophane flooded with 50 ml of phosphate buffered saline. The cells were suspended with a bent glass rod, removed and centrifuged at 10,000 x g for 15 min. The supernatant fluid which contained crude gamma lysin was retained and stored at -20°C. Because of technical difficulties, some experiments required that small petri dishes rather than the large trays be used. In this case, media and volumes were reduced proportionally.

Antibodies to gamma lysin were prepared in New Zealand white rabbits as described for alpha lysin by Packrell, Wiseman & Caird (1974). Before use, antisera were purified by the method of Campbell et al. (1970). Immunodiffusion and immunoelectrophoresis were performed according to methods described by these authors.

Buffers were prepared according to Gomori (1955). Phosphate saline buffer, 0.01M, pH 7.0, was used as a general
diluent, and contained NaCl at a concentration of 0.15M. In some experiments phosphate buffer which contained other concentrations of NaCl was required.

Protein in column effluents was expressed in terms of absorbance at 280 nm. Otherwise, the micro-Kjeldahl technique described by Markham (1942) was used to assay nitrogen and the value was multiplied by 6.25 which gave an estimate of the protein content of the sample.

Polyacrylamide disc gel electrophoresis used in the characterization of gamma lysin has been described elsewhere (Fackrell et al., 1974).

RESULTS

Production of gamma lysin. As shown in Table 1, no hemolysin was produced on semi-solid or solid media, although good growth was obtained. Some lysin was produced in liquid media as indicated in the Table, presumably because of the absence of agar, but titres were low.

The Birch-Hirschfeld technique, described in Materials and Methods, was used with ten varieties of solid media. Results, shown in Table 2, indicate that the highest yields of hemolysin were obtained with Dolman-Wilson and Gladstone media. The medium of Dolman and Wilson, because of its convenience of preparation, was used for subsequent production. It will be noted in the Table that a direct correlation was obtained between growth and lysin formation.
Cells were grown for various intervals so that the incubation period which corresponded to the highest yield of lysin could be ascertained. The hemolysin samples were stored at -20°C until required and then titrated against the same sample of blood. Gamma lysin produced on Dolman-Wilson medium reached a maximum titre after 24 h incubation, at the end of the logarithmic phase of growth (Fig. 1). Maximum growth was not reached for 48 h but by this time only 15% of the total hemolytic activity demonstrable at 24 h remained.

The rapid appearance of gamma lysin during the logarithmic phase of growth suggested that it may be an extracellular product. Cells of the Smith 5R strain were pelleted by centrifugation and washed twice in phosphate buffered saline. Both the supernatant and the washed cells were incubated with 1 mg/ml Lysostaphin (Mead-Johnson Co., Evansville, Indiana) for 15 min at 37°C and the fluid was then assayed for hemolytic and proteolytic activity. Proteolysis was used as an indicator of autolysis because the Smith 5R strain appeared to produce no extracellular protease until very late in the growth cycle. Intracellular hemolysin present in the washed pellet was detected after 8 h (Fig. 2) and reached a maximum at 16 h, in contrast with the extracellular hemolysin in the supernatant which reached highest levels after 24 h as shown in Figs. 1 and 2. Although protease was present intracellularly, it did not appear in the supernatant until 40 h had elapsed, which suggests that the cell membrane was intact when gamma lysin was released into the medium.
Cells of the Smith 5R strain grew well over a pH range of 5.0-8.5 (Fig. 3) but maximum titres of lysin were produced at pH 7.0. The buffer salts used in this experiment had no effect upon hemolysin; that is, they neither enhanced nor inhibited its activity since hemolysin dialyzed against buffer components at the same pH gave similar levels of activity when titrated. In another experiment, crude lysin was dialyzed against buffers of pH 4.0-9.5 but retained all of its activity even after storage for 4-6 weeks at 4°C. However, crude lysin was rapidly destroyed by exposure to pH levels below 3.5 or above 10.0 (data not shown).

Temperature influenced both growth and hemolysin production (Fig. 4). Maximal hemolysin production and growth occurred at 37°C but a shift of 3°C from this temperature resulted in a significant decrease in the amount of hemolysin found in the supernatant. No hemolysin was detected at 25°C or 50°C although slight growth occurred at 25°C.

Petri dishes were inoculated and placed in anaerobic jars, flushed with a range of mixtures of carbon dioxide in air, then sealed and incubated at 37°C for 24 h. Carbon dioxide concentration was measured at lower levels with a "Kwik-Chek" carbon dioxide analyzer (Burrell Corp., Pittsburgh, Pa.). The production of gamma lysin showed a marked dependence upon carbon dioxide tension (Fig. 5) with maximum yields of the lysin being achieved in an atmosphere of 10% of the gas. Growth gradually decreased at higher concentrations but an increase in growth was observed repeatedly at 90% levels.
Maximum growth occurred in air alone but the greatest amounts of hemolysin were formed in 10% carbon dioxide.

The possibility was considered that dissolved carbon dioxide at higher concentrations reduced the amount of hemolysin formed by changing the pH of the medium. In another experiment, however, pH remained constant in well-buffered media over the range of carbon dioxide tensions tested (data not shown).

**Purification of gamma hemolysin.** A series of Amicon membranes (Amicon Corp., Cambridge, Mass.); UM-10, PM-30, XM-50 and XM-100 was tested for its ability to concentrate and purify gamma lysin. Ten ml of the lysin were concentrated to one ml, diluted to 10 ml and the procedure repeated three times so that all lysin capable of traversing the membranes was removed. Both the retained material and the filtered fraction were assayed for gamma lysin and protein. Results shown in Table 3 indicate that hemolysin was completely retained by UM-10, PM-30 and XM-50 membranes but passed through the XM-100 membrane. A three-fold increase in specific activity was observed with the XM-50 membrane and the hemolysin was concentrated 10-20-fold. Consequently, this membrane was used as the first step in purification of gamma lysin.

Sephadex gel filtration was then investigated as a second step in the purification procedure. In a series of preliminary experiments, gamma lysin concentrated by ultrafiltration was fractionated on Sephadex columns which contained
G-50, G-75, G-100 or G-200 gels. The best increase in specific activity with 100% recovery was obtained with Sephadex G-75, and this method was subsequently used. As shown in Fig. 6, gamma lysin (Ve/Vo = 1.45) appeared in the effluent between two major protein peaks.

Ammonium sulphate fractionation was used as a third step in the purification procedure. Pooled active fractions obtained from the Sephadex G-75 column were divided into several parts and increased amounts of ammonium sulphate were added to each. The precipitate, after standing overnight at 4°C, was centrifuged and resuspended, then dialyzed against phosphate buffered saline and titrated for hemolysin and protein content. About 95% of the hemolysin was recovered in the precipitate of the 60% ammonium sulphate saturation (Table 4) fraction with a resulting increase in specific activity to 8431 hemolytic units/mg. Higher concentrations of ammonium sulphate caused precipitation of contaminating proteins. Subsequent experiments (not shown) demonstrated that no gamma lysin was precipitated by 25% ammonium sulphate. In summary, 25% ammonium sulphate was added to hemolysin eluted from a Sephadex G-75 column and this precipitate was discarded. More of the salt was added to give a final concentration of 60% and this second precipitate which contained the lysin was collected.

Gamma lysin fractionated by ammonium sulphate could be dissolved by dialysis against phosphate buffered saline. Subsequent dialysis of the dissolved lysin against distilled
water caused the formation of a white precipitate and loss of hemolytic activity. If dissolved lysin which contained protein, phosphate and pentose was treated with ribonuclease and then dialyzed against distilled water, the amount of precipitate was much less and contained only protein. This finding suggested that gamma lysin was coprecipitated with nucleic acids. Since nucleic acids are irreversibly denatured by dialysis against distilled water, an attempt was made to dissolve selectively the precipitated gamma lysin with NaCl.

After fractionation with ammonium sulphate, gamma lysin was dialyzed against distilled water for three days and various concentrations of NaCl in 0.01M phosphate buffer were added to suspensions of the precipitate. The treated suspensions were centrifuged to separate the insoluble material from the supernatant fluids. This insoluble material or pellet was then washed with 2.0M NaCl. Finally, both pellet and supernatant were assayed for hemolysin and protein. Results are summarized in Table 5 and show that all hemolytic activity is present in the precipitate when it is suspended in distilled water, but that the addition of phosphate buffer causes some of the hemolytic activity to appear in the supernatant fluid. As the NaCl concentration is increased, more hemolysin is dissolved and less activity remains in the precipitate. Although all hemolytic activity is recovered by the addition of 1.0M NaCl, 45% of the protein is still insoluble. This NaCl-extracted hemolysin contained protein but no phosphate or pentose.
Gamma hemolysin became unstable after NaCl extraction, all activity being lost after storage for 24 h at 4°C. If activity were to be retained, the hemolysin had to be preserved as a precipitate in ammonium sulphate solution. As a final step in the purification procedure, hemolysin was fractionated a second time with ammonium sulphate. The precipitate which occurred after addition of 25% of the salt was discarded and the activity collected by further addition of ammonium sulphate to 60% saturation. The active material was washed once and stored in saturated ammonium sulphate solution. This last procedure increased the specific activity about 10-fold.

Table 6 summarizes the procedure developed for the purification of gamma lysin. The lysin was purified 2736-fold with 74% recovery. Specific activity of the final product was \(10^5\) hemolytic units/mg protein.

Criteria of purity. Purified gamma lysin showed a single peak on polyacrylamide disc gel electrophoresis when 100 \(\mu\)g dry weight of material was applied to the column, as shown in Fig. 7. Immunoelectrophoresis of the lysin showed what appeared to be a single line of precipitation when the lysin was diffused against antibody to crude material (Fig. 8).

Sedimentation velocity analyses of gamma lysin, electrofocusing and determination of the N-terminal amino acid have also been used as criteria of homogeneity and are described in a companion paper (Fackrell & Wiseman, 1974).
DISCUSSION

This study has shown that growth and production of gamma hemolysin by the Smith 5R strain of *S. aureus* are not affected to the same degree by changes in pH or carbon dioxide tension. These findings resemble those obtained with the alpha, beta and delta lysins except that delta lysin production depends less upon the presence of carbon dioxide (Wiseman, 1970). It has not been possible to replace the 5R strain's carbon dioxide requirement with incubation of the cultures at acidic levels of pH, which indicates that reduced pH is not an explanation of the carbon dioxide requirement.

Our work has shown that gamma lysin is formed by the Smith 5R strain in the late log phase of growth and released into the medium. Although some lysin was found intracellularly, the bulk of it was in an extracellular location at a time when cell autolysis was not important. This raises the question of the function of the hemolysin in the metabolism of the bacteria which produce it.

The method of purification presented in this investigation has yielded gamma lysin purified 2700-fold with 75% recovery. The specific activity of $10^5$ hemolytic units/mg protein is about 100 times greater than that of the preparation of Guyonnet & Plommet (1970). These authors obtained evidence for two synergistic fractions of gamma lysin, each of which contained at least two other proteins. We were unable to
confirm their findings with the same Smith 5R strain. It may be that their two fractions were contaminated with beta and delta lysins which are known to act synergistically (Elek, 1959). Mollby & Wadstrom (1971) devised a method of purification of the gamma lysin based on ion exchange chromatography and isoelectric focusing. However, there is little or no evidence that their preparation was homogeneous.

ACKNOWLEDGMENT

This work was supported by the University of Manitoba and the Medical Research Council.
REFERENCES


Table 1. Growth and hemolysin production on liquid, semi-solid and solid media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth (O.D. 650 nm)</th>
<th>Hemolysin Titre (HU/ml)**</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liquid Medium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dolman-Wilson</td>
<td>0.51</td>
<td>78</td>
</tr>
<tr>
<td>Gladstone’s</td>
<td>0.11</td>
<td>0</td>
</tr>
<tr>
<td>Stolp &amp; Petzold</td>
<td>0.24</td>
<td>13</td>
</tr>
<tr>
<td>Brain Heart Infusion</td>
<td>0.48</td>
<td>23</td>
</tr>
<tr>
<td><strong>Semi-Solid Medium</strong>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dolman-Wilson</td>
<td>0.55</td>
<td>0</td>
</tr>
<tr>
<td>Gladstone’s</td>
<td>0.35</td>
<td>0</td>
</tr>
<tr>
<td>Stolp &amp; Petzold</td>
<td>0.30</td>
<td>0</td>
</tr>
<tr>
<td>Brain Heart Infusion</td>
<td>0.45</td>
<td>0</td>
</tr>
<tr>
<td><strong>Solid Medium</strong>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dolman-Wilson</td>
<td>0.63</td>
<td>0</td>
</tr>
<tr>
<td>Gladstone’s</td>
<td>0.59</td>
<td>0</td>
</tr>
<tr>
<td>Stolp &amp; Petzold</td>
<td>0.52</td>
<td>0</td>
</tr>
<tr>
<td>Brain Heart Infusion</td>
<td>0.40</td>
<td>0</td>
</tr>
</tbody>
</table>

*Media subjected to freezing and thawing.  
**Hemolytic Units/ml.
Table 2. Gamma hemolysin production on different media*

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth (O.D. 650 nm)</th>
<th>Hemolysin Titre (HU/ml±SX)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dolman-Wilson</td>
<td>0.810</td>
<td>453±21</td>
</tr>
<tr>
<td>Gladstone's</td>
<td>0.850</td>
<td>441±27</td>
</tr>
<tr>
<td>Brain Heart Infusion</td>
<td>0.650</td>
<td>263±14</td>
</tr>
<tr>
<td>Veal Infusion</td>
<td>0.710</td>
<td>205±20</td>
</tr>
<tr>
<td>Liver Infusion</td>
<td>0.790</td>
<td>152±10</td>
</tr>
<tr>
<td>Stolp &amp; Petzold</td>
<td>0.516</td>
<td>136±9</td>
</tr>
<tr>
<td>Blood Agar</td>
<td>0.610</td>
<td>86±6</td>
</tr>
<tr>
<td>Trypticase Soy</td>
<td>0.492</td>
<td>30±2</td>
</tr>
<tr>
<td>Nutrient Agar</td>
<td>0.320</td>
<td>15±1</td>
</tr>
<tr>
<td>Casamino Acids</td>
<td>0.116</td>
<td>6±1</td>
</tr>
</tbody>
</table>

*Overlaid with cellophane.
**Hemolytic Units/ml.
### Table 3. Ultrafiltration of gamma hemolysin

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Total Hemolysin (HU)*</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (HU/mg)</th>
<th>Purification (fold)</th>
</tr>
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<tr>
<td>None</td>
<td>125,000</td>
<td>90</td>
<td>1,400</td>
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<tr>
<td>UM-10</td>
<td>125,000</td>
<td>64</td>
<td>2,000</td>
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<tr>
<td>passed</td>
<td>0</td>
<td>15</td>
<td>-</td>
<td>-</td>
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<td>PM-30</td>
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<td>62</td>
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<td>-</td>
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<td>XM-50</td>
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<td>XM-100</td>
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<td>passed</td>
<td>90,000</td>
<td>66</td>
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*Hemolytic Units.
Table 4. Ammonium sulphate fractionation of gamma hemolysin

<table>
<thead>
<tr>
<th>Ammonium sulphate (% saturation)*</th>
<th>Precipitate</th>
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<tr>
<td></td>
<td>Total</td>
<td>Total</td>
<td>Specific</td>
<td>Purification</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemolysin (HU)</td>
<td>Protein (mg)</td>
<td>Activity (HU/mg)</td>
<td>(fold)</td>
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<tr>
<td>15</td>
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<td>0.205</td>
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<tr>
<td>30</td>
<td>2,200</td>
<td>0.325</td>
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<td>45</td>
<td>3,500</td>
<td>0.480</td>
<td>7,291</td>
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<td>60</td>
<td>4,300</td>
<td>0.510</td>
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<td>75</td>
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<td>90</td>
<td>4,300</td>
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<td>100</td>
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<td>2.010</td>
<td>2,238</td>
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Column effluent from Sephadex G-75
4,800 2.220 2,162 1.0

*Column effluent was divided into seven parts and each received the amount of ammonium sulphate indicated in the Table.
Table 5. Dialysis and salt extraction of gamma hemolysin

<table>
<thead>
<tr>
<th>NaCl (M)</th>
<th>Supernatant</th>
<th>Precipitate</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Hemolysin (HU/ml)</td>
<td>Protein (mg/ml)</td>
<td>Hemolysin (HU/ml)</td>
<td>Protein (mg/ml)</td>
</tr>
<tr>
<td>0 (distilled water)</td>
<td>0</td>
<td>0.045</td>
<td>1,300</td>
<td>0.305</td>
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<tr>
<td>0 (phosphate buffer, 0.01M)</td>
<td>270</td>
<td>0.104</td>
<td>990</td>
<td>0.245</td>
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<tr>
<td>0.01</td>
<td>320</td>
<td>0.130</td>
<td>880</td>
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<tr>
<td>0.03</td>
<td>470</td>
<td>0.135</td>
<td>795</td>
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<tr>
<td>0.06</td>
<td>520</td>
<td>0.140</td>
<td>630</td>
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<tr>
<td>0.10</td>
<td>640</td>
<td>0.150</td>
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<tr>
<td>0.30</td>
<td>970</td>
<td>0.154</td>
<td>275</td>
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<td>1,060</td>
<td>0.160</td>
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<tr>
<td>0.60</td>
<td>1,240</td>
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<td>1.00</td>
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<tr>
<td>2.00</td>
<td>1,300</td>
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Table 6. Purification of gamma hemolysin: Summary of data

<table>
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<tr>
<th>Purification Step</th>
<th>Total Hemolysin (HU)</th>
<th>Total Protein (mg)</th>
<th>Recovery %</th>
<th>Specific Activity (HU/mg)</th>
<th>Purification (fold)</th>
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<tr>
<td>Crude lysin</td>
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<td>3,844.0</td>
<td>100</td>
<td>40</td>
<td>1.0</td>
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<td>Ultrafiltration</td>
<td>148,750</td>
<td>1,214.0</td>
<td>98</td>
<td>123</td>
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<tr>
<td>Gel Filtration</td>
<td>128,000</td>
<td>198.9</td>
<td>84</td>
<td>643</td>
<td>16.3</td>
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<tr>
<td>(NH₄)₂SO₄ Fractionation</td>
<td>107,250</td>
<td>41.7</td>
<td>70</td>
<td>2,572</td>
<td>64.0</td>
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<tr>
<td>Dialysis</td>
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<td>15.8</td>
<td>76</td>
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<tr>
<td>NaCl Extraction</td>
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<td>9.8</td>
<td>76</td>
<td>11,737</td>
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<tr>
<td>(NH₄)₂SO₄ Fractionation</td>
<td>112,450</td>
<td>1.1</td>
<td>74</td>
<td>108,125</td>
<td>2,736.0</td>
</tr>
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</table>
CAPTIONS TO FIGURES

Figure 1  Effect of incubation time on production of gamma lysin by the Smith 5R strain. Hemolysin titres •••••; growth o o o.

Figure 2  Production of intracellular and extracellular gamma lysin by the Smith 5R strain. (A) intracellular lysin o o o; extracellular lysin ••••. (B) intracellular protease o o o; extracellular protease ••••; viable cell count x x x.

Figure 3  The effect of pH on production of gamma lysin by the Smith 5R strain. Hemolysin titres •••••; growth o o o. pH 4-6, succinate buffer; pH 6-8 phosphate buffer; pH 8-9 Tris buffer.

Figure 4  The effect of temperature upon gamma lysin production by the Smith 5R strain. Hemolysin titres •••••; growth o o o.

Figure 5  The effect of carbon dioxide concentration upon gamma lysin production by the Smith 5R strain. Hemolysin titres •••••; growth o o o.

Figure 6  Gel filtration of Smith 5R gamma lysin on Sephadex G-75. Hemolysin titres (_ _ _ _); Absorbance (_ _ _ _).

Figure 7  Densitometer tracing of crude and purified gamma lysin subjected to disc electrophoresis. The direction of migration is from left to right.

Figure 8  Immunoelectrophoresis of purified gamma lysin. Purified lysin is contained in the well, anti-gamma IgG in the trough.
Fig. 1
GAMMA LYSIN

EXTRACELLULAR

INTRACELLULAR

HEMOLYSIS (HU/ml)

0

500

1,000

0

24

48

TIME (hr)

Pig. 2

PROTEASE

VIABLE CELLS

0

0.2

0.4

0.6

0.8

1.0

0

24

48

72

96

TIME (hr)

INTRACELLULAR

EXTRACELLULAR

PROTEASE (O.D. 520nm)

Viable Cells (log)

Fig. 2
Fig. 3
Fig. 5
HEMOLYSIN (10^3 HU/ml) ———

Sephadex G-75

GAMMA LYSIN

FRACIION NUMBER (15 ml/tube)

ABSORBANCE (280 nm)

Fig. 6
Fig. 7
PROPERTIES OF THE GAMMA HEMOLYSIN OF 
STAPHYLOCOCCUS AUREUS "SMITH 5R"

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University of Manitoba, Winnipeg, Canada

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Introduction

The gamma hemolysin of Staphylococcus aureus, first discovered by Smith & Price (1938) has usually been confused with delta lysin (Elek, 1959) and consequently it has been poorly characterized until quite recently (Guyonnet & Plommet, 1970; Mollby & Wadstrom, 1971).

In the present study, the chemical and biological properties of purified gamma lysin have been compared with those of purified alpha, beta and delta lysins.

Materials and Methods

Production and purification of gamma lysin of the Smith 5R strain of S. aureus have been described in a companion paper by Fackrell & Wiseman (1974a); beta lysin by a modification of the method of Wiseman & Caird (1967) in which a column of Sephadex G-75 was equilibrated with the buffer of Hallander (1963). Hemolysin in the column effluent was pooled, concentrated and lyophilized. Delta lysin was purified according to the method developed by Caird & Wiseman (1970), and alpha lysin by the method given by Wiseman, Caird & Fackrell (1974).

Hemolysin titrations were performed as described by Wiseman and Caird (1972). Sheep blood was used in the titration of beta lysin, human blood with delta and gamma lysins and rabbit blood was employed with alpha lysin. All lysin titrations were supplemented with 0.001 M Mg++ as described elsewhere (Wiseman & Caird, 1967). The activity of beta hemolysin is enhanced in the presence of
Mg$^{++}$ ions, but they have no effect on alpha, gamma and delta lysins. Blood from other animal species was obtained from animals kept in the department or from National Biological Laboratories, Winnipeg.

Antibodies to the hemolysins were prepared in rabbits as described by Fackrell & Wiseman (1974b). Immunodiffusion, immunoelectrophoresis and quantitative precipitin tests were performed according to the methods of Campbell et al. (1970). The antibodies were purified according to a method described by these authors.

Cells of the C-6 line (Ida, Sairenji & Hinuma, 1972) were obtained from Dr. A. Wallbank of this department. They were grown in stationary cultures at 37°C in McCoy's 5A medium (Grand Island Biological Corp., New York, N.Y.) supplemented with 10% foetal calf serum and harvested at a concentration of $10^7$ cells/ml. The cells were washed with the McCoy medium which contained 2.5% calf serum and resuspended to the original volume in this medium.

Amino acid analysis was determined with the Auto-technicon amino acid analyzer and was performed by Dr. F. Labella and Dr. F. Stevens of this University.

The technique of Fraenkel-Conrat, Harris & Levy (1955) was applied to the determination of N-terminal amino acids with the following modification. Dinitrophenyl amino acids were chromatographed on glass plates coated with Silica Gel G (Merck and Co., Darmstadt) prepared as described by Stahl (1965). Water-soluble and ether-soluble
extracts were subjected to ascending chromatography in several solvent systems; n-propanol/ammonium hydroxide (7:3), chloroform/benzyl alcohol/acetic acid (70:30:3), chloroform/methanol/acetic acid (95:5:1), benzene/pyridine/acetic acid (80:20:2) and chloroform/t-amyl alcohol/acetic acid (70:30:3). These solvent systems have been recommended for separation of amino acids by Pataki (1968).

Acid-soluble phosphorus was determined by the method of Fiske & Subbarow as described by Leloir & Cardini (1955). Total phosphorus was based on a method described by the latter authors.

Additional techniques used in this investigation were nitrogen estimation by the Micro-Kjeldahl technique (Markham, 1942), polyacrylamide disc gel electrophoresis, electrofocusing and sedimentation velocity analysis, all of which are described in the paper by Fackrell et al. (1974).

Molecular weights obtained from Sephadex columns were determined by the method of Andrews (1964).

**Results**

Disc gel electrophoresis of purified gamma lysin along with purified samples of alpha, beta and delta lysins has shown that it cannot be distinguished by this method from the alpha lysin (Fig. 1), both of which have migrated toward the cathode. Beta lysin is displaced to the right of the alpha and gamma lysins while delta lysin has moved very little toward the cathode under the conditions of
the experiment.

A single peak \( S_{20w} = 2.6 \) was obtained in the ultracentrifuge for gamma lysin (Fig. 2a). Sedimentation coefficients of alpha and beta lysins were 1.4 S and 1.8 S respectively (Figs. 2b, c). If the alpha lysin was allowed to stand for several days at 4°C, the \( S_{20w} \) became 3.0 as reported elsewhere (Packrell et al., 1974).

The 1% extinction coefficients were calculated for the gamma lysin from the slope of the absorbance plot at 280 nm versus concentration (gm %), as shown in Fig. 3. The \( E_{280}^{1cm} \) for gamma lysin was calculated to be 28.35 compared to 29.08 for delta. The \( E_{280}^{1cm} \) for alpha lysin was 13.56 while that for beta lysin was 4.24.

Isoelectric focusing of the four hemolysins is shown in Fig. 4. The pI of beta and delta lysins are 9.5 and 9.6 respectively. The pI of alpha lysin is 8.5 while that for gamma lysin is 6.0. Only single peaks of protein were observed to correspond with the hemolytic activity of the purified preparations in each case. The molecular weight of the gamma lysin based on gel filtration was 45,000 daltons, indistinguishable from that of the alpha lysin (Fig. 5). The molecular weights of the beta and delta lysins by gel filtration were quite different. That for beta lysin was 26,000 daltons, half the value for alpha and gamma lysins while the molecular weight of delta lysin was beyond the resolution of the Sephadex column, probably of the order of 200,000 or greater.
An amino acid analysis of the gamma lysin is shown in Table 1. The data are characterized by a large number of aspartic and glutamic acid residues as well as a high glycine content. In this respect the gamma lysin is not different from the alpha, beta or delta lysins shown in the Table for comparison. Cysteine and methionine are absent or present in small amounts in the gamma lysin residues. The N-terminal amino acid of gamma lysin shown in Fig. 6 is methionine which as noted is present only in small quantities. Results shown in the Figure also confirm that histidine is the N-terminus of alpha lysin while proline is the N-terminus of delta lysin. No data are presently available for beta lysin.

Several immunological experiments were performed which serve to distinguish gamma lysin from the other hemolysins. The first of these is quantitative precipitation. As shown in Fig. 7a, gamma lysin was precipitated by its purified antibody with the formation of a characteristic precipitin curve. The other hemolysins were precipitated by their respective antisera and did not precipitate with anti-gamma lysin (Figs. 7b, c and d). Ouchterlony immunodiffusion tests of the gamma lysin (Fig. 8a,b,c,d) indicate that the lysin formed a single precipitin line with its homologous purified antibody as did beta, alpha and delta lysins with their antibodies, and that no cross-reactions were observed. These immunodiffusion experiments were repeated in agarose which contained 1% (v/v) washed erythrocytes. Purified hemolysins were diffused against
their respective purified antibodies for 2 hr. at 37°C after which the plates were cooled to 0°C and rapidly dried with blotting paper. In Fig. 9, it is shown that specific antibodies to the hemolysins neutralize their hemolytic activity and that no precipitin line is observed which does not correspond to the edge of the zone of hemolysis.

The effect of purified gamma lysin on mammalian cells of various types was investigated. Red cells obtained from eleven species of animals were used in the titration of the four hemolysins. As shown in Table 2, some differences in the hemolytic spectra of the hemolysins were noted. Rabbit erythrocytes were most sensitive to the alpha and gamma lysins in contrast with beta and delta lysins which preferentially lysed sheep and human cells respectively. Monkey and dog cells were most resistant to beta lysin and it is worth noting that pigeon cells were refractory to alpha, gamma and delta lysins.

Human leucocytes were incubated at 37°C with the gamma lysin. In Fig. 10 it is observed that the addition of 10 μg/ml gamma lysin to the leucocytes caused a relatively linear decrease in absorbance of the cells up to 10 min. Beyond that time, no further decrease was observed. Alpha lysin included for comparison showed a nearly linear reduction in absorbance over the 30 min. time period. The effect of gamma lysin on C-6 cells was also studied. Gamma lysin (50 μg) was dissolved in 0.5 ml medium and added to 4.5 ml cell suspension. At various times, 0.5 ml
of the suspension was removed and mixed with 0.2 ml trypan blue. At least 700 cells were counted and their ability to exclude the dye was recorded (Fig. 11). About 93% of the control cells excluded the dye but of the lysin-treated cells observed, only 15% excluded the dye after 4 hr. incubation. As shown in the Figure, the use of lower concentrations of hemolysin decreased the rate at which the C-6 cells were affected. At 10 μg concentration, the rate of killing appeared to be linear beyond 60 min.

The effect of gamma lysin upon mice, rabbits and guinea pigs was investigated. Ten mice per dilution were used and it was found that over the range of 0-100 μg, gamma, beta and delta lysins had no visible effect on the mice. As expected, the alpha lysin was lethal for mice; the LD$_{50}$ obtained by probit analysis was 0.68±0.19 μg or 27-34 μg/kg mouse tissue. In contrast with the negative results obtained for mice, gamma lysin killed guinea pigs instantly if they were injected intracardially with 50 μg amounts. The same result was obtained with alpha lysin but beta and delta lysins had no effect by this route of injection. Autopsy findings obtained for us by Dr. H. Sayed of this department were that guinea pigs which received gamma lysin intracardially showed massive haemorrhage of the kidney and serosal surfaces of the intestines and frank lysis of red cells in the major veins and arteries.

Subcutaneous injection of greater than one μg of alpha lysin caused dermonecrosis in rabbits and guinea pigs but 100 μg quantities of beta, gamma or delta lysins had no effect.
Discussion

A comparison of the physiochemical properties of gamma lysin in relation to the alpha, beta and delta lysins is shown in Table 3. Gamma lysin is in several respects similar to alpha lysin. For example, the molecular weights determined on Sephadex G-75 are identical. The two lysins also could not be separated on the basis of their behaviour when subjected to disc electrophoresis (Fig. 1). Other points worth noting in Table 3 are the low $E_{280}^{1cm}$ of beta lysin compared to other lysins and the similarity of its pI to that of the delta lysin. The sedimentation coefficient determined in our laboratory for beta lysin is close to the value obtained by Gow & Robinson (1969). The Table also indicates that the pI of gamma lysin is 6.0, which is not in agreement with the 9.5 value obtained by Mollby & Wadstrom (1971). However, the pI of alpha lysin (8.5) agrees well with that given for alpha-I by McNiven, Owen & Arbuthnott (1972) and for alpha-a published by Wadstrom (1968).

Amino acid analysis of the gamma lysin showed that it contained a large number of aspartic and glutamic acid residues, large amounts of ammonia and that cysteine was absent or not detected. The alpha, beta and delta lysins also contained high levels of aspartic and glutamic acids and ammonia. These lysins are basic proteins in contrast with the gamma lysin which appears to be acidic, at least
on the basis of its pI value of 6.0. From the amino acid analyses, one would also expect the gamma lysin to be basic, but presumably acidic or basic properties will in this case depend upon the presence or absence of some critical number of asparagine or glutamine residues. It is assumed that the high ammonia content is traced to the presence of aspartic and glutamic acids as asparagine and glutamine. Unfortunately, amino acid analyses of the beta and gamma lysins have not to our knowledge been published elsewhere and consequently it is not possible to make comparisons. Our amino acid analysis of the delta lysin, however, resembles those of several other workers (Heatley, 1971; Kantor, Temples & Shaw, 1972; Kreger et al., 1971). That is, arginine, proline, tyrosine and cysteine are absent or present in low concentration, while lysine, isoleucine and aspartic acids are present in all preparations in high concentration. Yoshida (1963) also presented an analysis of the delta lysin which in fact is similar to these analyses but his preparation was subsequently shown to be contaminated with other proteins (Gladstone & Yoshida, 1967). The amino acid analysis of alpha lysin compares reasonably well with those of Coulter (1966), Bernheimer & Schwartz (1963) and Six & Harshman (1973). However, the glycine content of our preparation is somewhat higher.

Molecular weight of beta lysin calculated from its behaviour on Sephadex was 26,000 daltons, somewhat higher than the value of 15,500 obtained by Chesbro & Kucic (1971) for the UNH-Donita strain. However, the minimal molecular
weight calculated from the amino acid analysis is 16,110 and gives better agreement. The molecular weight of beta lysin given by Wadstrom & Mollby is about twice the value obtained in our laboratory on Sephadex.

We have shown in this study that the gamma lysin is immunologically distinct from the alpha, beta and delta lysins. It is now abundantly clear from our own work and that of others (Guyonnet & Plommet, 1970; Mollby & Wadstrom, 1971) that Elek (1959) was incorrect in assuming that gamma and delta lysins were identical.

Generally speaking it has not been possible in the past to compare the hemolytic spectra of the four hemolysins in view of the fact that one seldom possessed highly purified preparations or had them together at the same time. It has been possible in the present study to make this comparison but the data must be interpreted with caution, since red cells are notoriously variable in their sensitivity to a given lysin. Bernheimer (1964) for example has reported that the difference in sensitivity of rabbit and human erythrocytes to alpha lysin may vary 15-150-fold.

With regard to toxicity studies, Wadstrom & Mollby (1972) reported that a dose of less than a milligram of partially purified gamma lysin is lethal for mice and rabbits in contrast with our own data. They also found that the LD₅₀ dose of delta lysin for mice was 125 mg although such a large LD₅₀ dose is meaningless except to suggest that the lysin is relatively nontoxic for mice. Calculations based on the data of Kreger et al. give an LD₅₀ dose of 2 mg delta
lysin for mice and 7.2 mg for guinea pigs. Contamination of their preparations with alpha lysin at a concentration of one part in 2000 or less would account for the findings (see Fackrell & Wiseman, 1974b).

**Summary**

Purified gamma hemolysin of *Staphylococcus aureus* was characterized in relation to the alpha, beta and delta hemolysins. The sedimentation coefficient of the gamma lysin was calculated to be 2.65, somewhat higher than the S20w values of 1.4 for freshly purified alpha lysin and 1.8 for the beta lysin. The molecular weight of gamma lysin determined by gel filtration was 45,000 daltons. The isoelectric point (pI) of gamma lysin was 6.0 while the pI of the alpha, beta and delta lysins ranged from 8.5-9.6.

The amino acid analysis of gamma lysin was characterized by low levels of methionine and histidine. Methionine was, however, the N-terminus which, in view of the small concentration of methionine present, suggested that all of the amino acid might be involved in the N-terminal group.

The gamma lysin was immunologically distinct from the alpha, beta and delta lysins on the basis of quantitative precipitin tests and in Ouchterlony agar gel diffusion tests, single lines of precipitation were observed which showed no evidence of cross-reactions amongst the four hemolysins.

Gamma, beta and delta lysins had no effect in mice when injected with increasing doses ranging from 0-100 μg.
The alpha lysin killed mice, the LD_{50} dose being 0.68±0.12 µg or 27-34 µg/kg mouse tissue. Gamma lysin was, however, lethal for guinea pigs when 50 µg quantities were injected intracardially. Gamma lysin also lysed human leucocytes and destroyed C6 (human lymphoblast) cells.

Acknowledgments

This study was supported by the University of Manitoba and the Medical Research Council of Canada.


TABLE 1

STAPHYLOCOCCAL HEMOLYSINS

AMINO ACID COMPOSITION*

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<thead>
<tr>
<th>AMINO ACID</th>
<th>ALPHA</th>
<th>BETA</th>
<th>GAMMA</th>
<th>DELTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>High</td>
<td>441.6</td>
<td>141.4</td>
<td>218.5</td>
</tr>
<tr>
<td>Aspartic Acid</td>
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<td>135.1</td>
<td>142.0</td>
<td>164.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>56.9</td>
<td>53.8</td>
<td>68.0</td>
<td>119.9</td>
</tr>
<tr>
<td>Serine</td>
<td>65.1</td>
<td>98.7</td>
<td>77.4</td>
<td>96.3</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>93.0</td>
<td>116.9</td>
<td>102.4</td>
<td>66.4</td>
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<tr>
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<td>81.2</td>
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<td>50.2</td>
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<tr>
<td>Glycine</td>
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<td>125.0</td>
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<td>Alanine</td>
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<td>78.2</td>
<td>63.2</td>
<td>75.2</td>
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<td>Valine</td>
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<td>39.6</td>
<td>38.6</td>
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*Residues/1,000 amino acid residues.
**TABLE 2**

**HEMOLYTIC ACTIVITY* OF STAPHYLOCOCCAL LYSINS**

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>ALPHA</th>
<th>BETA</th>
<th>GAMMA</th>
<th>DELTA</th>
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<tbody>
<tr>
<td>Rabbit</td>
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<td>81,300</td>
<td>113,210</td>
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<td>&lt;750</td>
<td>29,764</td>
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<td>2,497</td>
<td>4,960</td>
<td>44,041</td>
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<td>4,760</td>
<td>6,445</td>
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<td>758,830</td>
<td>89,906</td>
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*HU/mg protein
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<th>BETA</th>
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<th>DELTA</th>
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<tr>
<td>Sedimentation constant, $S_{20w}$</td>
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<td>1.8</td>
<td>2.6</td>
<td>2.8, 9.8*</td>
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<td>26,000</td>
<td>45,000</td>
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<td>Isoelectric point</td>
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<td>9.6</td>
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<td>N-terminal amino acid</td>
<td>histidine</td>
<td>***</td>
<td>methionine</td>
<td>proline</td>
</tr>
</tbody>
</table>

**Estimated.
***Not determined.
Figure 1. Densitometer scan of purified hemolysins subjected to disc electrophoresis. The direction of migration is from left to right.

Figure 2. Schlieren patterns obtained in the ultracentrifuge for alpha, beta and gamma hemolysins. (a) gamma lysin, (b) alpha lysin, (c) beta lysin.

Figure 3. Extinction coefficients (1%) of the purified hemolysins. The lines were determined by regression analysis.

Figure 4. Electrophoresing of purified hemolysins. The solid line represents hemolytic activity, the broken line absorbance and the dotted line, the pH gradient. (a) gamma lysin, (b) delta lysin, (c) alpha lysin, (d) beta lysin.

Figure 5. Molecular weight determination of purified hemolysins on a column of Sephadex G-75. Kav is the partition coefficient.

Figure 6. Thin layer chromatography of dinitrophenyl amino acid N-termini of the purified hemolysins. The solvent system in this case was benzene/pyridine/glacial acetic acid (80:20:2). The dotted line is the solvent front. Position 1 is histidine; 2, the N-terminus of alpha lysin and 3, that of gamma lysin. Position 4 is methionine; 5, the N-terminus of delta lysin and 6, proline.

Figure 7. Quantitative precipitation of purified hemolysins by homologous purified antibody. (a) precipitation of gamma lysin by antibody to gamma and delta lysins, (b) precipitation of delta lysin by antibody to gamma and delta lysins, (c) precipitation of alpha lysin by antibody to alpha and gamma lysins, (d) precipitation of beta lysin by antibody to beta and gamma lysins.

Figure 8. Ouchterlony double diffusion of purified hemolysins against homologous purified antibodies. The centre wells contain antibody and the peripheral wells contain the lysins. Well 1 contains alpha lysin; well 2, beta lysin; well 3 contains gamma lysin and well 4, delta lysin. (a) Centre well contains anti-alpha and anti-gamma lysins, (b) anti-beta and anti-gamma lysins occupy the centre well, (c) centre well contains anti-gamma and anti-delta lysins, (d) the centre well contains only anti-gamma lysin.
Figure 9. Double diffusion of purified hemolysins in blood agarose. Centre wells contain purified hemolysin, the lower peripheral well contains purified antibody. The light area which surrounds the centre well indicates hemolysis. Wells not designated are empty. (a) alpha lysin, (b) beta lysin: Note the typical hemolytic pattern, (c) gamma lysin: Hemolytic activity is not inhibited by agarose, (d) delta lysin.

Figure 10. Lysis of human leucocytes by gamma and alpha lysins.

Figure 11. Effect of gamma lysin on the exclusion of trypan blue by C-6 cells.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 4
GEL FILTRATION
SEPHADEX G-75

- Cytochrome C
- Myoglobin
- Trypsin
- Pepsin
- Beta Lysin
- Alpha, Gamma Lysin
- Hemoglobin
- Bovine Albumin
- IgG

Fig. 5
Fig. 7

(a) + ANTI-BETA LYSIN

(b) + ANTI-GAMMA LYSIN

(c) + ANTI-ALPHA LYSIN

PRECIPITATE (10^2 µgN)

ALPHA LYSIN (µgN)

BETA LYSIN (µgN)
Fig. 9
Fig. 10

CONTROL

+ ALPHA LYSIN

+ GAMMA LYSIN

TIME (min)

ABSORBANCE (650nm)
Fig. 11

The graph shows the trypan blue dye exclusion (%) over time (min) for different concentrations of a substance.

- **0 µg**
- **1.25 µg**
- **2.5 µg**
- **5 µg**
- **10 µg**

The time range is from 0 to 240 minutes, with key points at 60, 120, and 240 minutes.
MODE OF ACTION OF THE GAMMA HEMOLYSIN OF
STAPHYLOCOCCUS AUREUS "SMITH 5R"

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Introduction

The gamma hemolysin of Staphylococcus aureus has recently been distinguished from the alpha, beta and delta lysins and characterized (Guyonnet et al., 1968; Mollby & Wadstrom, 1971; Fackrell & Wiseman, 1974a,b). However, nothing is known of its mode of action.

The present investigation is a study of the reaction of gamma lysin with its substrate in the erythrocyte membrane.

Materials and Methods

Methods of production and purification of gamma lysin of the Smith 5R strain of S. aureus have been described in a companion paper by Fackrell & Wiseman (1974a). The characterization of gamma lysin and its differentiation from alpha, beta and delta lysins have been the subject of comment in another paper by Fackrell & Wiseman (1974b).

Human erythrocyte membranes were prepared by the method of Dodge, Mitchell & Hanahan (1963). The method of Rose & Oklander (1965) was used in the extraction of phospholipids from erythrocytes. Lipid-free membrane protein was prepared from red cells as described by Rosenberg & Guidotti (1968).

Total carbohydrate was estimated by the anthrone method as described by Rabat & Meyer (1961). Methods cited by these authors were also applied to the determination of pentose, deoxypentose, reducing sugar, hexosamine and sialic acid. Phosphorus was assayed by the Fiske-Subbarow method.
as described by Leloir & Cardini (1955). Nitrogen was estimated by the micro-Kjeldahl technique given by Markham (1942), while protein was assayed by Bailey's (1967) modification of the Lowry protein reaction.

Hemolytic activity of gamma lysin was assayed by the method of Wiseman & Caird (1972). Thin layer chromatography (TLC) was performed as described by Fackrell (1973) for detection of phospholipids which were identified by ascending chromatography on glass plates coated with Silica Gel G (Merck & Co., Darmstadt). Development was effected in Marinetti's (1962) solvent and the phospholipid spots were visualized with rhodamine 6G or ammonium molybdate sprays (Ansell & Hawthorne, 1964).

N-terminal amino acid analysis was performed by the Fraenkel-Conrat method as given by Wiseman, Caird & Fackrell (1974).

Pronase was obtained from Calbiochem, Los Angeles, Calif. Purified phospholipids were purchased from General Biochemicals, Chagrin Falls, Ohio. Human erythrocytes (Rh +, group 0) were supplied by the Canadian Red Cross in Winnipeg. Phospholipase C (Clostridium perfringens alpha toxin) was obtained from Worthington Corp., Freehold, N.J. Kinetic experiments were performed in an automated Pye-Unicam SP 800B double beam recording spectrophotometer the cuvette holder of which was equipped with a constant temperature water jacket set at 37°C. Washed red cells were suspended in 0.01 M buffered saline at pH 7.0 in a cuvette of 1 cm light path so that a 1:2 dilution of the
cells would have an absorbance of 1.8 at 650 nm. An equal volume of gamma lysin at the appropriate concentration was added to the cells after which the absorbance was monitored continuously or at specified time intervals as required. The slope of the line which depicted a decrease in absorbance versus time, expressed as absorbance change/min, reflected the concentration of hemolysin present. The effect of membrane phospholipid fractions, lipid-free membrane protein or red cell cytoplasm on hemolysis of red cells by the gamma lysin was measured in the same way. That is, the phospholipids or membrane proteins were added to the red cell suspension just before the addition of lysin, and absorbance changes were monitored in the usual manner. Controls were present and automatically compensated for by the double beam spectrophotometer.

An experiment was performed in which the effect of EDTA upon hemolysis of red cells in the presence of gamma lysin was measured, as follows: Equal volumes of EDTA dissolved in phosphate buffered saline at pH 7.0 were added to dilutions of hemolysin such that the combined volume was 1 ml. One ml of erythrocytes was added to each tube. Incubation and determination of the hemolytic end point were performed as described by Wiseman & Caird (1972).

In some titrations, solutions of cations were added to the gamma lysin dilutions in place of or along with the EDTA before addition of the erythrocytes.

In one experiment, sodium or potassium ions were added to the lysin dilution, and after the addition of red
cells, the rate of lysis was followed in the spectrophotometer as described for kinetic experiments.

**Results**

**Effect of gamma lysozyme on human erythrocyte membranes**

An attempt was made to show chemical differences in composition of red cell membranes treated with gamma lysozyme. The membranes were added to gamma lysozyme such that their final concentration in terms of nitrogen was 500 µg/ml while that of the lysozyme was 50 µg/ml (about 6000 hemolytic units/ml). The suspension was incubated at 37°C for 3 hr. at which time the reaction was stopped by the addition of trichloroacetic acid to a final concentration of 5 per cent (w/v). Control and test supernatant fluids showed no difference in total carbohydrate, pentose, deoxypentose, reducing sugars, hexosamines, sialic acid or protein as measured by the Lowry reagent (data not shown). Furthermore, no difference in absorbance at 260 or 280 nm was detected. However, nitrogen content of the membrane-lysozyme mixture increased by seven % in relation to a control. In addition, acid-soluble phosphorus was released from the membranes by the lysozyme.

These differences were further investigated as described except that nitrogen and phosphorus released from membranes were determined at various intervals of time ranging from 0-180 min. in the presence of twice the amount of substrate. As shown in Fig. 1, the rate of nitrogen liberation was linear over the 3 hr. period. The rate of release was,
however, not comparable to that obtained by treatment of the membranes with pronase. Results observed for release of acid-soluble phosphorus were similar (Fig. 2). The addition of phospholipase C to the membranes liberated a similar amount of phosphorus. The rate of liberation of phosphorus appeared to be greater than that of nitrogen.

The phospholipids of human erythrocyte membranes were dialyzed at a concentration of 1 mg/ml against Tris-saline buffer (pH 7.0) and incubated with 120 μg/ml (about $10^4$ hemolytic units/ml) gamma lysin. In contrast with results obtained for red cell membranes, no detectable phosphorus was released after 3 hr. incubation at 37°C. None of the phospholipids identified by TLC disappeared from the phospholipid preparation after treatment with gamma lysin. The lysin also did not hydrolyze sphingomyelin and was therefore not similar to beta lysin (Wiseman & Caird, 1967). The gamma lysin failed to degrade phosphatidylinositol and was thus unlike delta lysin (Wiseman & Caird, 1968). Gamma lysin had no effect upon phosphatidylserine, phosphatidylethanolamine or phosphatidylcholine.

A suspension of lipid-free membrane protein (two % w/v) in phosphate buffered saline at pH 7.0 was incubated with 120 μg/ml gamma lysin. No nitrogen was detected in the supernatant after 3 hr. incubation at 37°C which contrasts with observations made for membranes. No breakdown products were obtained by amino acid and N-terminal group detection on TLC plates.

It was thought that gamma lysin might act in a manner
similar to that reported for the alpha lysin by Wiseman & Caird (1972) and by Wiseman, Caird & Fackrell (1974). That is, alpha lysin is activated by red cell proteases and is then able to degrade sensitive erythrocytes or other suitable substrates such as p-toluene-sulphonyl-L-arginine methylester (TAME). However, gamma lysin incubated with red cell membranes known to activate alpha lysin failed to hydrolyze TAME.

Kinetics of substrate inhibition of hemolysis

The velocity of lysis of human red cells incubated with gamma lysin was measured over a range of red cell concentrations and also in the presence of dried red cell cytoplasm (1 mg/ml of reaction mixture). The results, expressed in the form of a Lineweaver-Burk plot, are shown in Fig. 3 and indicate that hemolysis was inhibited by as little as 20 μg N/ml of erythrocyte membranes. Cytoplasm had no effect on the rate of hemolysis. It will be noted that all curves have a common Y-intercept.

The experiment was repeated with phospholipid and lipid-free membrane protein components. In Fig. 4, it is observed that human erythrocyte phospholipids at several concentrations competitively inhibited hemolysis by the gamma lysin. By contrast, lipid-free protein had no effect.

Inhibition of gamma lysin by EDTA

Hemolysis of human erythrocytes in the presence of gamma lysin was increasingly inhibited at concentrations of EDTA 10^-6 M or greater, as shown in the Table. Inhibition
of hemolysis by EDTA was abolished if the solution of lysin and chelating agent was dialyzed against phosphate buffered saline at pH 7.0 for 24 hrs.

Titration experiments with the following ions showed no enhancement of lysis nor did they restore the activity of lysin-EDTA solutions when tested at concentrations ranging from $10^{-8}$ to 0.1 M: magnesium, calcium, iron, aluminum, zinc, manganese, nickel, cobalt, ammonia and chloride. However, a requirement for sodium or potassium was demonstrated and at concentrations of less than $7.5 \times 10^{-3}$ M NaCl, no lytic activity was present as shown in Fig. 5.

**Discussion**

Unfortunately, it has not been possible to elucidate the precise mode of action of gamma lysin at the present time. However, kinetics of hemolysis and inhibition are not incompatible with a catalytic reaction between gamma lysin and its substrate. Naturally, kinetic studies of a reaction in which erythrocytes are used as a substrate are not ideal, but we have found such studies useful and at best they are a true reflection of what is found when the substrate is identified and isolated.

Liberation of nitrogen and phosphorus from membranes treated with gamma lysin suggests that the lysin might be a phospholipase. This view is supported by the fact that membrane phospholipids competitively inhibited lysis but membrane proteins were unable to do so. Against this evidence is the observation that crude extracted or purified phospho-
lipids were not attacked by the gamma lysin. However, the extraction procedure could have denatured the substrate so that it is not susceptible to the action of the lysin, since many phospholipids are susceptible to oxidation and other factors. Another possibility is that conformation of the phospholipid substrate changes during extraction which renders it refractory to the action of gamma lysin.

The role of sodium or potassium ions in the interaction of gamma lysin with red cells is unclear. While there are many examples of enzymes which require sodium ions, we cannot at present reconcile the sodium requirement with the phospholipase-like activity of the gamma lysin.

**Summary**

Differences in the levels of nitrogen and phosphorus were detected in human erythrocyte membranes treated with gamma lysin of *S. aureus*, when compared to untreated cells. It was found that the rate of liberation of phosphorus and nitrogen in the presence of the lysin was linear over a three hour period. Gamma lysin incubated with phospholipids extracted from erythrocytes and with lipid-free membrane protein had no detectable effect on these preparations. However, it could be shown that the reaction was inhibited by erythrocyte membranes when these were added to lysin-red cell suspensions. Hemolysis was also inhibited by EDTA and activity could be restored by dialysis. A requirement of the hemolytic reaction for sodium ions was demonstrated.
Acknowledgment

This work was supported by the University of Manitoba and the Medical Research Council.
References


## TABLE 1

**INHIBITION OF GAMMA HEMOLYSIN BY EDTA**

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>EDTA (M)</th>
<th>TOTAL HEMOLYSIN (HU) *</th>
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</thead>
<tbody>
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<td></td>
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</tr>
<tr>
<td>10^-2</td>
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<tr>
<td>10^-3</td>
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<tr>
<td>10^-4</td>
<td>6,500</td>
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<tr>
<td>Untreated gamma lysin</td>
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*Hemolytic Units.*
Captions to Figures

Figure 1. Liberation of nitrogen from human erythrocyte membranes in the presence of gamma lysin. Membrane control, x—x; membranes treated with gamma lysin, •—•; membranes treated with pronase, o—o.

Figure 2. Liberation of acid-soluble phosphorus from human erythrocyte membranes in the presence of gamma lysin. Membrane control, x—x; membranes treated with gamma lysin, •—•; membranes treated with phospholipase C (Clostridium perfringens alpha toxin), o—o.

Figure 3. Lineweaver-Burk plot of reaction between human erythrocytes and gamma lysin. Gamma lysin-red cell control, •—•; gamma lysin-red cell control to which red cell cytoplasm has been added, x—x; gamma lysin-red cell suspensions (o) at a red cell concentration of 20 µg N/ml, (.....); 30 µg N/ml, (-----); 40 µg N/ml, (-----).

Figure 4. Lineweaver-Burk plot of reaction between human erythrocytes and gamma lysin in the presence of membrane phospholipids. Gamma lysin-human red cell control, •—•; gamma lysin-red cell control in the presence of membrane protein, x—x; gamma lysin-red cell suspensions (o) to which have been added membrane phospholipids at concentrations of 10 µg P/ml (.....); 20 µg P/ml (-----); 35 µg P/ml (-----).

Figure 5. The effect of NaCl concentration upon the rate of lysis of human erythrocytes in the presence of gamma lysin.
Fig. 1

Graph showing the effect of membranes + Pronase and membranes + gamma lysin on soluble nitrogen (percentage of total). The x-axis represents time (in minutes) ranging from 0 to 180, and the y-axis represents soluble nitrogen (% of total) ranging from 0 to 50. The graph compares the degradation over time for membranes with and without enzymes.

- Membranes + Pronase show a rapid increase in soluble nitrogen with time.
- Membranes + gamma lysin show a slower increase in soluble nitrogen over time.
- Membranes show no significant change in soluble nitrogen.

Fig. 1
Fig. 2

ACID SOLUBLE PHOSPHORUS (% of total)

membranes + phospholipase

membranes + gamma lysin

membranes

TIME (min)
Fig. 3

1/VELOCIY (ΔOD/min, 650nm)

1/ERYTHROCYTE CONC. (OD 650nm)

40μgN/ml

30μgN/ml

-20μgN/ml

+MEMBRANES

+CYTOPLASM

GAMMA LYSIN-CONTROL

Fig. 3
Fig. 4
Fig. 5

SODIUM CHLORIDE (x10^{-3} M)

VELOCITY (AOD/min, 650nm)
Some Characteristics of the Beta-Haemolysin of *Staphylococcus aureus*

BY

G. M. WISEMAN

Department of Bacteriology, University of Edinburgh
SOME CHARACTERISTICS OF THE BETA-HAEMOLYSIN OF STAPHYLOCOCCUS AUREUS

G. M. WIEMAN*

Department of Bacteriology, University of Edinburgh

The first description of the beta-lysin of Staphylococcus aureus as a distinct entity (Glenny and Stevens, 1935) drew attention to the fact that it was a "hot-cold" haemolysin for sheep erythrocytes, although Walbun (1922) had earlier described a haemolysin whose lytic activity was greatly enhanced if incubation at 37°C was followed by a period of holding at room temperature or at 4°C. Glenny and Stevens claimed that the crude beta-lysin was lethal for rabbits but not mice, and that it produced an erythematous flush rather than necrosis if injected intradermally into rabbits.

This work was followed by the investigation of Bryce and Rountree (1936) who noted that the beta-lysin was produced mainly by strains derived from animal sources, that it withstood heating at 60°C for 15 min. and that it was antigenic. They also confirmed the observations of Glenny and Stevens regarding its in-vitro activity in rabbits and mice. According to Bryce and Rountree, the erythrocytes of sheep and oxen were most susceptible to the beta-lysin, whilst those of the ferret, rabbit, rat, guinea-pig and koala bear were resistant. The lytin proved to be only feebly lytic to human erythrocytes. Roy (1937) and Kodama and Kojima (1939) claimed that the beta-lysin causes "hot-cold" haemolysis of human erythrocytes.

Data concerning the heat sensitivity of the beta-lysin are also conflicting. Various investigators (Bryce and Rountree; Flaum, 1938) found that beta-lysin resisted prolonged heating at 100°C and that autoclaving was necessary for its destruction. Others (Smith and Price, 1938) found a 40 per cent. loss of haemolytic activity after 1 hr at 55°C, and complete destruction after boiling. Still others (Kodama and Kojima) claimed that a small amount of activity remained after preparations were heated at 100°C for 30 min. These authors also stated that their beta-lysin preparation exhibited the paradoxical behaviour toward heat characteristic of the alpha-lysin.

The effect of mild reducing agents upon the haemolytic activity of the beta-lysin has also been disputed. Kodama and Kojima claimed that their beta-lysin preparation was not affected by ascorbic acid, whereas Thatcher and Matheson (1954-55) stated that enterotoxin preparations containing beta-lysin could be freed from it by boiling and subsequent incubation in the presence of ascorbic acid.

More recently, Jackson and Mayman (1958), working with crude beta-lysin, observed that its haemolytic activity was greatly reduced by dialysis and could be restored by the addition of 0·01M Mg++ or Mn++ ions but not by addition of Ca++ ions. They also found that citrate or ethylenediaminetetra-acetic acid (EDTA) would inhibit the haemolytic activity of the beta-lysin. Robinson, Thatcher and Gagnon (1958) partially purified two haemolysins from an enterotoxigenic strain of Staphylococcus, one of which was a "hot-cold" lytin for sheep erythrocytes: the activity of this lytin was enhanced by Co++, Mg++, Mn++, Ni++, and Fe++ ions.

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but not by Zn\(^{++}\) or Ca\(^{++}\) ions, and it was inhibited by EDTA. They were, however, unable to identify the lysozyme with the classical beta-lysin of Glenny and Stevens.

Heydrick and Chesbro (1962) claimed that their partially purified beta-lysin preparation was trypsin-resistant and, when activated by Mg\(^{++}\) ions, was lethal to guinea-pigs on intraperitoneal injection. Jackson (1963) partially purified the beta-lysin (c. 250-fold), but did not investigate its properties.

In view of the many conflicting reports on the thermostability, erythrocyte spectrum and in-vivo activity of the beta-lysin, most of which were based on experiments with crude material, the present work, which was done with partially purified preparations, was undertaken in order to re-investigate certain of the properties of the beta-lysin, particularly in the light of its behaviour in the presence of divalent metallic cations.

**Materials and methods**

**Strains of Staphylococcus.** Strains R1, G128, G143, and P92 were supplied by Professor J. W. McLeod, Edinburgh. Strain R1 was isolated from endemic furunculosis in horses and G128 from fomites. Strains G143 and P92 were taken from lesions in man. Strain 252F was supplied by Dr G. Fraser, Royal Dick School of Veterinary Studies, Edinburgh, and is of canine origin. All strains were coagulase-positive and fitted the description of *Staphylococcus aureus* given by Evans (1957). Immediately on receipt, the strains were subcultured on nutrient agar plates and single colonies from the plates were inoculated on nutrient agar slopes; the slope cultures after 24 hr were suspended in nutrient broth and freeze-dried.

**Reagents.** Chemicals employed were obtained from British Drug Houses, Poole, Dorset, and were of Analar or B.D.H. laboratory standard.

**Suspending fluids.** The following suspending fluids were employed: (1) physiological (0.85 per cent. NaCl) saline buffered with 0.01M phosphate at pH 7.0, (2) 0.01M borate buffer, pH 8.0 (Cruickshank et al., 1960) and (3) Tris-(hydroxymethyl)-aminomethane ("Tris ")-saline buffer of Jackson and Mayman (1958) adjusted to pH 7.0.

**Spectrophotometry.** This was carried out on a "Unicam" SP-500 spectrophotometer (Cambridge Instruments, Cambridge) employing a 1 cm. light path, and equipped with a constant-temperature waterjacket.

**Determination of the haemolysin spectrum of strains.** The method of Elek and Levy (1950), modified in some respects, was used. Instead of the medium described by these authors, that described by Dolman and Wilson (1940), containing 1.5 per cent. Davis New Zealand agar, was employed. Antiserum was Wellcome commercial staphylococcal antiserum containing 920 International Units of anti-alpha-lysin per ml. Instead of the 48-hr incubation period of Elek and Levy, a 24-hr period was used.

**Production of crude beta-lysin.** Dolman-Wilson medium containing 1.5 per cent. agar was employed in the preparation of crude beta-lysin. The technique of Birch-Hirschfeld (1933–34) was used in the inoculation of plates. Disks of cellophane cut to fit 1.5 × 10 cm. Petri dishes and sterilised by steaming at 100°C for 90 min. were layered on to plates containing 20 ml. solidified medium. The inoculum, which consisted of 0.1 ml. of a suspension of a 24-hr nutrient agar slope culture in 10 ml. saline, was placed on the cellophane and spread with a sterile bent glass rod. Approximately 100 plates were inoculated at one time and were placed, along with a beaker of water, in a glass tank measuring 30 × 30 × 60 cm. A Perspex lid containing an inlet and outlet for gas was then carefully sealed on to the tank with tape. The tank was gassed with a mixture of 25 per cent. CO\(_2\) in air for 15 min. before being placed in an incubator at 37°C. Plates were harvested after incubation for
24 hr. Two ml. of phosphate-buffered saline was added to each plate and the growth suspended with a bent glass rod and taken up with a Pasteur pipette. Growth from all plates was pooled, Gram stained and centrifuged at 3000g for 30 min. Merthiolate was added to give a concentration of 10 μg. per ml. in the supernatant crude lysins, which were then stored at -20°C until required.

Partial purification of beta-lysin with hydroxylapatite. The purification procedure employed in this investigation was that developed by Wiseman (1963). Hydroxylapatite was prepared according to the method of Tislevik, Hjertén and Levin (1956). Throughout the purification procedure, 0-1M phosphate buffer at pH 7-0 was employed. Twenty-four hours before use, the adsorbent was centrifuged at 250g for 10 min., the supernatant fluid was poured off, and the material was resuspended in 2-3 ml. buffer to give an "adsorbent slurry". Approximately 800 ml. crude beta-lysin was dialysed for 24 hr against 5 litres of buffer at 4°C. The adsorbent slurry was then added to this quantity of crude lyisin. It was found that little or no alpha-and delta-lysins were adsorbed if the amount of adsorbent in 800 ml. was kept to 9 g. (dry weight), if the pH was 7-0 and if the buffer concentration was 0.1M. The mixture was allowed to stand at 4°C for 1 hr with occasional shaking, although time was shown not to be an important factor in adsorption; it was then centrifuged at 250g for 10 min. The supernatant fluid was poured off and set aside for a further adsorption. The adsorbent was washed eight times with c. 200 ml. buffer to rid it of extraneous material, particularly alpha-lysin. The beta-lysin was eluted from the adsorbent by the addition of 100-150 ml. 2M-NaCl dissolved in buffer and adjusted to pH 7-0. The eluted lyisin was recovered by centrifuging the adsorbent at 250g for 10 min. The beta-lysin eluate was dialysed against running tap water at e. 10°C for 24 hr. A slight precipitate developed at this stage, and was removed by centrifugation at 40,000g for 45 min. The supernatant fluid, which was clear and colourless, was freeze-dried and stored at 4°C. The characterisation of the beta-lysin was carried out on this product.

Estimation of protein. Total protein was estimated by the method of Lowry et al. (1951).

Immunodiffusion analysis. This was carried out according to the technique of Ouchterlony (1958).

Preparation of erythrocyte suspensions for haemolysin titrations. Rabbit erythrocytes were obtained by bleeding four mature animals from the marginal ear vein. The blood from each rabbit was immediately added to an equal volume of Alsever's solution (Carpenter, 1956), pooled and stored at 4°C until required. Pooled human erythrocytes from 4 to 8 donors were obtained from the Blood Transfusion Unit of the Royal Infirmary of Edinburgh and stored in Alsever's solution at 4°C. Sheep's blood was obtained defibrinated from an abattoir in Edinburgh and treated in the same manner as rabbit and human blood. These bloods were replaced once weekly. Blood from other animal species was obtained as required. Erythrocytes stored in Alsever's solution were centrifuged at 1000g for 5 min., washed three times and resuspended to a final concentration of 2 per cent. in phosphate-buffered saline containing 0-002M-MgCl2 unless otherwise stated. Cell suspensions were always used on the day they were prepared.

Measurement of haemolysin. Culture supernatants and partially purified preparations of beta-lysin were assayed for haemolytic activity against rabbit, human and sheep red cells. Serial two-fold dilutions of lyisin, commencing at 1 in 2 or 1 in 10, were made in 1 ml. phosphate-buffered saline in tubes measuring approximately 1 x 8.5 cm. An equal volume of a suspension of 2 per cent. erythrocytes (prepared as described above) was added to each tube to give a final volume of 2 ml. The tubes were then agitated in racks and placed in a waterbath at 37°C for 60 min. The racks were removed, placed in a refrigerator at 4°C and examined at the end of a 16-hr period. The erythrocytes settled during this time and any haemolysis was clearly visible in the supernatant fluid.

The amount of haemolysin was expressed in terms of a 50 per cent. end-point.
The unknown was compared against a white illuminated background, with a standard test, showing 50 per cent. haemolysis. The end-point was defined as the reciprocal of the highest dilution of lysin causing 50 per cent. haemolysis of a suspension of 1 per cent. erythrocytes under the conditions specified. Generally, in the comparison of two or more single titrations, one-tube differences in end-point were not considered significant.

*Demonstration of the action of mild reducing agents on beta-lysin.* The effect of mild reducing agents upon haemolysis of erythrocytes in the presence of beta-lysin was investigated. Ascorbic acid was dissolved in phosphate-buffered saline in concentrations ranging from 0·1 to 0·0001 M, and L-cysteine hydrochloride at concentrations of from 0·01 to 0·0001 M; the pH was adjusted to 7·0. Equal volumes of partially purified beta-lysin from strains R1 and 252F were added to solutions containing from 0·0002 to 0·2 M ascorbic acid and others containing from 0·002 to 0·02 M cysteine, and the mixtures were incubated at 4°C for 18 hr. Haemolysin titrations in the presence of sheep erythrocytes were then made with this material. Control titrations showed that the reducing agents did not themselves alter the erythrocytes.

*Measurement of the heat stability of beta-lysin.* Studies were made of the sensitivity of partially purified beta-lysin to heat at 60°C and 100°C. Beta-lysin from strains R1 and 252F in 1·2 ml. quantities were added to 16 McCartney bottles, eight of which were placed in a waterbath at 60°C. Seven were placed in water in a metal trough and the water was heated to 100°C. Samples were removed at intervals of 3, 6, 9, 12, 15, 30, 60 and 120 min. The phosphate-saline buffer in which the preparations were dissolved was at pH 7·0, and its ionic strength was 0·21.

*Studies of the kinetics of haemolysis.* In the experiment on the relation of the extent of haemolysis to the time of interaction, accurately measured quantities of lysin ranging from 0·005 to 0·100 ml., or 0·005 to 0·50 ml. were added to a spectrophotometer cuvette. Phosphate-saline buffer was added to give a volume of 3·0 ml. and the reagents were warmed to 37°C in a waterbath. One ml. of a 4 per cent. suspension of sheep erythrocytes, also at 37°C, was added to the lysin in the cuvette, mixed thoroughly, and incubated for 5 min. at 37°C. The cuvette was then rapidly transferred in a beaker of water at 37°C to the spectrophotometer, the waterjacket of which was maintained at 25°C. The optical density at 650 mμ was read at zero time. Comparison with a normal red cell control indicated that haemolysis did not occur at 37°C in the presence of the beta-lysin of either strain during the transfer of the cuvette from the waterbath to the spectrophotometer. Commencing at zero time, readings were taken at 1 min. intervals, using a stopwatch, until 13 readings had been taken.

*Demonstration of the dermonecrotic property of haemolysins.* The skin-necrotising property of crude and partially purified staphylococcal lysins was demonstrated in mature rabbits, c. 2·5 kg. in weight. The back of the rabbit was closely shaved and the skin was divided into sections with a marking pencil. Each rabbit received intradermal injections of 0·05 ml. quantities of undiluted lysin. Two rabbits were used in each experiment. The dermonecrotic effect was measured in terms of the preparation of lysin that initiated a visible inflammatory reaction in the skin. As the lysins were not sterile, 5 units per ml. penicillin were incorporated in the material to be injected. It was found that the use of penicillin did not alter the result. Also, no inflammatory response was ever observed when non-sterile buffer controls containing penicillin were injected into the skin. The strains of *Staph. aureus* from which the material for injection was derived were shown by in-vitro disk sensitivity tests to be sensitive to 3 units per ml. penicillin. Reactions to the injected lysins were read and recorded every 24 hr for 3 days.

*Demonstration of the lethal property of haemolysins.* The lethal activity of crude and partially purified lysins was observed in mature rabbits weighing c. 2·5 kg. and in Swiss white mice weighing c. 25 g. In rabbits, 0·5–2·0 ml. lysin was injected
intravenously into the marginal ear vein and the animals were closely observed for 2 days. Only those deaths that occurred within a 24-hr period were recorded. In mice, 0·1 ml. lysis was injected intravenously into the tail vein, or 0·5 ml. intraperitoneally, and deaths occurring within 24 hr were recorded. Experiments with rabbits were repeated. In the case of mice, five animals were used with each injection. As in the demonstration of skin-necrotising properties of lysins, 5 units per ml. penicillin were employed in these experiments. The results were not influenced by the use of the penicillin.

RESULTS

Efficiency of the procedure of purification with hydroxylapatite

Measurements of the haemolytic activities of preparations taken at different stages in the purification of beta-lysin are shown in table I. Reference to the results for preparation no. 2 indicates that no haemolytic activity was lost during dialysis. A slight precipitate occurred during dialysis of preparation no. 4 and the removal of the precipitate accounts for the increase in specific activity at this stage. The purification procedure allows the recovery of 17 per cent. of the beta-lysin originally present, with a purification of c. 30-fold. The usefulness of hydroxylapatite employed at pH 7·0 in 0·1M buffer for the selective adsorption of beta-lysin is reflected in a comparison of the ratios of the rabbit and sheep cell haemolytic titres in preparations no. 1 and 5. The ratio for preparation no. 1 is 16 and that for no. 5 is 128. It thus appears that under these conditions of molarity and pH, little or no alpha-lysin is adsorbed to the hydroxylapatite. Similar results were obtained with preparations from strain 252F and two other beta-lysin-producing strains.

Immunodiffusion studies of crude and partially purified beta-lysin

An immunodiffusion analysis of crude and partially purified beta-lysins derived from strains R1 and 252F was carried out in order to ascertain the degree of purity obtained by this purification procedure. In addition, purified delta-lysin prepared according to the method of Yoshida (1963), and supplied by Dr G. P. Gladstone of the Sir William Dunn School of Pathology, Oxford, was also tested. The antiserum was the same as that used in the Elek and Levy antitoxin diffusion analysis. Results indicated that a 2-3-fold reduction in the number of precipitation lines was obtained when partially purified and crude beta-lysins were compared. The partially purified preparation of beta-lysin from strain 252F did not appear to contain any delta-lysin, whereas that from strain R1 did appear to be contaminated with delta-lysin. The amount of delta-lysin present, however, was probably small, as observations subsequently outlined in this paper show.

Effect of divalent metallic cations upon haemolysis

Preliminary experiments with chelating agents showed that the haemolytic activity of the beta-lysin could be inhibited. In view of this,
**Table I**

Haemolytic activities of preparations taken at different stages during the partial purification of beta-lysin from strain R1 by adsorption with hydroxylapatite and dialysis

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Haemolytic titre (units per ml.) with</th>
<th>Volume or weight of preparation</th>
<th>Total protein mg. per ml. preparation</th>
<th>Specific beta-lytic activity*</th>
<th>Per cent. recovery of beta-lysin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rabbit cells</td>
<td>human cells</td>
<td>sheep cells</td>
<td>mg. per ml. preparation</td>
<td>mg. per ml.</td>
</tr>
<tr>
<td>(1) Crude haemolysin</td>
<td>2560</td>
<td>5120</td>
<td>40,960</td>
<td>850 ml.</td>
<td>...</td>
</tr>
<tr>
<td>(2) Dialysed crude lysin</td>
<td>2560</td>
<td>5120</td>
<td>40,960</td>
<td>850 ml.</td>
<td>1·000</td>
</tr>
<tr>
<td>(3) NaCl eluate from adsorbent</td>
<td>1280</td>
<td>10,240</td>
<td>163,840</td>
<td>125 ml.</td>
<td>0·200</td>
</tr>
<tr>
<td>(4) Eluate dialysed against tap water</td>
<td>640</td>
<td>5120</td>
<td>81,920</td>
<td>125 ml.</td>
<td>0·068</td>
</tr>
<tr>
<td>(5) Lyophilised tap water dialysate (1·0 mg. per ml.)†</td>
<td>640</td>
<td>5120</td>
<td>81,920</td>
<td>67 mg.</td>
<td>0·070</td>
</tr>
</tbody>
</table>

* Haemolytic units (for sheep cells) per mg. protein.
† For preparation no. 5 the haemolytic titres and specific beta-lytic activity are stated as per mg. of protein. The tests were made on a solution containing 1·0 mg. of the dried preparation per ml. The reduction in the percentage recovery as compared with that for preparation no. 4 was probably due to the loss of some beta-lysin during lyophilisation.
the effect of divalent Mg, Mn, Co, Ni, Ca and Zn ions upon haemolysis of rabbit, human and sheep erythrocytes in the presence of partially purified beta-lysin from strains R1 and 252F and crude lysins from strain G143 was investigated. All metallic ions were employed as the chloride at a concentration of 0.001M in Tris-saline buffer; the Tris buffer was used in order to avoid the formation of insoluble phosphates in phosphate-saline buffer.

**Table II**

<table>
<thead>
<tr>
<th>Cation added</th>
<th>Haemolytic titres (units per ml.) of haemolysins from</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>strain R1</td>
<td>strain 252F</td>
</tr>
<tr>
<td></td>
<td>rabbit cells</td>
<td>human cells</td>
</tr>
<tr>
<td>Mg++</td>
<td>20</td>
<td>160</td>
</tr>
<tr>
<td>Co++</td>
<td>20</td>
<td>160</td>
</tr>
<tr>
<td>Mn++</td>
<td>20</td>
<td>160</td>
</tr>
<tr>
<td>Ni++</td>
<td>&lt;20</td>
<td>40</td>
</tr>
<tr>
<td>Ca++</td>
<td>&lt;20</td>
<td>40</td>
</tr>
<tr>
<td>Zn++</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td>None</td>
<td>&lt;20</td>
<td>40</td>
</tr>
<tr>
<td>Mg++ and phosphate buffer</td>
<td>40</td>
<td>160</td>
</tr>
</tbody>
</table>

* Partial haemolysis (<50 per cent.) at the dilution in brackets.
† Trace of haemolysis (<10 per cent.).

The haemolysins of strains R1 and 252F were partially purified preparations of beta-lysin. The haemolysin of strain G143 was a crude preparation; it appeared to contain alpha- and delta-lysins, but no beta-lysin.

Results are shown in table II. Beta-lysin (sheep-cell) titres of strains R1 and 252F were greatly enhanced in the presence of Mg++, Co++ and Mn++ ions, but not with Ni++ and Ca++ ions. There was no haemolytic activity in the presence of Zn++ ions, probably because of the complexing of beta-lysin with this metal.

Strain G143 presented a contrast to the other two strains. It produced no detectable beta-lysin, but it did appear, according to the antitoxin diffusion test of Elek and Levy (1950), to produce alpha- and delta-lysins. It is evident that the rabbit, human and sheep erythrocyte titres of this strain were unaffected in the presence of any of the ions.

Since it was intended to use phosphate rather than Tris buffer routinely, phosphate-saline buffer containing Mg++ ions was tested to discover whether the same enhancement of titres would be observed as with Tris buffer. Reference to the bottom line of table II indicates.
that the Mg++ ions are fully as active in phosphate, and that the phosphate salts themselves exert no detectable effect on the haemolysin titres.

*Optimal concentrations of magnesium and cobalt ions for enhancement of haemolytic activity*

Concentrations of Mg++ and Co++ ranging from 0-000001 to 0-1M were tested for their effect upon the activity of the beta-lysin of strains R1 and 252F. Table III shows that the greatest enhancement of the sheep-cell haemolytic titres of these lysins occurs with Mg++ and Co++ ions at concentrations of 0-01 and 0-001M.

**Table III**

*Effect of concentration of magnesium and cobalt cations on the sheep-cell haemolytic titres of the beta-lysins of strains R1 and 252F*

<table>
<thead>
<tr>
<th>Molarity of added cation</th>
<th>Sheep-cell haemolytic titres (units) per ml. in presence of added cation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg++</td>
</tr>
<tr>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>0-1</td>
<td>40</td>
</tr>
<tr>
<td>0-01</td>
<td>2560</td>
</tr>
<tr>
<td>0-001</td>
<td>2560</td>
</tr>
<tr>
<td>0-0001</td>
<td>320</td>
</tr>
<tr>
<td>0-00001</td>
<td>80</td>
</tr>
<tr>
<td>0-000001</td>
<td>40</td>
</tr>
</tbody>
</table>

*Action of chelating agents upon the haemolytic activity of beta-lysin*

The effect of disodium ethylenediaminetetra-acetate (EDTA) and citric acid in concentrations ranging from 0-000001 to 0-01M in Tris buffer upon the haemolytic activity of the beta-lysins derived from strains R1 and 252F was investigated and the results are shown in table IV. In the absence of added Mg++ ions the beta-lysin activity of both strains was completely inhibited by EDTA at a concentration of 0-01M. Inhibition tended to decrease at 0-001 and 0-0001M EDTA and it disappeared altogether at 0-00001M. In the presence of Mg++ ions (0-001M) there was no inhibition at a concentration of EDTA of 0-0001M; this suggests that Mg++ ions are required for full expression of the beta-lysin haemolytic activity. Results with citric acid were similar to those with EDTA. In contrast to these data, it was found that 0-00001-0-01M EDTA did not alter the rabbit-, human- or sheep-cell haemolytic titres of the crude lysin (alpha and delta) of strain G143. It thus appears that of the known staphylococcal lysins, only the beta-lysin is inhibited by the chelating agents tested.
Influence of 0.001M magnesium ions upon crude and partially purified lysins

Partially purified lysin preparations derived from strains P92, G128, R1 and 252F and crude lysins from R1 and 252F were titrated against rabbit, human and sheep erythrocytes in the presence and absence of 0.001M Mg$^{++}$ ions.

**Table IV**

*Effect of the chelating agents disodium ethylenediaminetetra-acetate (EDTA) and citric acid on the sheep-cell haemolytic titres of partially purified beta-lysins from strains R1 and 252F in the presence and absence of added magnesium ions*

<table>
<thead>
<tr>
<th>Chelating agent</th>
<th>Molarity of chelating agent</th>
<th>Sheep-cell haemolytic titre (units per ml.) of lyso</th>
<th>without added Mg$^{++}$</th>
<th>with 0.001M Mg$^{++}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R1 lysin</td>
<td>252F lysin</td>
<td>R1 lysin</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.01</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>20</td>
<td>&lt;20</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>0.0001</td>
<td>80</td>
<td>20</td>
<td>10,240</td>
</tr>
<tr>
<td></td>
<td>0.00001</td>
<td>640</td>
<td>40</td>
<td>10,240</td>
</tr>
<tr>
<td></td>
<td>0.000001</td>
<td>1280</td>
<td>40</td>
<td>10,240</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1280</td>
<td>40</td>
<td>10,240</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.01</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>40</td>
<td>&lt;20</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>0.0001</td>
<td>320</td>
<td>20</td>
<td>2560</td>
</tr>
<tr>
<td></td>
<td>0.00001</td>
<td>1280</td>
<td>40</td>
<td>10,240</td>
</tr>
<tr>
<td></td>
<td>0.000001</td>
<td>1280</td>
<td>40</td>
<td>20,480</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

* Haemolysis at this concentration in the controls.

The effect of Mg$^{++}$ ions on the haemolytic titres of the preparations is illustrated in table V. With all preparations there was a considerable enhancement of the haemolytic titres for rabbit, human and sheep cells in the presence of Mg$^{++}$ ions. The degree of enhancement was not the same in all titrations, however, the net increase with the three red cell species being greatest with the beta-lysin from strain R1, intermediate with the lysins from strains P92 and G128, and least with that from strain 252F. It should also be pointed out that in the case of some preparations, namely the partially purified beta-lysins from strains P92, G128 and 252F, there was little or no haemolytic activity against rabbit or human red cells in the absence of Mg$^{++}$ ions. The addition of 0.001M Mg$^{++}$ did, however, dramatically alter the sensitivity of these erythrocytes to beta-lysin. The rabbit-cell haemolytic titre of the crude preparation from strain R1 was not enhanced by the Mg$^{++}$ ions, but that of the partially purified beta-lysin was enhanced 8-fold.

It may be assumed that the rabbit-, human- and sheep-cell haemolytic
titres obtained for these partially purified preparations in the presence of 0-001M Mg\(^{++}\) ions are measures of beta-lysin activity, since results obtained with lysis from strain G143 indicate that the haemolytic activity of alpha- and delta-lysins is unaffected by Mg\(^{++}\) ions.

**Table V**

**Influence of addition of 0-001M magnesium ions on the haemolytic titres of haemolysins prepared from different strains of Staph. aureus in tests with rabbit, human and sheep erythrocytes**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Haemolysin preparation</th>
<th>Addition of magnesium ions</th>
<th>Haemolytic titres (units per ml.) with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>rabbit cells</td>
</tr>
<tr>
<td>P92</td>
<td>Partially purified beta-lysin</td>
<td>-</td>
<td>&lt;20</td>
</tr>
<tr>
<td></td>
<td>Partially purified beta-lysin</td>
<td>20</td>
<td>160</td>
</tr>
<tr>
<td>G128</td>
<td>Partially purified beta-lysin</td>
<td>-</td>
<td>&lt;20</td>
</tr>
<tr>
<td></td>
<td>Partially purified beta-lysin</td>
<td>20</td>
<td>640</td>
</tr>
<tr>
<td>R1</td>
<td>Crude lysis</td>
<td>-</td>
<td>2560</td>
</tr>
<tr>
<td></td>
<td>Crude lysis</td>
<td>2560</td>
<td>3120</td>
</tr>
<tr>
<td></td>
<td>Partially purified beta-lysin</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Partially purified beta-lysin</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Partially purified beta-lysin</td>
<td>160</td>
<td>320</td>
</tr>
<tr>
<td>252F</td>
<td>Crude lysis</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Crude lysis</td>
<td>160</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>Partially purified beta-lysin</td>
<td>-</td>
<td>&lt;20</td>
</tr>
<tr>
<td></td>
<td>Partially purified beta-lysin</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

* Trace of haemolysis (<10 per cent.).

Sensitivity of different species of erythrocytes to beta-lysin

There is some agreement that staphylococcal beta-lysin lyses the erythrocytes of ox, sheep and goat, but not those of rabbit, ferret, guinea-pig, monkey, horse, rat and mouse (Elek, 1959). Reports of the sensitivity of the erythrocytes of man are conflicting. Virtually all investigations, however, have been carried out in ignorance of the effect of certain metal ions upon haemolysis by beta-lysin.

In the present work, the erythrocytes of 13 animal species were tested with partially purified beta-lysins from strains R1 and 252F. Phosphate-saline buffer containing 0-002M Mg\(^{++}\) ions was employed as the suspending fluid for the erythrocytes. Results are presented in table VI. Horse, guinea-pig and dog erythrocytes were not lysed by the beta-lysin of either strain. The highest titres occurred with sheep, ox and human erythrocytes, in descending order, and the red cells of the cat, rabbit and pig occupied an intermediate position in susceptibility to lysis.
Action of mild reducing agents upon haemolysis

Results shown in table VII indicate that ascorbic acid inactivates the beta-lysins of strains R1 and 252F, and that for both lysin preparations the inactivation is proportional to the concentration of the ascorbic acid and is virtually complete at a concentration of 0.1M.

TABLE VI
Spectrum of erythrocyte sensitivity to partially purified beta-lysins of strains R1 and 252F

<table>
<thead>
<tr>
<th>Erythrocyte species</th>
<th>Haemolytic titres (units per ml.) of beta-lysin from</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R1</td>
<td>252F</td>
</tr>
<tr>
<td>Horse</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Guinea-pig</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Dog</td>
<td>&lt;4 (4)*</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Frog</td>
<td>&lt;4 (4)</td>
<td>&lt;4 (4)</td>
</tr>
<tr>
<td>Mouse</td>
<td>&lt;4 (8)</td>
<td>&lt;4 (8)</td>
</tr>
<tr>
<td>Rat</td>
<td>&lt;4 (32)</td>
<td>&lt;4 (32)</td>
</tr>
<tr>
<td>Fowl</td>
<td>&lt;4 (64)</td>
<td>&lt;4 (64)</td>
</tr>
<tr>
<td>Pig</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Rabbit</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>Cat</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Man</td>
<td>256</td>
<td>128</td>
</tr>
<tr>
<td>Ox</td>
<td>512</td>
<td>512</td>
</tr>
<tr>
<td>Sheep</td>
<td>2048</td>
<td>512</td>
</tr>
</tbody>
</table>

* Trace of haemolysis at dilution stated in brackets.

Cysteine at a concentration of 0.01M markedly inactivated the beta-lysin of strain R1, but it showed little effect on that of strain 252F, the difference observed between control and test titres being within the range of experimental error.

In an attempt to reverse the inactivation of beta-lysin by ascorbic acid, 5-ml. mixtures of the lysin and reducing agent were dialysed for 24 hr against a 3-litre volume of phosphate-buffered saline at 4° C. If the inactivation were reversible, an increase in haemolytic titre could be expected. Results indicated that the action of ascorbic acid upon the beta-lysin of these strains was not reversed by dialysis of the ascorbic acid-lysin complex against isotonic buffer for 24 hr. This finding contrasts with that of Mercier (1938) regarding the inhibition of alpha-lysin by ascorbic acid.

Heat stability of the beta-lysin

The sensitivity of partially purified beta-lysins from strains R1 and 252F to heating at 60° and 100° C was studied.

Results (table VIII) show that heat-inactivation of the R1 lysin proceeded slowly at 60° C until only c. 3 per cent. of the haemolytic
activity remained after heating for 2 hr. On heating at 100°C inactivation was more rapid, and no activity remained after 1 hr.

**Table VII**

*Effect of the reducing agents ascorbic acid and cysteine on the haemolysis of sheep erythrocytes by partially purified beta-lysin from strains R1 and 252F*

<table>
<thead>
<tr>
<th>Molarity of reducing agent</th>
<th>Sheep-cell haemolytic titre (units per ml.) of lyisin in presence of reducing agent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ascorbic acid</td>
</tr>
<tr>
<td></td>
<td>R1 lyisin</td>
</tr>
<tr>
<td>0·1</td>
<td>20</td>
</tr>
<tr>
<td>0·01</td>
<td>1280</td>
</tr>
<tr>
<td>0·001</td>
<td>2560</td>
</tr>
<tr>
<td>0·0001</td>
<td>5120</td>
</tr>
<tr>
<td>0</td>
<td>20,480</td>
</tr>
</tbody>
</table>

With the lysin of strain 252F, relatively little inactivation occurred over a 2-hr period of heating at 60°C, but all haemolytic activity was destroyed by heating for 1 hr at 100°C.

**Table VIII**

*Heat sensitivity of partially purified beta-lysin from strains R1 and 252F*

<table>
<thead>
<tr>
<th>Time of heating at stated temperature (min.)</th>
<th>Haemolytic titres with sheep cells (units per ml.) of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R1 lyasin after heating at 60°C</td>
</tr>
<tr>
<td>0</td>
<td>2048</td>
</tr>
<tr>
<td>3</td>
<td>2048</td>
</tr>
<tr>
<td>6</td>
<td>1024</td>
</tr>
<tr>
<td>9</td>
<td>1024</td>
</tr>
<tr>
<td>12</td>
<td>1024</td>
</tr>
<tr>
<td>15</td>
<td>1024</td>
</tr>
<tr>
<td>30</td>
<td>256</td>
</tr>
<tr>
<td>60</td>
<td>128</td>
</tr>
<tr>
<td>120</td>
<td>64</td>
</tr>
</tbody>
</table>

Since Kodama and Kojima had stated that the beta-lysin may be subject to the same anomalous behaviour toward heat as the alpha-lysin, an experiment was made to investigate this possibility. Two samples of beta-lysin from strain R1 were employed. The first was
heated at 60° C for 2 hr and then at 100° C for 3 min. The second received the same treatment but was heated for 6 min. at 100° C. It appeared that denaturation resulting from heating at 60° C for 2 hr could not be reversed by heating at higher temperatures (100° C) for 3–6 min.

*Effect of trypsin upon the haemolytic activity of beta-lysin*

In view of the observation of Heydrick and Chesbro, the sensitivity of partially purified beta-lysins from strains R1 and 252F to trypsin was investigated. Beta-lysin adjusted to pH 8-0 was added in equal volume to 0-01M borate buffer, pH 8-0, containing 0-5 mg. per ml. of crystalline trypsin (British Drug Houses), and the mixture was incubated in a waterbath at 37° C for 6 hr. Lysin controls were incubated at pH 8-0 for the same length of time without showing any loss of activity.

Results indicated that the beta-lysins of strains R1 and 252F are trypsin-sensitive, only 0-4-0-8 per cent. of their haemolytic activity remaining after incubation for 6 hr.

*Kinetic studies of haemolysis of sheep erythrocytes by beta-lysin*

The interrelationships of time, extent of haemolysis, and haemolysin concentration have been studied with partially purified R1 and 252F beta-lysins.

Results shown in fig. 1 indicated that for the R1 lysin, the plot of time versus extent of haemolysis over lysin concentrations ranging from 0-005 to 0-100 ml. lysin per 4 ml. mixture is a sigmoid curve. A common curve was obtained with 0-04-0-10 ml. amounts of lysin, and also for 0-02 and 0-03 ml. amounts, although the similarity in the latter case is no doubt the result of experimental error. A decrease in lysin concentration tended to decrease the slope of the linear portion of the curve and at very low lysin concentrations (0-005 ml. per 4 ml.), the slope approached zero.

Reference to fig. 2 shows that the curve obtained when the rate of haemolysis is plotted against the concentration of lysin is linear at low concentrations (0-0-03 ml. lysin per 4 ml. mixture). At higher concentrations (0-04-0-10 ml. per 4 ml.), the reaction velocity is constant. The rate of haemolysis was determined by calculating the slope of the mid-portion of the linear section of the sigmoid curves of the time-haemolysis plot.

Results obtained with beta-lysin from strain 252F were similar.

*In-vivo activity of beta-lysin*

*Effect of beta-lysin on mice.* Samples of crude and partially purified beta-lysin from strains R1 and 252F were separately injected into mice, either intravenously or intraperitoneally. The haemolytic titres of the preparations with rabbit, human and sheep erythrocytes
were determined. The partially purified materials contained relatively little activity against mouse red cells (table VI).

![Graph showing the relation between the extent of haemolysis of sheep erythrocytes in different concentrations of partially purified R1 beta-lysin and the duration of holding of the red cell/lysin mixture at 25°C. The extent of haemolysis is indicated by the decrease in the optical density of the mixture. The lyasin concentration is given as ml. of lyasin preparation per 4 ml. mixture.]

**Fig. 1.**—Relation between the extent of haemolysis of sheep erythrocytes in different concentrations of partially purified R1 beta-lysin and the duration of holding of the red cell/lysin mixture at 25°C. The extent of haemolysis is indicated by the decrease in the optical density of the mixture. The lyasin concentration is given as ml. of lyasin preparation per 4 ml. mixture.

It was found that intravenous or intraperitoneal injection of partially purified beta-lysin from either strain was not lethal to mice at the dosages employed. Some of the mice given intravenous injections of crude lyasin from strain R1 died, but these deaths were probably not
due to the content of beta-lysin in the crude lysin, since the sheep-cell and human-cell haemolytic titres of the crude lysin were only a small fraction of those of the partially purified lysin, although the rabbit-cell titres of the crude and partially purified lysins were identical.

With intraperitoneal injection the results were similar, a lethal effect being obtained with crude, but not with partially purified R1 beta-lysin.

Effect of beta-lysin on rabbits. Results obtained from the intravenous inoculation of rabbits suggested that partially purified R1 and 252F beta-lysins were not lethal at the doses employed. In contrast, the rabbit receiving crude lysins died within 12 hr. As in the experiments on intravenous inoculation of mice, the human-cell and sheep-cell haemolytic titres of the crude material were considerably less than those of the partially purified preparation, although the titres with rabbit red cells were similar.
On intradermal inoculation in rabbits, the partially purified preparations from both strains elicited erythema with slight swelling at the site of injection. This reaction appeared to be very mild. Injection of crude R1 lysis produced very severe necrosis, but injection of crude 252F lysis produced a mild reaction similar to that obtained with the partially purified material.

**Discussion**

The preparations of beta-lysin partially purified by the method of adsorption on hydroxylapatite and dialysis contained little or no alpha- and delta-lysins. Reference to Table V indicates that the preparations from strains R1 and 252F did not lyse guinea-pig or horse red cells, which, according to Williams and Harper (1947), are attacked by delta-lysin. Agar gel diffusion studies carried out on partially purified R1 and 252F beta-lysin preparations and on purified delta-lysin indicated that the 252F preparation did not contain delta-lysin. Gladstone (unpublished observation) has told me that purified delta-lysin initiates a severe dermonecrosis in rabbits, but I observed no such reaction with either of the beta-lysin preparations and this suggests that they did not contain either alpha- or delta-lysin in any quantity.

Crude lysis from strain R1, but not the partially purified beta-lysin derived from it, was lethal for rabbits and mice. This finding further confirmed the absence of significant quantities of alpha-lysin from the partially purified beta-lysin preparation. In addition, the haemolytic activity of the partially purified beta-lysins towards rabbit and human red cells was almost completely inhibited by EDTA and citric acid, whilst, as was also shown by Robinson, Thatcher and Montford (1960) and Jackson and Little (1958), the activity of the alpha- and delta-lysins was unaffected by these chelating agents.

Since both EDTA and citric acid inhibited the haemolytic activity of the partially purified R1 and 252F beta-lysins, it was thought that metallic cations might play some role in haemolysis. The haemolytic activity of the two beta-lysins was considerably intensified in the presence of Mg++, Co++ and Mn++ ions, but not in that of Ni++, Ca++ or Zn++ ions. The effect of these ions cannot be explained by the position of the elements in the periodic table. It was surprising that neither Ni++ nor Ca++ ions enhanced the haemolytic activity of the beta-lysin, as would be expected from the relationships of Ni to Co and Ca to Mg. The data acquired about the effects of these ions and chelating agents suggest that the "hot-cold" haemolysin affecting sheep red cells isolated by Robinson, Thatcher and Gagnon (1958) was almost certainly the beta-lysin, although the haemolytic activity of their preparation was enhanced by Ni++ ions. Dialysis of preparations of beta-lysin for 24 hr against running water did not result in the same loss of activity as that reported by Jackson and Mayman (1958), but since these authors did not record the length of time for which their material was dialysed, the discrepancy may merely be due to a difference in the time of dialysis.
Differences in the degree of hardness of the water in the two laboratories may also have been important.

The present work has shown that earlier reports on the red-cell spectrum of the beta-lysin must be re-interpreted in the light of the lysin's sensitivity to metal ions. As this investigation has shown in the case of the partially purified beta-lysins from two strains, the presence of 0.001M Mg\(^{++}\) ions greatly alters the sensitivity of rabbit, human and sheep erythrocytes to the lysin. The alteration is so great that beta-lysin preparations that did not lyse rabbit and human erythrocytes in the absence of Mg\(^{++}\) ions, did so when these ions were added. However, the activity of beta-lysin with rabbit red cells is low in comparison to that with sheep cells. Elek has stated that reports of the sensitivity of human erythrocytes to beta-lysin are variable, but in the present study it has been found that both of the purified preparations of beta-lysin caused lysis of human erythrocytes in the presence of Mg\(^{++}\) ions. Finally, regarding the "hot-cold" haemolytic effect, it should be noted that these two preparations did not lyse human, rabbit or sheep erythrocytes at 37\(^\circ\) C, but lysed all three species of cells after a period of holding at 4\(^\circ\) C. This finding provides support for the observations of Roy (1937) and Kodama and Kojima (1938) that beta-lysin causes "hot-cold" haemolysis of human red cells.

Conflicting reports about the thermostability of the beta-lysin may be due to (1) variations in the pH and ionic strength at which the test is conducted, (2) the use of preparations of differing degrees of purity, and (3) the existence of more than one kind of beta-lysin. Data acquired in a study of the sensitivity of beta-lysin to heat indicate that, under the conditions specified, the lysin from strain R1 was inactivated slowly at 60\(^\circ\) C, and was almost completely destroyed in 2 hr, whereas the lysin from strain 252F was little affected by heating at 60\(^\circ\) C. The activity of both preparations, however, was destroyed by heating at 100\(^\circ\) C for 1 hr. Contrary to statements by Kodama and Kojima, and Elek, there is reason to believe that the beta-lysin is not subject to the paradoxical behaviour to heat that characterises the alpha-lysin. In view of the fact that Lominski and Arbuthnott (1962) found that crude preparations of alpha-lysin were reversibly inactivated at 60\(^\circ\) C, whereas partially purified preparations were irreversibly denatured, the contradiction between my data and those obtained by Kodama and Kojima may be explained in terms of the degree of purity of the beta-lysins employed.

Kinetic studies of the haemolysis of sheep erythrocytes during incubation with partially purified beta-lysin at 25\(^\circ\) C indicated that the time-haemolysis curve is similar to that of most of the other lytic agents of bacterial origin that have so far been investigated. The sigmoid shapes of these curves may be due to the differences in sensitivity of individual members of the erythrocyte population to lysis. When the rate of haemolysis is plotted against the concentration of lysin, the reaction velocity obtained with both preparations is linear at low
concentrations of lysin, but levels out at higher ones. These observa-
tions of mine on beta-lysin are similar to those of Robinson, Thatcher and Gagnon on their unidentified, metal-ion-dependent sheep-cell lysin, and to those of Lominski and Arbuthnott on alpha-lysin. Lominski and Arbuthnott have interpreted this curve as indicative of an enzymic relationship between the haemolysin and the substrate in the red cell. This may be true, but such plateaux could be the result of an experimental error in measuring reaction velocities that are greater than a certain figure, and the presence of the plateaux would in that case depend upon the limited sensitivity of the method used to obtain the curves. The difficulty of making such studies with the beta-lysin is increased by the possibility that the curves obtained might not reflect the actual reaction between the beta-lysin and the red-cell substrate. The lysin-substrate reaction presumably takes place at 37°C without the occurrence of lysis and the curve observed may merely reflect the kinetics of the cold-shock at 25°C that precipitates the lysis. Bernheimer (1947) found that for non-bacterial lysins the relation between lysin concentration and rate of haemolysis was exponential and that the relation for bacterial haemolysins was a directly proportional one. He also stated that since all the bacterial haemolysins showing this latter relation are proteins, they are probably enzymes. Since the two beta-lysins employed were sensitive to destruction by trypsin they appear to be proteins, and if the occurrence of haemolysis can be assumed to mirror the reaction between the lysin and its red-cell sub-
strate, the results of the kinetic studies on the two products suggest that they are enzymes.

It has been shown that the mild reducing agents, ascorbic acid and cysteine, will inactivate the beta-lysin of both strains, although the effect of cysteine upon the 252F lysin was less evident than its effect on the R1 lysin. In contrast with the observation of Mercier that ascorbic acid reversibly inhibited the haemolytic activity of the alpha-
lysin, no such finding was observed in the case of the beta-lysin. Jackson and Benns (1961) claimed that so-called "gamma"-lysin could be differentiated from delta-lysin because the former was inactivated by ascorbic acid and cysteine, and the latter was not. It thus appears that these reducing reagents may be useful in the classification of staphylococcal and other bacterial haemolysins.

In the investigation of the effect of beta-lysin on a small number of rabbits and mice, it was found that partially purified beta-lysins from strains R1 and 252F had no lethal effect upon either animal at the dosage employed. With respect to the lack of toxicity in rabbits, these findings are at variance with the observations of a number of investigators. Reasons for the difference might be: (1) variations in the sensitivity of the animals, (2) differences in the numbers of animals used, (3) incomplete removal of the alpha-lysin from the preparations found toxic, (4) the existence of toxic factors other than haemolysins in some strains, (5) differences in dosage, and (6) the existence of more than one beta-lysin.
In the present study, erythema with slight swelling was observed in rabbits given intradermal injections of partially purified R1 and 252F preparations. In the case of the R1 preparation, it could be argued that small amounts of alpha- or delta-lysin were present and that these were responsible for the tissue reaction. However, the 252F preparations, which gave the same type of reaction, were shown to be free from alpha- and delta-lysins in tests by the technique of Elek and Levy and the immunodiffusion method of Ouchterlony, and also in the finding that their haemolytic actions on rabbit, human and sheep red cells were completely inhibited by EDTA.

In the report of Heydrick and Chesbro that beta-lysin, when activated by Mg++ ions, is lethal to guinea-pigs on intraperitoneal injection, no reference was made to the in-vivo level of Mg++ ions. The Mg++ level in the plasma in mice and rabbits averages 0.0010 and 0.0013 respectively (Eveleth, 1937) and, in the present investigation, beta-lysin preparations were dissolved in buffer containing 0.001M Mg++ ions. It would appear, therefore, that the in-vivo conditions in rabbits and mice are optimal for the enhancement or activation of haemolytic activity but, as already stated, no such activity would be observed at 37°C. The implication is that if beta-lysin has any toxic action, this is not related to the haemolytic activity, and it must require some other factor than Mg++ ions for its activation.

During the course of the experimental work, certain differences were observed between the beta-lysin preparations from strains R1 and 252F. These were (1) in the degree of enhancement of their haemolytic activity in the presence of metallic ions, (2) in the degree of their inactivation with cysteine, and (3) in their thermostability at 60°C. The reason for these differences may be either experimental error or the existence of different kinds of beta-lysin. It was found that antiserum prepared against the 252F beta-lysin would neutralise the R1 beta-lysin. If therefore, more than one kind of beta-lysin exists, one is faced with the concept of two lysins that are serologically similar but possess different chemical characteristics. Robinson, Thatcher and Gagnon made similar observations in connexion with two haemolysins that were not the same in character but were serologically identical. The work of these authors and that of Thaysen (1948), Heydrick and Chesbro (1962) and Riaz-ul-Haque and Baldwin (1963) indicates that the existence of a second beta-lysin cannot be excluded.

Summary

Beta-lysin from several strains of *Staphylococcus aureus*, some of which also produced alpha- and delta-lysins, was partially purified by selective adsorption to hydroxylapatite and dialysis.

The haemolytic activity of the partially purified beta-lysin was inhibited by the chelating agents citric acid and EDTA, and was greatly enhanced by the presence of certain divalent metallic cations, Mg++ and Co++ in particular. The degree of enhancement with Mg++ ions
was, however, not identical for all the preparations of beta-lysin investigated.

The erythrocyte sensitivity spectrum of beta-lysin was reviewed in the light of its behaviour in the presence of Mg++ ions. The erythrocytes of sheep, ox and man were most sensitive, and those of horse, guinea-pig and dog were not affected. The erythrocytes of the cat, rabbit, pig, fowl, rat, mouse and frog occupied an intermediate position.

The haemolytic activity of the beta-lysin preparations was irreversibly inhibited by the mild reducing agents ascorbic acid and cysteine, and it is suggested that these reactions may be useful in the classification of bacterial haemolysins.

The beta-lysin prepared from one strain of Staph. aureus, but not that prepared from another strain, was almost completely inactivated by heating at 60° C for 2 hr; both lysins were inactivated by heating at 100° C for 1 hr.

In contrast with another report, beta-lysin was found to be sensitive to inactivation by crystalline trypsin.

Kinetic studies of the haemolytic action of partially purified beta-lysin at 25° C showed that the time-haemolysis curve was sigmoid and that when the rate of haemolysis was plotted against lyasin concentration, the curve obtained was linear at low concentrations but levelled out at higher ones. Such a curve may be indicative of an enzymic reaction.

Intravenous injection of partially purified beta-lysin had no lethal effect on either rabbits or mice. Erythema with slight swelling was, however, observed in rabbits receiving intradermal injections.

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BETALYSIN OF STAPHYLOCOCCUS AUREUS

FACTORS AFFECTING THE SENSITIZATION OF SHEEP ERYTHROCYTES TO STAPHYLOCOCCAL BETA LYSIN

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Abstract

It is well known that the beta hemolysin of Staphylococcus aureus is unable to cause lysis of erythrocytes at 37 °C but will do so when the temperature is lowered. Sheep erythrocytes sensitized to partially purified beta lysin were, however, lysed at 37 °C by NaCl at concentrations less but not greater than 0.80-0.85%. Increasing acidity rather than increasing alkalinity was also effective in causing lysis of erythrocytes at this temperature. These observations suggest that the beta lysin-sensitized red cells are much more sensitive to osmotic swelling than are normal cells.

Kinetic studies of the effect of time, lysoin concentration, pH, and temperature on sensitization of sheep erythrocytes to beta lysin indicate that the reaction may be enzymatic. This concept is further supported by the Mg++ ion requirement of the beta lysin and its sensitivity to the action of trypsin.

Introduction

The hemolysins of Staphylococcus aureus, particularly the alpha and delta lysins, have for some time occupied the attention of a number of investigators. Relatively little consideration has been given to the nature of the beta lysin, however, or to the fact that erythrocytes sensitized to this lysoin will not lyse at 37 °C, but only when the temperature is lowered. Bigger (2) believed that interfering substances resulting from the use of unsuitable media, prolonged incubation, or “too alkaline a reaction” were the cause of this “hot-cold” lytic effect. Pulsford (7) also dealt with this phenomenon as it appeared on sheep blood agar. He found that the red cells incubated in the presence of beta lysin became fragile, hemolysis being effected at 37 °C by rapid changing of the pH or rendering of the NaCl concentration less or greater than 0.85%.

Doery et al. (3) claim that the beta lysin is an enzyme attacking sphingomyelin, but it is not known how this relates to the “hot-cold” phenomenon of hemolysis.

In the present paper, Pulsford’s observations have been confirmed and extended to include a study of the kinetics of the reaction between partially purified beta lysin and its substrate in the erythrocyte surface.

Materials and Methods

The R-1 strain of S. aureus, methods of production and partial purification of beta lysin, preparation of erythrocyte suspensions, and quantitative hemolysin titrations have been described elsewhere (10). In the present investigation, sheep erythrocytes were employed, and the suspending fluid was 0.01 M phosphate–saline buffer at pH 7.0 containing MgCl₂ unless otherwise specified. All other buffers were used at the same concentration and also contained Mg++ ions. When required, the NaCl content was varied, or inhibitors added, and the pH readjusted to neutrality. Purification of the beta lysin was about 30-fold.
and the product appeared not to contain alpha or delta lysins when tested with rabbit and human erythrocytes.

**Experiments on Red Cell Fragility after Sensitization with Beta Lysin**

(a) In the study of the effect of NaCl concentration upon sensitization of erythrocytes, NaCl ranging from 0.43 to 2.50% was tested in phosphate buffer. A Bausch and Lomb Spectronic-20 colorimeter was employed in the determination of the degree of hemolysis, which was expressed as % transmittance at 650 μm. For this purpose a control suspension of 1% red cells was standardized to zero in terms of % transmittance. Complete hemolysis of normal and beta lysin-treated red cells generally gave a reading of 57 ± 3% transmittance.

A 0.90 ml volume of isotonic phosphate-buffered saline was added to a series of colorimeter tubes containing 0.10 ml beta lysin at a potency of 2560 hemolytic units (HU)/ml. These tubes and all reagents were incubated in a water bath at 37 °C for 15-20 minutes until temperature equilibrium was reached. Then 1.00 ml of 4% sheep erythrocytes was rapidly added and the tube agitated in the bath. After incubation for 1 minute to ensure complete sensitization, 2.00 ml of phosphate buffer containing 0-4.15% NaCl was quickly added, the tube agitated again at 37 °C for 10 seconds, and a reading immediately taken in the colorimeter. All tests were carried out in duplicate.

(b) The effect of rapid changes in pH was studied in a similar manner. A 0.5 ml amount of beta lysin was added to a 200 ml "milk dilution" bottle, followed by 19.5 ml of isotonic phosphate buffer at pH 7.0, and 20.0 ml of 2% erythrocytes. The contents were mixed and incubated at 37 °C for 1 minute at which time the appropriate amount of 1 N acid or alkali was added to vary the pH from 7.0 to 6.0 and 9.8 respectively. The reactants were further agitated and incubated for 1 minute at the temperature of the bath before a reading was taken in the colorimeter. The amount of acid or alkali required to change the pH to the desired levels was predetermined so that all final test volumes could be made identical. The change in ionic strength due to the addition of the acid or alkali was calculated to be very small.

(c) The effect of inhibitors on hemolysis of sensitized red cells was investigated using NaCl and glucose. The latter was employed because various sugars have been reported to inhibit hemolysis of erythrocytes in the presence of bacterial lysins (Bernheimer (1), Jackson and Mayman (5), and others). Sodium chloride will also inhibit hemolysis at certain concentrations. The method employed was identical with that in (a), except that 2.00 ml of inhibitor dissolved in isotonic phosphate buffer was added, the tubes agitated and incubated further at 37 °C for 1 minute, and then plunged into cold water (about 10 °C) for 10 minutes. Readings were taken in the colorimeter as usual. Concentrations of NaCl and glucose ranged from 0.5 to 3.0%, and of beta lysin, 2560 HU/ml.

**Kinetic Studies of the Sensitization of Erythrocytes to Beta Lysin**

(d) The effect of time and lysin concentration upon sensitization was studied employing final concentrations of beta lysin ranging from 8 to 64 HU/ml. The method used was the same as under (a) above, with the exception that 2.00 ml of 0.66% NaCl in phosphate buffer was added to the system.
This resulted in a final concentration of 0.745% NaCl which lysed sensitized but not control red cell suspensions at 37 °C. Readings taken in the colorimeter from 0 to 5 minutes at 15-second intervals were recorded.

(e) The effect of temperature on rate of sensitization was investigated according to the procedure in (a). Observations were made over the range of 5–45 °C in a water bath. In this experiment, a constant amount of beta lysin (64 HU/ml) was used.

(f) In an experiment designed to determine the relationship of pH to rate of sensitization, observations were made over a pH range of 5.0–9.0 at 0.5-unit intervals employing a constant amount of beta lysin as in (e). Acetate and phthalate buffers were employed at a pH of 5.0–6.0, phosphate from 6.0 to 7.5, borate from 8.0 to 9.0, and veronal from 7.0 to 9.0. Erythrocytes and beta lysin were incubated together as previously described under (a) at the appropriate pH and after reaction had occurred, buffer at the same pH containing 0.66% NaCl was added, the contents of the tube mixed, and a reading taken. It has already been pointed out that the use of hypotonic saline showed whether sensitization had taken place.

Calculation of Reaction Rates
This was done by determining the reciprocal of the time in seconds required for the reactants to reach the level of 30% transmittance (% T₃₀).

Results
Effect of Rapid Changes in Salt Concentration and pH on Sensitized Sheep Erythrocytes
Results in Table I indicate that sheep erythrocytes which have been sensitized to beta lysin are unstable, even though no hemolysis has occurred. Final salt concentrations ranging from 0.43 to 0.75% caused nearly complete hemolysis of sheep red cells, but none was observed at concentrations greater than 0.80%. It thus appears that hypotonic but not hypertonic saline solutions are effective in causing lysis of such red cells, a decrease in concentration of as little as 0.05% less than 0.80% NaCl producing virtually complete hemolysis.

<table>
<thead>
<tr>
<th>Final % NaCl</th>
<th>% transmittance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.43</td>
<td>54.5*</td>
</tr>
<tr>
<td>0.55</td>
<td>52</td>
</tr>
<tr>
<td>0.66</td>
<td>53</td>
</tr>
<tr>
<td>0.745</td>
<td>55.5</td>
</tr>
<tr>
<td>0.80–2.50</td>
<td>0</td>
</tr>
</tbody>
</table>

*Complete lysis corresponds to 37 ± 3. No lysis observed in NaCl controls.

With regard to rapid changes in pH, it is evident from Table II that alterations from neutral to acid pH (6.9–6.0) are capable of lysing sensitized erythrocytes, the effect being greatest when the pH is changed one unit from 7.0 to
6.0. Smaller changes also caused some hemolysis but not to the same extent. Changes in pH from neutral to alkaline conditions did not affect the sensitized red cells over the range tested.

**Effect of Inhibitors on Hemolysis of Sensitized Sheep Erythrocytes**

Both NaCl and glucose solutions were tested for their ability to inhibit hemolysis of red cells sensitized to beta lysin. Glucose varying in concentration from 0.5 to 3.0% (Fig. 1) caused increasing inhibition. In the case of NaCl, the degree of inhibition was much greater over the range of concentrations tested, a maximum being observed between 2.0 and 2.5%. At levels above 2.5%, inhibition decreased.

**Effect of Lysin Concentration, pH, and Temperature on Kinetics of Sensitization**

Once it was established that erythrocytes sensitized to beta lysin were lysed by hypotonic saline, a study of the kinetics of the reaction between beta

---

**TABLE II**

Effect of pH gradient on sheep erythrocytes sensitized to beta lysin

<table>
<thead>
<tr>
<th>Final concn. beta lysin</th>
<th>Final pH</th>
<th>% transmittance</th>
</tr>
</thead>
<tbody>
<tr>
<td>64</td>
<td>6.0</td>
<td>48</td>
</tr>
<tr>
<td>&quot;</td>
<td>6.3</td>
<td>25</td>
</tr>
<tr>
<td>&quot;</td>
<td>6.5</td>
<td>13</td>
</tr>
<tr>
<td>&quot;</td>
<td>6.6</td>
<td>3</td>
</tr>
<tr>
<td>&quot;</td>
<td>6.9-9.8</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>6.0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>9.8</td>
<td>0</td>
</tr>
<tr>
<td>64</td>
<td>7.0</td>
<td>60*</td>
</tr>
</tbody>
</table>

*Chilled to about 10 °C after exposure to lysin for 1 minute at 37 °C. This control was included to show that the beta lysin employed was fully active at this concentration.

---

**Fig. 1.** Effect of inhibitors on hemolysis of sheep erythrocytes sensitized to staphylococcal beta lysin. The degree of inhibition increases with a decrease in % transmittance.
Wiseman: Sheep Erythrocytes

lysin and its substrate became possible. In Fig. 2, the time-hemolysis curves obtained at concentrations of beta lysin ranging from 12 to 64 HU/ml show that a decreasing lysin concentration caused a corresponding increase in the time required to lyse 50% Tₜ₀ of the red cells. When lysin concentration was plotted against the reaction rate (Fig. 3), a set of points approximating a straight line was obtained, indicating that the velocity of the reaction is directly proportional to lysin concentration between 12 and 64 HU/ml. These results are similar to those obtained with phospholipase C of Clostridium perfringens acting on horse erythrocytes by Ikezawa and Murata (4).

Fig. 2. Relationship of lysin concentration to time required for sensitization of sheep erythrocytes to beta lysin.

Fig. 3. Relationship of beta lysin concentration to the rate of its reaction with sheep erythrocytes. See text for calculation of reaction rates.
Concerning the effect of pH on constant amounts of beta lysin (64 HU/ml) in respect to time, curves like those of Fig. 2 were obtained. In Fig. 4, a plot of reaction rate vs. pH showed a maximum between pH 7.0 and 8.0, with con-

![Figure 4](image)

**Fig. 4.** The effect of variation of pH on the rate of reaction of beta lysin with sheep erythrocytes.

![Figure 5](image)

**Fig. 5.** The effect of temperature variations on the rate of reaction of beta lysin with sheep erythrocytes.
Wiseman: Sheep Erythrocytes

Considerable decrease in rate above and below this range. This experiment was carried out employing phthalate and veronal buffers in the appropriate pH ranges. Another experiment in which acetate, phosphate, and borate buffers were used gave similar results. Beta lysin and red cell controls incubated at the extremes of pH used in the experiments and then employed in quantitative titrations indicated that pH values over the range tested did not cause detectable deterioration of either. The buffer salts themselves did not appear to have any effect on hemolysin titers.

The effect of temperature on the time-hemolysis curves was similar to those for pH and lysin concentration. An increase in temperature from 20 to 45°C progressively decreased the time required for sensitization. Reference to Fig. 5 indicates that the velocity of the reaction is directly proportional to temperature over the range tested. From the Arrhenius plot in Fig. 6, the activation energy, \( \mu \), of beta lysin was calculated to be 14,100 cal.

![Fig. 6. Arrhenius plot of data on sensitization of sheep erythrocytes to beta lysin. The value of \( \mu \) was calculated from the slope.](image)

**Discussion**

Hemolysis of erythrocytes at 37°C is not characteristic of the beta lysin of *S. aureus*. However, it can be made to occur if the physical environment of the beta lysin-sensitized red cell is altered. Thus it has been shown that hypotonic saline at concentrations less than 0.80% will, if added to erythrocytes treated with beta lysin, cause hemolysis at 37°C. Concentrations of NaCl greater than this level (0.80-2.50%) do not similarly cause lysis of the erythrocytes. Increasing acidity rather than increasing alkalinity has also been shown to cause hemolysis of the red cells in the presence of beta lysin at 37°C. These observations generally agree with those of Pulsford whose work was mainly
concerned with such effects on blood agar, except that he noted hemolysis of erythrocytes at hypertonic salt concentrations. The reason for this discrepancy is not clear. Hemolysis occurring under conditions of increasing acidity and at NaCl concentrations less than physiological is probably related to an increase in the volume of the red cell under such conditions (Ponder (6)); and inhibition of hemolysis in the presence of glucose and hypertonic NaCl and under alkaline conditions, would tend to support this view. The disparity of the results of Fig. 1 regarding the effect of glucose and NaCl on inhibition of hemolysis is, no doubt, due only to the fact that the latter compound has the smaller molecular weight and is ionized. The degree of osmotic pressure exerted on the cell membrane by each substance would therefore be different.

Sensitization of the red cell, then, appears to increase its susceptibility to conditions which cause an increase in cell volume insofar as the effects of salt and pH are concerned. It is more difficult, however, to explain the effect of a sudden decrease in temperature on sensitized red cells on this basis, since such an increase in volume would presumably not occur as the temperature is lowered. If "hot-cold" hemolysis were due to a sudden contraction in cell volume, one would also expect to observe hemolysis under conditions of hypertonicity and alkalinity. It has been shown in the present study that this is not the case. Nevertheless, the cell volume might change more rapidly with a sudden decrease in temperature than it does when alkali or NaCl at hypertonic concentrations is added to the system, and this alone might be responsible for the "hot-cold" lytic effect.

It can therefore be argued that the beta lysin of *S. aureus* is not a true hemolysin like the alpha and delta hemolysins since the external environment of the erythrocyte has to be altered before lysis occurs. In the case of alpha and delta lysins, hemolysis is a primary effect; that is to say, the lysins act at all temperatures on a substrate directly involved in the maintenance of cellular integrity, while the beta lysin, on the other hand, reacts with a substrate only indirectly implicated in the preservation of the intact erythrocyte. The latter could perhaps be termed a "false" or secondary hemolysin.

From a consideration of the kinetics of sensitization, it appears that the rate of the reaction is directly proportional to lysin concentration and temperature. Also, the value of $\mu$ for beta lysin was calculated to be 14,100 cal and is within the range of 1000–25,000 cal observed for most enzyme-catalyzed reactions (Sizer (8)). By way of comparison, Bernheimer found the values of $\mu$ for pneumolysin, streptolysin S, and *Clostridium perfringens* theta toxin to be 23,800, 14,600, and 19,500 cal respectively.

In conclusion, it is suggested that staphylococcal beta lysin acts in the manner of an enzyme on the basis of its reaction kinetics, its established requirement for Mg$^{++}$ ions (Jackson and Mayman (5), Wiseman (10)), and its protein nature (10). In view of the fact that magnesium is the natural activator of most enzymes attacking phosphorylated substrates, it is reasonable to suppose that the beta lysin is a phospholipase other than lecithinase, since Turner (9) has shown that sheep erythrocytes contain no lecithins. Doery *et al.* (3) have recently produced evidence that at least two phospholipases are present in a number of staphylococcal culture filtrates. Using fractions of undefined purity,
they found that one of these hydrolyzes sphingomyelin and is closely correlated with the presence in the filtrate of beta lyasin. Thus it is now possible, in view of these results and those obtained in the present paper, to put forward a case for the enzymatic nature of this lyasin. Final proof, however, must await its more complete purification.

References

I. MODE OF ACTION

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Received August 5, 1966

The mode of action of highly purified beta hemolysin derived from the R-1 strain of \textit{Staphylococcus aureus} has been investigated. Sphingomyelin was absent from lipid extracts of sheep erythrocytes treated with beta hemolysin when compared to normal cells. A correlation was also established between the sphingomyelin content of other erythrocyte species and their sensitivity to beta hemolysin. Further investigations revealed that sphingomyelin is hydrolyzed to yield phosphorylcholine and N-acyl sphingosine. Thus, the mode of action of the beta hemolysin is like that of phospholipase C. Various phosphate compounds other than sphingomyelin, including RNA, glycerophosphate, phenylphosphate, phosphatidylethanolamine, and phosphatidylincholine were tested as substrates, but virtually no hydrolysis was observed. In contrast with the results of other workers, R-1 beta hemolysin did not release detectable amounts of carbohydrate from staphylococcal cell walls.

Introduction

Concurrent with the present chemical approach to problems of microbial pathogenicity and virulence, the exotoxins of \textit{Staphylococcus aureus} have received close attention. The beta hemolysin has recently attracted some interest as a result of the suggestion that it is a phospholipase which hydrolyzes sphingomyelin with the liberation of the two nitrogenous bases, sphingosine and phosphorylated choline (5). Chesbro et al. (2) found that treatment of rabbit erythrocytes and staphylococcal or streptococcal cell walls with the beta hemolysin released a variety of compounds including polysaccharides, mucoproteins, glucose, rhamnose, N-acetylhexosamine, and an unidentified monosaccharide. Their preparation was not tested for phospholipase activity.

The object of the present investigation was to study the mode of action of staphylococcal beta hemolysin in the light of these discrepancies.

Materials and Methods

Descriptions of the R-1 and 252F strains of \textit{S. aureus}, methods of production and quantitative titration of the beta hemolysin have been published elsewhere (17). The E-delta strain of \textit{S. aureus} employed as a source of cell wall material was obtained from Professor S. Elek of St. George's Hospital Medical School, London, England.

Purification of the Beta Hemolysin

The first step in the procedure involved adsorption of the beta hemolysin onto hydroxylapatite followed by elution with NaCl, dialysis, and freeze-drying (17). The freeze-dried material was then dissolved and dialyzed at 4°C against 0.01 M acetate buffer, pH 4.0, for 24 h with two changes of buffer. A slight precipitate developed at this stage and was removed by centrifugation at 40,000 g for 30–45 min. The clear supernatant fluid was
retained and dialyzed against two changes of distilled water for 24 h at 4 °C and freeze-dried. Further purification was achieved on a column of Sephadex G-100. The freeze-dried hemolysin preparation was dissolved in 0.05 M phosphate buffer at pH 7.0 and applied in 10-ml volumes to a column measuring 2 X 60 cm. Ten-millimeter fractions were collected and it was found that the active material began to appear in the effluent after 70 ml of buffer had passed through the column. Fractions containing the greatest amount of hemolytic activity were pooled, dialyzed against distilled water for 24 h, and freeze-dried. Storage of the dialyzed solution of hemolysin at -20 °C also proved to be an acceptable method of preservation.

When crude and partially purified material were compared, the overall increase in specific activity (hemolytic units of hemolysin per milligram of protein) was about 70-fold. Analysis of the fractions by agar gel diffusion indicated the presence of two antigens, but since additional tests of homogeneity were not carried out, it was difficult to be certain that other antigens were not present. No detectable alpha and delta hemolysins, coagulase, lipase other than the one under study, hyaluronidase, or deoxyribonuclease were present in the partially purified material. Alpha and delta hemolysins were assayed according to the method of Wiseman (17), coagulase by the method of Jackson and Little (7), lipase according to that of Marks (10), and hyaluronidase and deoxyribonuclease by the techniques of Tolksdorf et al. (15) and Bernheimer and Schwartz (1) respectively.

Reagents
Silicic acid (SIL-R grade), with which papers used in phospholipid chromatography were impregnated, DL-alpha lecithin (dipalmitoyl), phosphatidylethanolamine, and ribonucleic acid substrates were obtained from the Sigma Chemical Co. of St. Louis, Mo. Sodium beta glycerophosphate was purchased from British Drug Houses, Toronto, Ont. Highly purified sphingomyelin from beef brain, and DL-alpha cephalin (dipalmitoyl) were obtained from Mann Biochemicals, New York, N.Y. Disodium phenylphosphate was purchased from Eastman Organic Chemicals, Rochester, N.Y.

Buffers were prepared according to the instructions of Cruickshank (3). In experiments requiring phosphorus determinations, tris(hydroxymethyl)aminomethane buffer (Tris) was used.

Phosphorus and Nitrogen Determinations
Phosphorus was assayed by the method of Fiske and Subbarow (6), using a Beckman DU spectrophotometer at 680 mg with a 1-cm light path. Nitrogen was determined by a microKjeldahl method in which the Aminco nitrogen apparatus (American Instrument Co., Silver Springs, Md.) was used.

Chromatography of Phospholipids
Phospholipid extracts of erythrocytes were prepared by the method of Rose and Oklander (13). Phospholipids in the extracts were chromatographed according to the method of Marinetti (9).

Preparation of Cell Wall Suspensions of Staphylococci
Cell walls were prepared by the method of Chesbro et al. (2).
Results

Experiment 1. Effect of Beta Hemolysin on Sheep Erythrocytes

Quantities of sheep erythrocytes were washed three times in 0.01 M phosphate-buffered saline, pH 7.0, by centrifugation at 1000 g such that the yield of packed cells was 5 ml. To this volume of cells was added beta hemolysin to give a final concentration of 500 hemolytic units (HU/ml.). The mixtures were then incubated for 1 h at 37 °C and the phospholipids subsequently extracted from the intact cells (in the control) or from the cell debris in the erythrocyte–hemolysin mixture and separated chromatographically.

Results illustrated in Fig. 1 show that cephalin, lecithin, and sphingomyelin were detected in extracts obtained from intact sheep erythrocytes, although only cephalin and lecithin were detected in extracts obtained from hemolysin-treated cells.

Similar results were obtained with beta hemolysin isolated from cultures of the 252F strain of S. aureus.

Experiment 2. Beta Hemolysin Sensitivity Spectrum of Mammalian Erythrocytes

Red cells of the various animal species used were obtained either locally or from the Colorado Serum Co., Denver, Colo. The cells were prepared and the experiment carried out as described above, except that Tris was substituted for phosphate buffer. Sphingomyelin in the extracts was located by chromatography and then eluted with methanol. Elution was quantitative and areas of paper which had contained sphingomyelin no longer fluoresced under ultraviolet light after treatment with the solvent. Phosphorus determinations were carried out on the extracts.
The phosphorus content of the sphingomyelin spot was compared to the total phosphorus of the initial extract in Table I. A correlation between sphingomyelin content and sensitivity of red cells to β-hemolysin has been established except for goat cells. The values obtained for sphingomyelin content of sheep, ox, and goat cells are in close agreement with those of Dawson (4). There is a difference in the figures for horse cells, however.

**Experiment 3. Identification of Reaction Products of Sphingomyelin Treated with Beta Hemolysin**

Beta hemolysin (500 HU/ml) or buffered saline as a control was added to a suspension of sphingomyelin (2 mg) in 1 ml Tris buffer at pH 7.0. Samples were extracted with 1 ml of chloroform initially and after 20 h incubation at 37 °C. The aqueous and chloroform layers were separated by centrifugation and the nitrogen and phosphorus content of each determined.

Data presented in Table II show that phosphorus and nitrogen were recovered quantitatively in the chloroform extract of samples collected initially in the presence or absence of beta hemolysin. These data show that sphingomyelin is soluble in chloroform, insoluble in water, and that no products of hydrolysis were detectable in unincubated reaction mixtures. After the reaction with β-hemolysin had proceeded for 20 h at 37 °C, phosphorus was no

**TABLE I**

<table>
<thead>
<tr>
<th>Erythrocyte species</th>
<th>µg P in sphingomyelin spot</th>
<th>% sphingomyelin*</th>
<th>Hemolytic titer (HU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>31.0</td>
<td>50.5</td>
<td>1810</td>
</tr>
<tr>
<td>Ox</td>
<td>30.0</td>
<td>45.0</td>
<td>1280</td>
</tr>
<tr>
<td>Man</td>
<td>10.2</td>
<td>21.6</td>
<td>256</td>
</tr>
<tr>
<td>Rabbit</td>
<td>7.1</td>
<td>19.7</td>
<td>160</td>
</tr>
<tr>
<td>Goat</td>
<td>14.0</td>
<td>38.0</td>
<td>113</td>
</tr>
<tr>
<td>Horse</td>
<td>5.5</td>
<td>15.0</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>2.0</td>
<td>16.6</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Monkey</td>
<td>4.1</td>
<td>11.4</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

*% phospholipid as sphingomyelin = (x/y)*100 when x = µg P in sphingomyelin spot and y = total µg P in lipid extract.

**TABLE II**

End-product analysis of the reaction of beta hemolysin with sphingomyelin

<table>
<thead>
<tr>
<th>Test</th>
<th>Incubation time (h)</th>
<th>% phosphorus</th>
<th>% nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aqueous extract</td>
<td>Chloroform extract</td>
<td>Aqueous extract</td>
</tr>
<tr>
<td>Sphingomyelin control</td>
<td>0</td>
<td>94</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>5</td>
<td>87</td>
</tr>
<tr>
<td>Sphingomyelin and beta lysin</td>
<td>0</td>
<td>95</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>93</td>
<td>0</td>
</tr>
</tbody>
</table>

*Gives color with ninhydrin.
longer found in the chloroform extract, but rather in the aqueous extract. This is consistent with the formation of phosphorylcholine, which is soluble in water but not in chloroform. With respect to nitrogen, 52% was recovered in the aqueous and 65% in the chloroform extracts. This confirms the formation of phosphatidylcholine which is soluble in water but not in chloroform. The latter is soluble in chloroform but not in water. The chloroform extract also gave a color with ninhydrin, characteristic of sphingosine, in contrast with the parent compound sphingomyelin.

**Experiment 4. Specificity of Action of the Beta Hemolysin**

Equal amounts (2 mg/ml) of phosphatidylethanolamine (cephalin), ribonucleic acid, sodium beta glycerophosphate, sodium phenylphosphate, phosphatidylcholine (lecithin), and sphingomyelin as a positive control were suspended or dissolved in Tris buffer. After the addition of beta hemolysin to each tube, the reactants were incubated for 24 h at 37 °C. Samples were taken for phosphorus estimations at 1-, 4-, and 24-h periods. After each sample had been removed, the reaction was stopped by the addition of 2 N trichloroacetic acid and heated at 100 °C for 5 min. The tubes were then centrifuged and the amount of acid-soluble phosphorus estimated.

Results expressed in Table III show that sphingomyelin is the only substrate attacked to an appreciable extent, with a six- to eight-fold increase in phosphorus observed at 24 h.

**Experiment 5. Action of Beta Hemolysin on Suspensions of Staphylococcal Cell Walls**

Equal volumes of cell wall material were incubated at 37 °C for 8 h in the presence of beta hemolysin. To clear the suspensions, the samples were centrifuged after incubation. Samples of 100 μl were applied to Whatman No. 1 paper (20 X 50 cm) and chromatographed overnight in n-butanol/pyridine/water 6:4:3. The papers were removed and dried for 1 h in a fume chamber and then stained with Trevelyan’s reagent (16).

Reference to Fig. 2 shows that six compounds reacting with Trevelyan’s reagent are present in the untreated cell walls. Incubation of the purified cell wall suspension with beta hemolysin had no visible effect on the intensity or distribution of these spots.

**TABLE III**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>µg P released after incubation for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>0.0</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>0.0</td>
</tr>
<tr>
<td>Ribonucleic acid</td>
<td>0.6</td>
</tr>
<tr>
<td>Beta glycerophosphate</td>
<td>0.0</td>
</tr>
<tr>
<td>Phenylphosphate</td>
<td>0.4</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>3.9</td>
</tr>
</tbody>
</table>
Fig. 2. Chromatogram of staphylococcal cell wall suspensions. Extracts of normal and beta hemolysin-treated cell walls were chromatographed in n-butanol/pyridine/water 6:4:3. The paper was then stained with Trevelyan’s reagent.

Discussion

It was shown that purified beta hemolysin preparations derived from the R-1 and 252F strains of *S. aureus* will hydrolyze sphingomyelin. On the basis of results presented in Table 1, a correlation has been established between the hemolytic titer of the erythrocyte species tested and their sphingomyelin content. The reason for the exceptional behavior of goat cells in this case is obscure.

Evidence obtained indicates that beta hemolysin hydrolyzes sphingomyelin to yield phosphorylcholine and N-acyl sphingosine. Phosphorus analyses of the end products revealed that the phosphorus had become water-soluble, since the total amount was recovered from the aqueous layer. Nitrogen, which was contained in the chloroform extract of the sphingomyelin controls, was recovered in an approximate 1:1 ratio from both aqueous and chloroform extracts of the hydrolyzed sphingomyelin. The complete recovery of phosphorus and about 50% of the nitrogen from the aqueous extract of test preparations is consistent with the formation of phosphorylcholine. The recovery of the balance of the nitrogen from the chloroform extract is in keeping with the formation of N-acyl sphingosine. The latter gave a color with ninhydrin which is characteristic of sphingosine but not of sphingomyelin. The mode of action of beta hemolysin is thus like that of phospholipase C. The results reported here are not in agreement with those of Doery *et al.* (5), who noted complete recovery of the nitrogen in the chloroform extract of hydrolyzed sphingomyelin. Their observation is inconsistent with the formation of N-acyl sphingosine and phosphorylcholine, since all of the phosphorus and choline nitrogen should be recovered from the aqueous extract. Sphingosine nitrogen will be present in the chloroform layer.
With regard to the specificity of the beta hemolysin, phosphate bonds of RNA, lecithin, cephalin (phosphatidylethanolamine), beta glycerothosphate, and phenylphosphohosphate are not attacked to any extent even after prolonged incubation. In reality the beta hemolysin would appear to be a highly specific phosphatase which hydrolyzes only the phosphate bond of sphingomyelin. In contrast, the alpha toxin of Clostridium perfringens, which was shown by Macfarlane and Knight (8) to be a phospholipase C, preferentially hydrolyzes lecithin and does not attack phenylphosphate, beta glycerothosphate, or nucleic acid. It has, however, been reported to hydrolyze sphingomyelin slowly.

The observation of Chesbro et al. (2) that beta hemolysin attacks staphylococcal cell walls could not be confirmed. They claimed that, in cell walls treated with beta hemolysin, two spots were observed which reacted with carbohydrate spray reagents alone and one which was also ninhydrin- and carbohydrate-positive. These compounds did not appear in untreated suspensions. In our own investigation, chromatograms of cell wall suspensions of the E-delta strain of S. aureus incubated in the presence of beta hemolysin revealed six compounds of \( R_g \) values varying from 0.15 to 1.40. However, chromatograms of control preparations were identical. Spots observed by Chesbro had values of 0.15, 0.58, and 0.75. It is unfortunate that their material was not tested for phospholipase activity. It seems likely that the activity of Chesbro’s beta hemolysin is due to contaminating enzymes.

It is also necessary to comment on the fact that hydrolysis of purified sphingomyelin with the production of detectable amounts of aqueous phosphorus proceeds more slowly than actual hemolysis of red cells in the presence of beta lysin. While some might ascribe this discrepancy to the use of partially purified preparations high in hemolytic activity and containing traces of another protein having sphingomyelinase activity, it is most likely due, in our case, to the use of buffers without added magnesium ions in experiments using purified sphingomyelin. This reduces the rate of hydrolysis. Apart from this observation, Doery et al. (5) have noted that hydrolysis of natural in contrast with purified phospholipid substrates proceeds more slowly.

In conclusion, it is possible that the results obtained by Chesbro are due not to contamination but to the existence of at least two distinct staphylococcal hemolysins each possessing in part the characteristics of the classical beta hemolysin. Support for this view is to be found in the work of Thaysen (14), Haque and Baldwin (11), Robinson et al. (12), and Wiseman (17). Until the question of the existence of multiple beta hemolysins is clarified, it is suggested that enzyme preparations of S. aureus hydrolyzing sphingomyelin be referred to as beta hemolysin A.

Addendum

Since this paper was accepted for publication, Doery et al. (personal communication) on the basis of further experimental work, now agree with our finding that hydrolysis of sphingomyelin by the beta hemolysin allows recovery of nitrogen from the aqueous and chloroform extracts in an approximate 1:1 ratio.

References

Phospholipase Activity of the Delta Hemolysin of *Staphylococcus aureus* (33029)

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The alpha, beta, and delta hemolysins of *Staphylococcus aureus* are implicated in the pathogenicity of this microorganism for man and animals. However, only the mode of action of the beta hemolysin is known with certainty. This enzyme hydrolyzes sphingomyelin, a common constituent of cellular membranes, resulting in the liberation of water-soluble phosphorylcholine and water-insoluble N-acyl sphingosine (1,2). In the present experiments, we have shown that two highly purified preparations of the delta hemolysin liberate organic phosphorus from phospholipid extracts of mammalian erythrocytes. Of several phospholipids investigated as substrates, phosphatidylinositol was the most susceptible to degradation by the delta hemolysin.

**Materials and Methods.** Crude delta hemolysin from the Newman and E-delta strains of *S. aureus*1 was prepared in stainless steel trays of Dolman-Wilson agar (3) overlain with sterile cellophane and covered with aluminum foil. The cellophane surface was inoculated with 2 ml of a saline suspension of a 24-hour agar slope culture of the organisms, which was spread with a glass rod. After 24-hours incubation at 37°C in a sealed plexiglass tank in an atmosphere of 25% CO₂ in air, 0.01 M phosphate buffered saline at pH 7.0 was added to the trays. The growth was taken up in the buffer, pooled, and centrifuged at 11,000g for 30 min. The supernatant fluid containing the hemolysin was stored at −20°C.

Crude delta hemolysin prepared in this manner was dialyzed against 0.05 M acetate buffer at pH 4.0 for 48 hours. The resulting precipitate which contains the active material was collected by centrifugation and dissolved in 0.05 M tris (hydroxymethyl) aminomethane (Tris) buffer at pH 9.0 to effect solution. Further dialysis of the preparation was then carried out against 0.1 M phosphate buffer at pH 7.0 for 48 hours. It was centrifuged and the supernatant fluid was added to hydroxylapatite (4) in the amount of 10 ml/0.2 gm of dry weight of adsorbent. Elution of activity was accomplished after 1-hour standing at 4°C by addition of 2 M NaCl in buffer to the adsorbent, followed by centrifugation.

Further purification of the delta hemolysin was achieved on a column of diethylaminoethyl (DEAE) cellulose equilibrated with 0.02 M phosphate buffer at pH 7.0. The hemolysin was eluted from the column against a linear gradient of NaCl (0–0.5 M) in phosphate buffer prepared in a Varigrad.2 About 40 mg of protein was applied to the column and 10 ml fractions were collected, the active material appearing in the first of several peaks eluted.

A 30-fold increase in specific activity was achieved by this method when crude hemolysin was compared to that eluted from the DEAE column. One strong line of precipitation was observed in Ouchterlony plates when purified hemolysin was incubated with a rabbit-produced antiserum to crude material. However, Jackson and Little (5) and Gladstone and Yoshida (6) have observed that protein components of normal serum strongly inhibit hemolytic activity and the latter workers were also unable to demonstrate antigenicity. We think it unlikely that the single line of precipitation we have observed with our preparations is due to anything other than delta hemolysin.

**Results.** Purified Newman and E-delta hemolysins lysed human erythrocytes to a

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1 The Newman strain was obtained from Dr. G. P. Gladstone, Sir William Dunn School of Pathology, Oxford University, and the E-delta from Professor S. D. Elek of St. George's Hospital Medical School, London, England.

2 Buchler Instruments, Inc., Fort Lee, New Jersey.
**Delta Hemolysin Phospholipase Activity**

Table I. Relationship of Degree of Hemolysis to Organic Phosphorus Released by Delta Hemolysin from Erythrocytes.

<table>
<thead>
<tr>
<th>Erythrocyte species</th>
<th>Hemolytic titer (HU/ml)</th>
<th>Total P released (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man</td>
<td>256</td>
<td>82</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>64</td>
<td>57</td>
</tr>
<tr>
<td>Rabbit</td>
<td>64</td>
<td>54</td>
</tr>
<tr>
<td>Bovine</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>Goat</td>
<td>32</td>
<td>43</td>
</tr>
<tr>
<td>Sheep</td>
<td>32</td>
<td>39</td>
</tr>
<tr>
<td>Horse</td>
<td>16</td>
<td>30</td>
</tr>
</tbody>
</table>

*The titer of hemolysin, given as hemolytic units/ml, is the highest dilution which lyse 50% of the red cells.*

*Phosphorus content of controls has been subtracted from each value.*

greater degree than those of other species. Both hemolysins were strongly inhibited by 0.1% rabbit and human sera and to a lesser extent by 0.1% human gamma globulin, bovine albumin, and gelatin, which is in keeping with Jackson's observations (5) and those of Gladstone (6, 7). Hemolytic activity was also inhibited fourfold by cholesterol at a concentration of 2 mg/ml.

Phospholipid extracts of various erythrocyte species were prepared according to the method of Rose and Oklander (8). Sphingomyelin, phosphatidylinositol (fraction I of Folch), phosphatidylserine, and phosphatidylcholine, obtained from the Sigma Chemical Co., St. Louis, Mo., were used as substrates. Delta hemolysin activity in the presence of these substrates was assayed as follows: One ml of a 1 mg/ml suspension of phospholipids in 0.01 M Tris buffer at pH 7.0 was placed in a test tube to which 1 ml of purified hemolysin containing 250 hemolytic units/ml of activity was added (9). In some experiments, ethylenediaminetetraacetic acid disodium (EDTA) or MgCl₂ was included at a final concentration of 0.001 M. After incubation of the reaction mixture for 1 hour at 37°C, 2 ml of 10% trichloroacetic acid was added. The contents of the tube were centrifuged and the supernatant fluid was assayed for organic phosphorus by the method of Fiske and Subbarow (10).

In Table I, seven species of erythrocytes are arranged in order of decreasing sensitivity to the delta hemolysin. Erythrocytes of man were most sensitive to the hemolysin and from their extracts the largest amounts of organic phosphorus were released. Horse red cells were quite resistant with only 30% of the total phosphorus liberated. It thus appears that with the possible exception of bovine cells, hemolytic activity and release of organic phosphorus are significantly correlated.

In another experiment (Table II), delta hemolysin activity was compared with that of beta hemolysin in the presence and absence of Mg²⁺ ions and EDTA. Results obtained with beta hemolysin show a 30–40% enhancement of its phospholipase activity in the presence of Mg²⁺. The inclusion of 0.001 M EDTA resulted in a twofold inhibition of beta hemolysin activity. By contrast, neither Mg²⁺ ions nor EDTA had any appreciable effect upon delta hemolysin activity compared to the control.

We also incubated sphingomyelin, phosphatidylinositol, phosphatidylserine, and phosphatidylcholine with the delta hemolysin. The hemolysin liberated 20 μg of phosphorus from the phosphatidylinositol, 2.3 μg from phosphatidylserine, a trace from phosphatidylcholine and none from sphingomyelin. We carried out limited kinetic studies with hemolysin from the E-delta strain using Sigma phosphatidylinositol as substrate. When hemolysin concentration was plotted against the reaction velocity, a straight line was obtained. The activation energy, determined from an Arrhenius plot, was 18,750 cal/mole.

Table II. Liberation of Organic Phosphorus from Extracts of Sheep Erythrocytes by Staphylococcal Hemolysins.

<table>
<thead>
<tr>
<th>P (μg) released by</th>
<th>Beta strains</th>
<th>Delta strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R-1</td>
<td>Foggie</td>
</tr>
<tr>
<td>Additions*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>38</td>
<td>44</td>
</tr>
<tr>
<td>Mg²⁺ ions</td>
<td>54</td>
<td>58</td>
</tr>
<tr>
<td>EDTA</td>
<td>20</td>
<td>27</td>
</tr>
</tbody>
</table>

*EDTA and Mg²⁺ ions are present in 0.001 M concentrations.*
cal, within the range of 1000-25,000 cal characteristic of most enzymes as determined by Sizer (11).

Discussion. On the basis of the evidence presented, it is likely that the delta hemolysin is an enzyme. In view of the fact that aqueous organic phosphorus is released from phospholipid substrates by the hemolysin, it may have a mode of action similar to that of phospholipase C. Unlike the beta hemolysin, which is a phospholipase C and releases water-soluble phosphorylcholine from sphingomyelin, the delta hemolysin does not attack this substrate. Its activity is not affected by Mg²⁺ ions and is uninhibited by EDTA in contrast with the beta hemolysin.

Although its substrate in the erythrocyte has not yet been clearly identified, the delta hemolysin releases water-soluble organic phosphorus from phosphatidylinositol and to a lesser degree from phosphatidylserine. We have detected the presence of small amounts of phosphatidylinositol in the erythrocytes used in this study when their extracts were chromatographed using Marinetti's technique (12). The same species of erythrocytes have also been shown to contain phosphatidylinositol and in addition, phosphatidylserine, according to a recent report by Nelson (13).

Summary. Purified delta hemolysin from the Newman and E-delta strains of S. aureus liberates aqueous organic phosphorus from phospholipid extracts of various species of mammalian erythrocytes. Of several phospholipids investigated as substrates, phosphatidylinositol is most susceptible to degradation by the delta hemolysin. Phosphatidylserine was to a lesser extent attacked by the enzyme. In contrast with the beta hemolysin of S. aureus, delta hemolysin does not hydrolyze sphingomyelin and its activity is unaffected by EDTA or Mg²⁺ ions.


The nature of staphyloccocal beta hemolysin.  
II. Effect on mammalian cells

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Purified beta hemolysin A was toxic to human amnion and monkey kidney and KB cells on 
the basis of trypan blue uptake, absence of pH changes in the medium, and inability of the cells 
to adhere to glass surfaces. Cells treated with the hemolysin were smaller than controls with 
granular cytoplasm and prominent nuclei. Rabbit and sheep leucocytes were unable to 
reduce the dye dichlorophenol-indophenol in the presence of the hemolysin but their ability 
to take up bacteria seemed unimpaired. Morphological changes in other mammalian cells were 
not found in leucocytes.

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Recent work suggests that the beta hemolysin of *Staphylococcus aureus* is an enzyme which 
hydrolyzes sphingomyelin (2, 13), liberating N-acyl sphingosine and phospholylcholine. In view of the fact that highly 
purified preparations have only recently been obtained, it is not surprising that there is lack 
of agreement with regard to the toxicity of the beta hemolysin (5, 6, 14). Preparations 
hydrolyzing sphingomyelin are herein referred to as beta hemolysin A, since the evidence 
indicates that there may be more than one type of beta hemolysin possessing, in common, 
certain characteristics of the classical hemolysin (9, 11, 14). The object of the present study was to assess the toxicity of 
beta hemolysin A for mammalian cells other than erythrocytes.

Beta hemolysin A was purified according to methods described elsewhere (13). Its specific 
activity was about 75-fold greater than that of crude material. Primary human amnion cells 
were prepared according to the methods of Wilt et al. (12). Monkey kidney cells were 
purchased from Connaught Laboratories, Toronto, Ont., and KB cells (3) were obtained 
from the tissue culture laboratory of this department. Methocell (Dow Chemical Co., 
Midland, Mich.) in the amount of 3 ml of 4% (w/v) solution was added to each 100 ml of 
Syverton medium (10) in which all cells were suspended. Trypan blue uptake in cells was 
measured by the method of McLimans et al. (8).

Beta hemolysin A (0.25 ml) was added to 9.75 ml of Syverton medium such that its 
final concentration was 64 hemolytic units per milliliter. The hemolysin was then added to a 
pellet of cells washed three times in Hank’s balanced salt solution which were resuspended 
to a concentration of 100,000 cells per milliliter. An initial viable count was made and the 
suspensions were rotated in Universal bottles at 120 r.p.m. on an Eberbach rotary shaker 
at 37°C for 12 h. The suspensions were removed, another viable count carried out, 
and 1.5-ml volumes were placed in “roller” tubes and incubated at 37°C for at least 3 
days.

It was found after 12 h incubation that 7% of control human amnion cells took up trypan 
blue in contrast with 82% of those treated with beta hemolysin A. A similar high 
uptake of the dye was also noted with KB and monkey kidney cells incubated with 
hemolysin. Dye uptake by all cells was correlated with non-adherence to glass surfaces 
and absence of pH changes in the medium in the roller tubes after 3 days. Under the microscope 
(4 mm objective), these hemolysin-treated cells were smaller than controls, with 
granular cytoplasm and prominent nuclei. Some differences were noted between test and 
control cells after 120 min incubation, but the
effect was not as well developed as it was after 12 h. Cell damage as reflected by trypan blue uptake was directly proportional to log hemolysin concentration as shown in Fig. 1.

As part of the same experiment, a phospholipid extract of 2 ml of packed human amnion cells was made after treatment with 64 hemolytic units per milliliter of the hemolysin for 12 h. The extract was prepared and chromatographed, and the sphingomyelin spot identified in relation to a standard as described elsewhere (13). Elution of the sphingomyelin and of the initial extract from the paper was achieved quantitatively. Results indicated that the sphingomyelin concentration of treated cells fell from 17% to 3.5% of total cell phospholipids over the 12-h period. The sphingomyelin content of the controls was unchanged.

Leucocytes were harvested from rabbit and sheep blood freshly collected in Alsever’s solution (1). The leucocyte layer was carefully removed from centrifuged blood and the cells were washed three times in Woodin’s buffer (15) containing 0.002 M Mg++ ions. Suspensions were standardized to an optical density of 0.100 at 650 μm. Leucotoxicity was assessed by Woodin’s method in which the dye dichlorophenol-indophenol is used as an indicator of viability, and also by the ability of the cells to take up bacteria. The latter was studied using Staphylococcus epidermidis which had been washed three times and resuspended to an optical density of 0.05 in Woodin’s buffer. Beta hemolysin A (0.05 ml) was added to a tube containing 0.1 ml of sheep or rabbit leucocytes to give a final concentration of 64 hemolytic units per milliliter. The tubes were incubated for 1 h and then each received 0.05 ml of the bacterial suspension. Incubation then proceeded for a further 15 min. Smears of the suspensions were prepared and stained with Wright’s stain, and polymorphonuclear leucocytes to which cocci adhered were counted. The dye reduction test was performed in a similar manner, except that an equal volume of oxidized dichlorophenol–indophenol solution was added to the leucocyte suspension in place of the cocci. The tests were scored visually for reduction of dichlorophenol–indophenol to the colorless state after 1-h incubation at 37 C. Of 400 control sheep leucocytes counted (Table I), 97% took up cocci and reduced the dye. Leucocytes treated with the hemolysin showed no differences with regard to phagocytosis, but were unable to reduce the dye. Crude hemolysin included in the experiment at the same concentrations (with respect to sheep erythrocytes) lysed leucocyte suspensions and prevented reduction of the dye. Results obtained with rabbit leucocytes were similar to those with sheep cells.

In this investigation it would appear that the purified beta hemolysin A preparations are toxic to suspensions of human amnion, KB, and monkey kidney cells as revealed by trypan blue uptake, inability to attach to glass, and absence of acid production in the medium. With respect to leucocyte experiments, the cells were unable to reduce the dye when treated with crude or purified hemolysin in contrast with controls. There was, however, no alteration in morphology where purified material was used, as observed with other types of cells. It was thought that leucocytes unable to reduce dichlorophenol–indophenol would also show a reduction in the numbers of bacteria ingested or adhering to the membrane, but this was not borne out. It may be that the dichlorophenol–indophenol reduc-

![Graph](image-url)

**Fig. 1.** Effect of beta hemolysin A concentration on trypan blue uptake by human amnion cells. Points on the graph are the means of two determinations.
tion test as applied to leucocytes does not reflect viability but instead indicates a change in the cells unrelated to it. Edwards and Ball (4) have, however, observed that phospholipases of which beta hemolysin A is an example inhibit electron transport in cells.

Even though the beta hemolysin A preparations used in this study appear to have some toxicity, the injection of relatively large doses of the same material into rabbits is without serious visible effect (7, 14) even when it is known that their blood contains no circulating antibody and that the addition of rabbit serum to hemolysin titrations tends to enhance titers. The explanation of the discrepancy might be found in the differences in cell–hemolysin ratios in vivo and in vitro. Nevertheless, possession by S. aureus of an enzyme which hydrolyzes a constituent of cell membranes would seem to be of some advantage to the organism in gaining a foothold in the host.

Chapter 6

The Beta- and Delta-Toxins of Staphylococcus aureus

GORDON M. WISEMAN

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I. Beta-Toxin

Glenny and Stevens (1935), investigating two preparations of a-toxin, observed that with the first, dermonecrotic and hemolytic activities were neutralized by equal amounts of antiserum as expected. For the second toxin, ten times as much antiserum was required for neutralization of its dermonecrotic activity. They further observed that following incubation at 37°C, the hemolytic effect of this second toxin was greatly intensified on standing for a period at room temperature or at 4°C. This “hot-cold” hemolysin, antigenically distinct from the a-toxin, was referred to by Glenny and Stevens as the β-toxin.

Bryce and Rountree (1936) found that the β-toxin was produced largely by Staphylococcus aureus strains of animal origin. According to their investigations, the erythrocytes of sheep and the ox were susceptible to its action while those of the ferret, rabbit, rat, guinea pig, and koala bear were resistant. The toxin had slight activity in the presence of human erythrocytes.

A. TOXICITY

1. EFFECT ON WHOLE ANIMALS

Early work on the toxicity of the β-toxin was limited to the observation of Glenny and Stevens that their preparation was lethal for rabbits but not
mice. The toxin also caused no necrosis when injected subcutaneously into rabbits but did give rise to a mild erythema. Bryce and Rountree confirmed its toxicity for rabbits.

Thatcher and Matheson (1955), in a study of the kitten test for staphylococcal enterotoxin, made the claim that β-toxin, like enterotoxin, could cause emesis in cats. In contrast with enterotoxin, it could be inactivated by boiling with subsequent incubation in the presence of ascorbic acid.

Following the discovery by Jackson and Mayman (1958) that the hemolytic activity of the β-toxin is activated by Mg$^{2+}$, Heydrick and Chesbro (1962) claimed that intraperitoneal injection of the toxin was lethal for guinea pigs only in the presence of these ions. The work of Wiseman (1965a), who used a partially purified preparation of β-toxin from the R-1 and 252F strains of S. aureus, was at variance with earlier work which claimed that the toxin was lethal for rabbits. He found that mice and guinea pigs were insusceptible to the toxin whether or not it was injected intravenously in the presence and absence of Mg$^{2+}$. Subcutaneous inoculation of rabbits caused only a mild erythema. Maheswaran et al. (1967) were unable to demonstrate necrosis in the skin of rabbits when a highly purified preparation was injected with Mg$^{2+}$. Although hemolytic activity of β-toxin is enhanced in the presence of Mg$^{2+}$, there is, however, no evidence that parallel results are obtained in vivo.

2. Effect on Mammalian Cells in Culture

Much of the more recent work concerning toxicity has utilized tissue culture and suspensions of nucleated mammalian cells. Chesbro et al. (1965) have shown that β-toxin produced by the UNH-Donita strain of S. aureus has a leukocidal effect on suspensions of guinea pig macrophages. The cells' ability to reduce phenolindone-2,6-dichlorophenol (PIP) to a colorless compound was lost in the presence of toxin, and Giemsa-stained smears of such preparations revealed that the leukocytes were either disintegrated or swollen and distorted. Wiseman (1968) has also observed that β-toxin derived from S. aureus R-1 is toxic to suspensions of human amnion, KB, and monkey kidney cells on the basis of trypan blue uptake, absence of acid production in the medium, and inability to attach to glass surfaces. In addition, rabbit and sheep leukocytes were unable to reduce PIP in the presence of the toxin, but their ability to take up Staphylococcus epidermidis cells seemed unimpaired. Bernheimer and Schwartz (1964) were unable to disrupt rabbit leukocyte lysosomes or rabbit liver lysosomes as indicated by failure of β-toxin to reduce turbidity of a suspension or release lysosomal enzymes. In a later publication (1965), these authors found that the β-toxin attacked rabbit blood platelets. Korbecki and Jeljaszewicz (1965) observed that β-toxin prepared by fractional precipitation of culture supernates of S. aureus Wood 46 exerted a toxic ef-
fect on KB and monkey kidney cells. According to their report, two hemolytic units of β-toxin per milliliter added to the medium caused detachment of KB cells from glass after 24 hours of incubation, along with the appearance of vacuolation and some disintegration. Monkey kidney cells treated with the toxin were granular in appearance, but the number of intact cells remaining was greater than was the case with KB cells. Histochemical studies of KB cells incubated with β-toxin (Jeljaszewicz et al., 1965) revealed no alteration in their alkaline phosphatase and 5'-nucleotidase activity, but the number of cells hydrolyzing β-naphthyl acetate and 5-bromoindoxyl acetate decreased significantly. The toxin also increased the number of cells containing lipids staining with Sudan B and markedly reduced the proportion showing acid phosphatase activity.

According to Gladstone and Yoshida (1967), the addition of crude β-toxin to HeLa, L, HL, FL, HeP, chick fibroblasts, and rat heart connective tissue cells had no effect after 2 hours. After incubating β-toxin with human, bovine, and monkey kidney cells, Hallander and Bengtsson (1967) were unable to show uptake of neutral red by the cells after 6 hours, indicating that the toxin was without effect.

3. CURRENT STATUS OF TOXICITY

In summary, early work with β-toxin cannot be relied upon to give an accurate picture of its toxicity for laboratory animals, since results were obtained with crude material. The presence of δ-toxin in these preparations could not be ruled out, as its presence in staphylococcal culture filtrates was undetected before 1947. Points upon which there is agreement at present are that the β-toxin is not lethal for mice and is not necrotic when injected subcutaneously into rabbits, but rather produces an erythematous flush. All of the early crude preparations killed rabbits, but partially purified material free of α- and δ-toxins did not (Wiseman, 1965a). It is difficult to compare the effects of the toxin upon laboratory animals with work done using cultures of mammalian cells, since the dose per cell is enormous in these tissue culture systems compared to that in whole animals. Studies of various investigators concerned with the effect of β-toxin on tissue culture cells are also not easily compared owing to the fact that observations on the cells were made at different times after addition of the toxin. Furthermore, toxin concentration and purity were not uniform.

B. PRODUCTION AND PURIFICATION

1. PRODUCTION

In a study of four strains of S. aureus, Wiseman (1963) found that β-toxin titers were higher on media containing 1.5% agar if the plates were incubated in a mixture of 25% carbon dioxide in air rather than 25% car-
TABLE I

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gas mixture</th>
<th>Growth (O.D. 650 μμ)</th>
<th>Sheep red cell titer (HU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>252F</td>
<td>Air</td>
<td>0.84</td>
<td>1,016</td>
</tr>
<tr>
<td></td>
<td>CO₂-oxygen</td>
<td>0.76</td>
<td>1,280</td>
</tr>
<tr>
<td></td>
<td>CO₂-air</td>
<td>0.91</td>
<td>2,560</td>
</tr>
<tr>
<td>R-1</td>
<td>Air</td>
<td>0.58</td>
<td>2,560</td>
</tr>
<tr>
<td></td>
<td>CO₂-oxygen</td>
<td>0.43</td>
<td>20,480</td>
</tr>
<tr>
<td></td>
<td>CO₂-air</td>
<td>0.59</td>
<td>32,510</td>
</tr>
<tr>
<td>P-92</td>
<td>Air</td>
<td>0.58</td>
<td>5,120</td>
</tr>
<tr>
<td></td>
<td>CO₂-oxygen</td>
<td>0.51</td>
<td>16,260</td>
</tr>
<tr>
<td></td>
<td>CO₂-air</td>
<td>0.54</td>
<td>65,010</td>
</tr>
<tr>
<td>G-128</td>
<td>Air</td>
<td>0.62</td>
<td>20,480</td>
</tr>
<tr>
<td></td>
<td>CO₂-oxygen</td>
<td>0.48</td>
<td>16,260</td>
</tr>
<tr>
<td></td>
<td>CO₂-air</td>
<td>0.64</td>
<td>130,100</td>
</tr>
</tbody>
</table>

*a* After Wiseman (1963).

*b* Values are the mean of two experiments. Plates were layered with cellophane, inoculated, and incubated for 24 hours. Two milliliters of 0.01 M phosphate-saline buffer were added to each plate and the growth was removed. Hemolysin titrations were carried out on the supernatant fluid after centrifugation and in the presence of 0.001 M Mg²⁺.

Carbon dioxide in oxygen or air alone as shown in Table I. Growth was depressed slightly in the carbon dioxide and oxygen mixtures. Haque and Baldwin (1964) have also investigated the effect of various mixtures of carbon dioxide in air and oxygen on β-toxin formation. Carbon dioxide concentrations of 10, 20, 40, and 80% in air were of equal value in increasing titers in heart infusion broth to which 0.3% agar was added. In contrast with Wiseman’s data, they found that 20% carbon dioxide in oxygen was as effective in increasing titers as mixtures of carbon dioxide in air. Haque and Baldwin also found that aeration of broth cultures by agitation or sparging was ineffective in improving yields.

A neutral pH favors growth with production of highest titers of toxin (Wiseman, 1963) as shown in Table II. Haque and Baldwin also studied the influence of pH and the addition of fermentable carbohydrates upon growth and toxin production, finding that best yields were obtained when pH was initially adjusted to 5.2–5.8. The pH rose to 8.5 after 48 hours of incubation, indicating absence of strong buffering action in the media, making these results difficult to compare with Wiseman’s data. The addition of carbohydrates to the basal medium did not improve yields.

Chesbro *et al.* (1965) used a completely dialyzable medium for β-toxin production, stating that the addition of 0.5% L-arginine enhanced its formation. They employed 20% carbon dioxide in oxygen and incubated their cultures at 35°C for 24 hours with shaking, replenishing the gases
6. BETA- AND DELTA-TOXINS OF S. AUREUS

### Table II
Effect of pH on Production of $\beta$-Toxin on Solid Medium

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Growth (O.D. 650 m(\mu))</th>
<th>Sheep red cell titer (HU/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>5.0</td>
<td>0</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Phosphate</td>
<td>6.0</td>
<td>0.75</td>
<td>81,920</td>
</tr>
<tr>
<td>Phosphate</td>
<td>7.0</td>
<td>0.87</td>
<td>130,000</td>
</tr>
<tr>
<td>Borate</td>
<td>8.0</td>
<td>0.36</td>
<td>10,240</td>
</tr>
<tr>
<td>Borate</td>
<td>9.0</td>
<td>0</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

*Values are the mean of two experiments. Buffers were 0.01 M with respect to Dolman-Wilson (1940) medium containing 1.5% agar. The pH did not change by more than 0.3 units after 24 hours of growth. The buffer salts themselves were not responsible for the effects upon growth and toxin production. After Wiseman (1963).

After 6 and 12 hours, Maheswaran et al. (1967) extracted toxin from medium containing 1.5% agar after incubation in an atmosphere of 50% carbon dioxide in oxygen.

Wiseman (1963) found that the rate of toxin production was maximal in strains R-1 and 252F during the early logarithmic growth phase (Fig. 1). Highest titers were reached after 24 hours of incubation with little or no decline detectable after 48 hours, which supports the observations of Haque and Baldwin (1964) based on work with semisolid media. As part

![Graph](https://example.com/graph.png)

**Fig. 1.** Rate of $\beta$-toxin formation with respect to growth of strains R-1 and 252F. The toxins were produced on solid media and titrated in the presence of sheep erythrocytes and 0.001 M Mg$^{2+}$. The gaseous environment was 25% carbon dioxide in air. (Redrawn from Wiseman, 1963.)
of the same study, Wiseman also investigated amino acid requirements of growth and \( \beta \)-toxin production in the R-1 strain (Table III). He used the basal medium of Gladstone (1938) to which various amino acids and growth factors were added. For this strain, an absolute requirement for arginine, proline, and glycine was observed, growth and toxin formation being negligible if these three amino acids were omitted when compared to complete medium. Toxin titers were reduced if valine alone or aspartic and glutamic acids or cystine and methionine in combination were omit-

<table>
<thead>
<tr>
<th>Omissions from complete medium</th>
<th>Growth (O.D. 650 m( \mu ))</th>
<th>Sheep red cell titer (HU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine, valine, leucine, glycine</td>
<td>0.47</td>
<td>80</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.71</td>
<td>640</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.37</td>
<td>640</td>
</tr>
<tr>
<td>Valine</td>
<td>0.59</td>
<td>160</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.26</td>
<td>40</td>
</tr>
<tr>
<td>Proline, hydroxyproline, tryptophan, histidine</td>
<td>0.10</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>1.66</td>
<td>640</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.53</td>
<td>320</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.57</td>
<td>320</td>
</tr>
<tr>
<td>Proline</td>
<td>0.22</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Aspartic acid, glutamic acid</td>
<td>1.43</td>
<td>40</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>1.54</td>
<td>320</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.69</td>
<td>320</td>
</tr>
<tr>
<td>Cystine, methionine</td>
<td>0.35</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Cystine</td>
<td>1.14</td>
<td>160</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.60</td>
<td>640</td>
</tr>
<tr>
<td>Phenylalanine, tyrosine</td>
<td>1.68</td>
<td>640</td>
</tr>
<tr>
<td>Lysine, arginine</td>
<td>0.13</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.74</td>
<td>640</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.30</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Thiamine, nicotinamide</td>
<td>0.53</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.67</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>0.85</td>
<td>20</td>
</tr>
<tr>
<td>Complete medium</td>
<td>1.62</td>
<td>640</td>
</tr>
</tbody>
</table>

*aOptical density (O.D.) measurements were made on suspensions diluted 1:20. The value obtained was multiplied by 20 to give the figures in the table.
Aspartic and glutamic acids were interchangeable, but full growth and toxin production were not restored when cystine was substituted for methionine. The growth factors thiamine and nicotinamide were indispensable. In this study there was no clear distinction between amino acid requirements for growth and toxin production except possibly in the omission of both aspartic and glutamic acids. Growth, but not toxin production, in their absence nearly equaled that in the complete medium.

2. Purification

In an early attempt to free β-toxin from other exocellular products, Bryce and Rountree (1936) inactivated α- but not β-toxin in their preparations by heating at 60°C for 15 minutes. Kodama and Kojima (1939) were able to precipitate the active β-toxin by treatment with ethanol, methanol, or acetone. The feasibility of acetone as a precipitating agent was confirmed by Fulton (1943), who obtained maximum recovery if precipitation was carried out at pH 9.0.

The application of recent advances in methods of separation of proteins has facilitated recovery of highly purified β-toxin (Table IV). Robinson et al. (1958) combined ethanol precipitation with adsorption to calcium phosphate gel and subsequent electrophoresis, obtaining a 670-fold increase in specific activity. Yields were not reported, and tests of homogeneity were not carried out. The highest yields of any procedure to date have been achieved by Jackson (1963) using ethanol precipitation and complexing with Zn²⁺ followed by adsorption to hydroxyapatite. Recovery was about 50–60% with an overall 255-fold increase in specific activity. Chesbro et al. (1965) purified β-toxin from their UNH-Donita strain of S. aureus about 150-fold in a single step on cellulose phosphate, eluting the toxin by application of a buffer concentration gradient. They reported the presence of two antigens in their purified preparations. Doery et al. (1965) employed ammonium sulfate precipitation of β-toxin from strain 1062-17 followed by fractionation on hydroxyapatite. Like Chesbro et al., they found two antigenic components in their best preparations. Wise- man and Caird (1967) achieved a 70-fold increase in specific activity by adsorption of crude β-toxin of the R-1 strain to hydroxyapatite and precipitation of impurities from the eluate at pH 4.0. Passage of the supernatant fluid through Sephadex G-100 resulted in a β-toxin preparation containing two antigens, with overall recovery of about 20–25%. Maheswaran et al. (1967) twice precipitated β-toxin with ammonium sulfate, obtaining a 23-fold purification. A further twofold increase was effected on Sephadex G-100. The active effluent was then passed through carboxymethyl-cellulose equilibrated with 0.005 M phosphate buffer at pH 7.0. β-Toxin was retained on the column and eluted with a phosphate buffer gra-
TABLE IV
COMPARISON OF METHODS FOR PURIFICATION OF $\beta$-TOXIN

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Step</td>
<td>Specific activity</td>
<td>Step</td>
<td>Specific activity</td>
</tr>
<tr>
<td>Crude</td>
<td>1</td>
<td>Crude</td>
<td>1</td>
</tr>
<tr>
<td>Ethanol precipitation</td>
<td>15</td>
<td>Zinc-ethanol precipitated</td>
<td>3</td>
</tr>
<tr>
<td>Calcium phosphate treatment</td>
<td>30</td>
<td>Zinc-mercuric acetate precipitated</td>
<td>10</td>
</tr>
<tr>
<td>Starch electrophoresis</td>
<td>670</td>
<td>Hydroxyapatite</td>
<td>255</td>
</tr>
</tbody>
</table>

gradient containing 3% sodium chloride. They did not specify the concentration of buffer–sodium chloride which removed the toxin. Maheswaran’s group reported an overall 245-fold increase in specific activity for the three-step procedure, but percentage recovery was not reported. Agar gel diffusion, immunoelectrophoresis and disk electrophoresis on polyacrylamide gel indicated the presence of one antigen in their purified toxin preparation. It is of interest to note that Sephadex G-100 in their investigations also resulted in an active effluent consisting of two antigenic components as observed by Wiseman and Caird.

In spite of the fact that several of the methods of purification reported have permitted recovery of highly purified $\beta$-toxin containing only one or two antigens, the application of rigorous criteria of homogeneity to high toxin concentrations has not been carried out. Criteria presently available include immunodiffusion, immunoelectrophoresis, disk electrophoresis, analytical ultracentrifugation, and N-terminal analysis. Recycling on an ion exchanger or Sephadex would also give some indication of purity, but these procedures have not as yet all been applied to a single toxin preparation.

Purified $\beta$-toxin was reported to be stable for as long as 4 months if freeze-dried (Maheswaran et al., 1967), but activity was lost rapidly if kept as a solution at 4°C. Chesbro et al. (1965) also noted marked instability of toxin in solution or if filtered or shaken. Jackson’s toxin (1963) when freeze-dried lost no activity over a 6-month period, nor did that of Wiseman and Caird (1967).
C. CHARACTERISTICS

1. THE "HOT-COLD" REACTION

The property which first drew attention to the \( \beta \)-toxin was the classic "hot-cold" hemolytic phenomenon characteristic of the toxin (Bigger et al., 1927). Hemolysis is incomplete at the lowest dilutions of toxin or is absent at 37°C and only becomes evident when the titration is further incubated at a lower temperature, hence the application of the term "hot-cold." If colonies of \( S. \) aureus producing \( \beta \)-toxin are incubated on sheep blood agar at 37°C, a zone of darkening is apparent which progresses to hemolysis if the plates are cooled. Concentric ring effects are frequently noticed surrounding the colony before the zone of red cells affected by the \( \beta \)-toxin begins to lyse. Flamm (1957) attributed this to the Liesegang phenomenon, which is a complex relationship between the growing colony producing the exocellular agent, its diffusion through the supporting gel, and its reaction with other microorganisms or with erythrocytes (Pulvertaft et al., 1947).

The nature of the hot-cold effect has been the subject of some speculation as reflected in the work of Pulsford (1954) and Wiseman (1965b). Pulsford dealt with this phenomenon as it appeared on sheep blood agar, finding that the red cells incubated with \( \beta \)-toxin could be lysed at 37°C by rapid alteration of the pH or sodium chloride concentration above or below 0.85%. Wiseman confirmed Pulsford's findings for concentrations of sodium chloride below 0.80% in a fluid system, but above this level no lysis occurred. He furthermore observed that rapid adjustment of the pH from 6.9 downward resulted in increasing lysis of sensitized cells. Changes in pH from 6.9 to 9.8 did not cause hemolysis. It was also shown that glucose (0.5-3.0%) and sodium chloride (1.0-2.5%) inhibited lysis of sensitized sheep cells. The explanation of these findings may depend upon an increase in the cell volume under such conditions (Ponder, 1948), and inhibition of the lytic reaction by glucose and hypertonic sodium chloride tends to support this view. Since it is not known whether lysis of erythrocytes brought about by \( \beta \)-toxin is the result of membrane perforations or its disintegration, it could also be argued that hemolysis at 37°C caused by rapid pH changes or hypotonic sodium chloride concentrations is effected through enlargement of the perforations. Similarly, the explanation of the hot-cold phenomenon may be that sudden contraction of the red cell membrane as a result of lowered temperature has the effect of increasing the circumference of the perforations, allowing hemoglobin to leak out at some critical point.
2. THE ERYTHROCYTE SPECTRUM

There is some agreement that staphylococcal \( \beta \)-toxin lyses erythrocytes of the ox, sheep, and goat, while those of the rabbit, ferret, guinea pig, monkey, horse, rat, and mouse are quite resistant (Elek, 1959). Reports of the sensitivity of human erythrocytes are variable. The early investigations were, however, carried out with crude toxin and in ignorance of the effect of metal ions upon hemolysis of red cells in the presence of \( \beta \)-toxin. Wiseman (1965a) has tested the sensitivity of 13 species of erythrocytes to beta toxin from the R-1 and 252F strains of \textit{S. aureus} (Table V) in the presence of \( \text{Mg}^{2+} \). The highest titers in both cases were with sheep, ox, and human erythrocytes, while those of the horse, guinea pig, dog, frog, mouse, rat, and fowl were quite resistant. Pig, rabbit, and cat red cells occupied an intermediate position. In other work done some time later, Wiseman also found that monkey erythrocytes were resistant to the R-1 toxin, while goat red cells were about as sensitive as those of the rabbit.

Haque and Baldwin (1964) were unable to detect lysis of human, rabbit, or horse erythrocytes in the presence of purified \( \beta \)-toxin derived from their Parisi strain. There was some difference in their method of titration.

### TABLE V

<table>
<thead>
<tr>
<th>Erythrocyte species</th>
<th>Hemolytic titers (HU/ml) of ( \beta )-toxins from strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R-1</td>
</tr>
<tr>
<td>Horse</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Dog</td>
<td>&lt;4&lt; a</td>
</tr>
<tr>
<td>Frog</td>
<td>&lt;4&lt; a</td>
</tr>
<tr>
<td>Mouse</td>
<td>&lt;4&lt; a</td>
</tr>
<tr>
<td>Rat</td>
<td>&lt;4&lt; a</td>
</tr>
<tr>
<td>Fowl</td>
<td>&lt;4&lt; a</td>
</tr>
<tr>
<td>Pig</td>
<td>32</td>
</tr>
<tr>
<td>Rabbit</td>
<td>64</td>
</tr>
<tr>
<td>Cat</td>
<td>64</td>
</tr>
<tr>
<td>Man</td>
<td>256</td>
</tr>
<tr>
<td>Ox</td>
<td>512</td>
</tr>
<tr>
<td>Sheep</td>
<td>2048</td>
</tr>
</tbody>
</table>

*°Slight hemolysis, less than 50%, was observed at these dilutions. Titrations were performed in the presence of 0.001 \( M \text{ Mg}^{2+} \) in 0.01 \( M \) phosphate-saline buffer at pH 7.0. All species showed the classic hot-cold effect after incubation for 1 hour at 37°C followed by standing overnight at 4°C.*
from that of Wiseman, however. Tests were incubated for 80 minutes at 37°C followed by refrigeration for 30 minutes, when readings were taken.

The effect of Mg\(^{2+}\) on \(\beta\)-toxin titers with rabbit, human, and sheep red cells is observed in Table VI (Wiseman, 1965a). In the absence of Mg\(^{2+}\), rabbit and human red cells are relatively insensitive to \(\beta\)-toxin, but lysis is greatly enhanced if these ions are present at 0.001 \(M\) concentrations, although lysis is still greater in sheep red cells. Roy (1937) and Kodama and Kojima (1939) also found that \(\beta\)-toxin lysed human red cells.

3. Effect of Metal Ions

Jackson and Mayman (1958) showed that inactivation was the result of dialysis of \(\beta\)-toxin but that its hemolytic activity was restored in the presence of 0.01 \(M\) Mg\(^{2+}\) or Mn\(^{2+}\). Calcium ions had no effect. The addition of citrate or EDTA to the toxin had the same effect as dialysis; that is, hemolytic activity was lost.

Robinson et al. (1958) reported that hemolytic activity of their \(\beta\)-toxin preparation in the presence of sheep erythrocytes was enhanced by Co\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\), and Fe\(^{2+}\), but not by Zn\(^{2+}\) or Ca\(^{2+}\) (Table VII). They have further reported that hemolysis of sheep cells in the presence of \(\beta\)-toxin is optimal at 0.00025 \(M\) concentrations of Mg\(^{2+}\) and Co\(^{2+}\). Wiseman (1965a) confirmed this for Co\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), and Ca\(^{2+}\) but noted that Ni\(^{2+}\) had no effect on R-1 and 252F toxins. Zinc ions at 0.001 \(M\) concentrations inhibited hemolysis. He also found that the activity optimum was in the range of 0.01–0.001 \(M\) Mg\(^{2+}\) and Co\(^{2+}\) in contrast with Robinson’s data.

Other investigators have all noted increased hemolytic activity with Mg\(^{2+}\) (Haque and Baldwin, 1964; Chesbro et al., 1965). The former au-

### TABLE VI

**Influence of Addition of 0.001 \(M\) Mg\(^{2+}\) on Hemolytic Titers of \(\beta\)-Toxins from Different Strains of *Staphylococcus aureus***

<table>
<thead>
<tr>
<th>Strains</th>
<th>Mg(^{2+}) added</th>
<th>Rabbit (HU/ml)</th>
<th>Man (HU/ml)</th>
<th>Sheep (HU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-92</td>
<td>-</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>20</td>
<td>160</td>
<td>1,280</td>
</tr>
<tr>
<td>G-128</td>
<td>-</td>
<td>&lt;20</td>
<td>&lt;20</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>40</td>
<td>640</td>
<td>2,560</td>
</tr>
<tr>
<td>R-1</td>
<td>-</td>
<td>20</td>
<td>40</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>160</td>
<td>320</td>
<td>10,240</td>
</tr>
<tr>
<td>252F</td>
<td>-</td>
<td>&lt;20</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>20</td>
<td>80</td>
<td>320</td>
</tr>
</tbody>
</table>

*Partially purified \(\beta\)-toxin from these strains was tested.
TABLE VII

<table>
<thead>
<tr>
<th>Cations added</th>
<th>Robinson et al.(^a) (0.0005 M)</th>
<th>Wiseman(^b) (0.001 M)</th>
<th>Haque and Baldwin(^c) (0.001 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co(^{2+})</td>
<td>40</td>
<td>20,480</td>
<td>—</td>
</tr>
<tr>
<td>Ni(^{2+})</td>
<td>24.5</td>
<td>640</td>
<td>—</td>
</tr>
<tr>
<td>Fe(^{2+})</td>
<td>20.0</td>
<td>—</td>
<td>640</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>18.7</td>
<td>20,480</td>
<td>2,560</td>
</tr>
<tr>
<td>Mn(^{2+})</td>
<td>20.0</td>
<td>10,240</td>
<td>—</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>0</td>
<td>320</td>
<td>40</td>
</tr>
<tr>
<td>Zn(^{2+})</td>
<td>0</td>
<td>20</td>
<td>—</td>
</tr>
<tr>
<td>No additions</td>
<td>0</td>
<td>640</td>
<td>160</td>
</tr>
</tbody>
</table>

\(^a\)Robinson et al. (1958). Activity was expressed as hemin (\(\mu g\))/protein (\(\mu g\)).
\(^b\)Wiseman (1965a). Activity was expressed as hemolytic units per milliliter, which were defined as the highest dilutions of toxin lysing 50% of red cells.
\(^c\)Haque and Baldwin (1964). Their hemolytic units were similar to those of Wiseman.

Authors confirmed the absence of increased hemolytic activity in the presence of Ca\(^{2+}\). The effect of all these ions cannot be explained by their position in the periodic table, as would be expected from the relationships of nickel to cobalt and calcium to magnesium. Activation by a metal cation was the first evidence that the \(\beta\)-toxin was an enzyme. There is no record in the literature of anions enhancing hemolytic activity of the \(\beta\)-toxin.

D. Mode of Action

1. In Vitro Mode of Action

   a. Analysis of End Products. In a study of ten strains of \(S.\) aureus producing \(\alpha\)- and \(\beta\)-toxins, Doery et al. (1963) found that all hydrolyzed sphingomyelin, a common constituent of cell membranes. Ten strains producing only \(\alpha\)-toxin had no effect on this phospholipid. Furthermore, the distribution of certain electrophoretic fractions corresponded with those containing \(\beta\)-toxin. They also demonstrated that when phospholipid extracts of erythrocytes were chromatographed, sphingomyelin disappeared if the cells had been treated with the toxin before the extract was made.

   The mode of action was determined by Doery's group using sphingomyelin prepared from ox brain as substrate. Their partially purified \(\beta\)-toxin was added to an emulsion of sphingomyelin at pH 7.5 and incubated at 37°C for 2.5 hours. Trichloroacetic acid was added to stop the reaction. The supernatant fluid was subjected to electrophoresis on paper at pH 6.5, pyridine-acetic acid-water (25:1:225) being used as solvent. It was noted
that a spot staining with reagent specific for phosphorus had the same $R_f$ value as an authentic sample of phosphorylcholine chromatographed simultaneously. This led them to suggest the following reaction:

$$\text{Sphingomyelin + water} \xrightarrow{\text{β-toxin}} N\text{-acylsphingosine} + \text{phosphorylcholine}$$

Wiseman and Caird (1966, 1967) and Maheswaran and Lindorfer (1966, 1967) were able to confirm Doery's results. The data of Wiseman and Caird (Table VIII) show that phosphorus and nitrogen were recovered quantitatively in the chloroform extract of samples initially collected in the presence and absence of toxin. Since sphingomyelin is insoluble in water and soluble in chloroform, it is evident that no hydrolysis products could be detected in unincubated reaction mixtures. After 20 hours of incubation with toxin, nearly all of the phosphorus was recovered from the aqueous rather than the chloroform extract. With regard to nitrogen, 52% was found in the aqueous and 65% in the chloroform extracts. Thus, the presence of phosphorylcholine in the aqueous extract is confirmed. These data also support the suggestion of the formation of chloroform-soluble $N$-acylsphingosine as the other product of hydrolysis. The chloroform solution of this compound, unlike its parent compound sphingomyelin, gave a color with ninhydrin. Wiseman and Caird (1967) also showed that the occurrence of greater amounts of sphingomyelin in various species of erythrocytes was correlated with increased hemolysis in the presence of β-toxin.

b. Specificity of the reaction. Doery et al. (1965) studied the action of β-toxin only on one phospholipid other than spingomyelin, noting that the toxin also hydrolyzed lysophosphatidylcholine with the liberation of phosphorylcholine and a monoglyceride. Wiseman and Caird (1967) found that phosphatidylethanolamine, phosphatidylcholine, ribonucleic acid, sodium β-glycerophosphate, and disodium phenylphosphate were resistant to attack. It appears that the toxin is a quite specific enzyme, attacking, so far as is known, only sphingomyelin and lysophosphatidyl-

**TABLE VIII**

<table>
<thead>
<tr>
<th>Test</th>
<th>Incubation time (hours)</th>
<th>Phosphorus (%)</th>
<th>Nitrogen (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aqueous extract</td>
<td>Chloroform extract</td>
</tr>
<tr>
<td>Sphingomyelin control</td>
<td>0</td>
<td>0</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>5</td>
<td>87</td>
</tr>
<tr>
<td>Sphingomyelin + β-toxin</td>
<td>0</td>
<td>0</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>93</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* After Wiseman and Caird (1967).
choline. The mode of action of the \(\beta\)-toxin strongly suggests that it is similar to phospholipase \(C\). \(C.\) \emph{Clostridium perfringens} \(\alpha\)-toxin, which is a phospholipase \(C\), preferentially hydrolyzes phosphatidylcholine, resulting in the release of phosphorylcholine and a diglyceride, but it does not attack phenylphosphate, \(\beta\)-glycerophosphate, or nucleic acid. It has been reported to hydrolyze sphingomyelin slowly (van Heyningen, 1950), an observation which has been confirmed by Wiseman and Caird (unpublished data).

c. Effect of Inhibitors and Activators. A study of the effect of inhibitors and activators on systems in which sphingomyelin is incubated with purified \(\beta\)-toxin has in general confirmed data obtained when erythrocyte stromata were used as substrate. Doery et al. (1965) showed that the hydrolysis of sphingomyelin by \(\beta\)-toxin is activated by \(\text{Co}^{2+}\) and \(\text{Mg}^{2+}\), \(\text{Co}^{2+}\) being more effective than \(\text{Mg}^{2+}\). Calcium ions inhibited the reaction. Using sphingomyelin as substrate in the presence of toxin, Maheswaran and Lindorfer (1967) confirmed that \(\text{Mg}^{2+}\) activated hydrolysis of the phospholipid. Furthermore, the addition of EDTA prevented release of aqueous organic phosphorus from this substrate, an observation also made by Jackson and Mayman (1958), Robinson et al. (1958), and Wiseman (1965a) on the toxin's hemolytic activity. These authors also found that citrate would inhibit the reaction, not an unexpected result in view of the demonstrated activation of \(\beta\)-toxin by cations. Chesbro et al. (1965) found that the thiol-inactivating agents \(p\)-chloromercuribenzoate and iodoacetate inhibited hemolytic activity of the toxin. Maheswaran and Lindorfer (1967) observed that these reagents prevented hydrolysis of sphingomyelin by the toxin.

2. In Vivo Mode of Action

There is no evidence at present that \(\beta\)-toxin attacks sphingomyelin in vivo. Normal rabbit serum \textit{in vitro} does not inhibit lysis of sheep erythrocytes, but rather enhances it slightly at a concentration of 0.05-1.0%. Bovine serum albumin (0.05-0.1%) also increases hemolysis, but inhibition rather than enhancement is observed with similar concentrations of fibrinogen (Wiseman, 1965a).

Working with rabbits, Corkill (1955) showed that injection of \(\beta\)-toxin increased blood sugar levels. Toxoided material when injected did not give rise to an elevated blood sugar. In connection with this observation it is interesting to note that Smith (1965) found that blood glucose in human patients dying of staphylococcal infections was significantly elevated the day before death. However, carcass analysis of mice dead of staphylococcal infection showed a decrease of total glucose. Bergman et al. (1965) reported that intravenous injection of rabbits and cats produced instanta-
neous biphasic changes of blood pressure followed by a slow decline to zero level. Their β-toxin killed both species of animal and stimulated respiration just before death. Both Bergmann et al. and Corkill used a β-toxin preparation of undefined purity, and the presence of other toxic products causing these effects cannot be ruled out. The relationship of their observations to phospholipase activity of the β-toxin is obscure. There is some evidence (Edwards and Ball, 1954) that phospholipases A, C, and D inhibit electron transport in mammalian cells. It has been shown recently by Wiseman (1968) that β-toxin destroys the ability of leukocytes to reduce the dye dichlorophenol-indophenol, but a specific study of inhibition in cell-free systems has not been made.

E. ANTIGENICITY

Bryce and Rountree (1936) reported that in their experience the α- and β-toxins were antigenically distinct. Williams and Harper (1947) observed that δ-toxin could not be neutralized on sheep blood agar plates by antiserum to β-toxin. Thaysen (1948), using culture filtrates of S. aureus isolated from cases of furunculosis in dogs, differentiated on serological grounds a toxin which he referred to as β₂. He claimed that the β₂-toxin differed from the classic β-toxin in its antigenic properties and characteristic dose-time curve. Thaysen also stated that both toxins occur together in ordinary filtrates and that normal dog serum contains relatively large amounts of β₂-antitoxin. Although his observations were never confirmed, support for the existence of two toxins with similar characteristics comes from the work of Maheswaran et al. (1967) and Haque and Baldwin (1963) who reported separation of “anionic” and “cationic” β-toxins on DEAE-cellulose. Although Wiseman (1965a) found that β-toxins derived from the R-1 and 252F strains of S. aureus manifested slight differences in several properties, he noted that antiserum to 252F toxin would neutralize the R-1 toxin. Further work in this area is required before the concept of multiple forms of the toxin can be supported.

F. RELATIONSHIP OF BETA-TOXIN TO PATHOGENICITY OF S. AUREUS

There is general agreement that β-toxin is produced only by strains which are coagulase positive, or rather, it has never been reported in coagulase-negative strains of S. aureus. Since coagulase is closely associated with pathogenicity, by implication β-toxin must also play a role in the disease process or its initiation. Of course, this has not been proved, nor has it been for any other toxin of S. aureus for that matter.

Although β-toxin production is associated with coagulase-positive
strains, it is uncommon in strains isolated from lesions in man (Elek, 1959). Microorganisms taken from animals, however, generally produce it (Stamatin et al., 1949; Burns and Holtman, 1960). In particular, β-toxin production is characteristic of S. aureus isolated from bovine mastitis (Slanetz and Bartley, 1953). Elek and Levy (1950) in a study of 59 coagulase-positive animal strains, noted that 88% produced detectable quantities of β-toxin on sheep blood agar (Table IX) in contrast with 74% and 86% for α- and δ-toxins, respectively. In the same study, they found that 59% of animal pathogens tested produced all three hemolysins. Only 7% of strains produced β-toxin alone.

Marandon and Oeding (1966) confirmed the results of Rountree (1947), which showed that strains of S. aureus producing β-toxin do not generally form staphylokinase and are most commonly isolated from animals. The reverse was also true. Marandon and Oeding further noted that strains producing β-toxin in the absence of staphylokinase were susceptible to phage 42D. Thus, staphylokinase-negative, β-toxin-positive staphylococci are characteristic of strains from animal sources (particularly bovine mastitis) and staphylokinase-positive, β-toxin-negative organisms are chiefly associated with disease in man. The significance of these findings in reference to the question of pathogenicity and virulence of staphylococci has not as yet been appreciated. Winkler et al. (1965) have shown that lysogenization of strains of S. aureus producing β-toxin by certain serological group F phages has resulted in loss of β-toxin production and gain in staphylokinase production. This appears to be a true lysogenic conversion of the type controlling toxigenicity in Corynebacterium diphtheriae and

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of strains examined</th>
<th>Occurrence of hemolysin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Coagulase-positive human pathogens</td>
<td>200</td>
<td>α 96  β 11  δ 97  ε 0  nil 0</td>
</tr>
<tr>
<td>II. Coagulase-positive animal strains</td>
<td>59</td>
<td>α 74  β 88  δ 86  ε 0  nil 2</td>
</tr>
<tr>
<td>III. Coagulase-negative skin strains</td>
<td>77</td>
<td>α 0  β 0  δ 0  ε 95  nil 5</td>
</tr>
</tbody>
</table>

*After Elek and Levy (1950).*
Streptococcus pyogenes. The study of mutants of this type might shed light on the role of β-toxin in infection.

It is to be expected that possession of enzymes hydrolyzing components of the cell membrane would offer an advantage to staphylococci. However, in the case of β-toxin, in vivo sphingomyelinase activity has not yet been demonstrated. It is true that mammalian cell membranes generally contain sphingomyelin in varying concentrations and that certain tissues, notably brain, are rich in this phospholipid, but there is no evidence that such tissues are targets for β-toxigenic S. aureus.

II. Delta-Toxin

Twenty years after the discovery of the β-toxin, Williams and Harper (1947) observed that with various strains of S. aureus grown on sheep blood agar in the presence of α- and β-antitoxins, hemolysis was not suppressed. The substance responsible for this hemolytic activity was called δ-toxin. The δ-toxin was shown to have a wider spectrum of hemolysis than α- and β-toxins, acting on erythrocytes of rabbit, sheep, man, monkey, horse, rat, mouse, and guinea pig. Marks and Vaughan (1950), who confirmed the existence of δ-toxin, added that it acted synergistically with β-toxin on human and sheep erythrocytes.

A. Toxicity

Until recently, little definite knowledge was available regarding the toxicity of δ-toxin. Jackson and Little (1956, 1957), working with crude toxin, thought that it might be identical with the leukocidin that acts on human leukocytes. They compared leukocidal activity of α-toxin with that of δ-toxin which had been freed of the former by filtration. In contrast with α-toxin, which caused swelling of the leukocytes, δ-toxin was lytic. Gladstone and van Heyningen (1957) noted that the leukolytic action of the δ-toxin ran parallel with its hemolytic action on human and horse red cells and, like hemolysis, was inhibited by cholesterol. In their experience, the δ-toxin affected the leukocytes of man, rabbit, guinea pig, and mouse. Gladstone and Yoshida (1967) found that polymorphonuclear leukocytes, lymphocytes, and blood macrophages of the rabbit, man, mouse, rat, guinea pig, pigeon, and fowl were all susceptible to the action of crystalline δ-toxin. HeLa, L, HLM, FL, and HEp cells, including chick fibroblasts and rat heart connective tissue cells, all showed essentially similar cytopathic changes in the presence of the toxin according to these authors. The δ-toxin also liberated aldolase and β-glucuronidase from HeLa cells, indicating that the toxin acted on both the cell membrane and on
lysozymes. Unfortunately, after the work was completed, Gladstone and Yoshida found that their δ-toxin preparation was contaminated with β-toxin and ribonuclease. Their results, therefore, await confirmation with the use of toxin of demonstrated purity. Bernheimer and Schwartz (1964), using the same toxin preparation employed by Gladstone and Yoshida, found that it did not release significant amounts of β-glucuronidase and acid phosphatase from a large granule fraction of rabbit liver.

Data regarding toxicity of δ-toxin for laboratory animals are scant. Marks and Vaughan (1950) reported that it was dermonecrotic when injected into the skin of rabbits; this was confirmed by Gladstone (1966). Gladstone further observed that intravenous injection of mice with a 55% purified preparation was without visible effect.

B. PRODUCTION AND PURIFICATION

1. PRODUCTION

Williams and Harper (1947) reported that δ-toxin production was not possible in a fluid medium and that carbon dioxide is not essential for its formation on solid media. This was confirmed by Marks and Vaughan (1950). Wiseman (1963) found that on solid media, carbon dioxide concentrations varying from 25 to 75% in air had little effect on growth and toxin production. α-Toxin titers were increased 13-fold in the same strain at 50% concentrations of carbon dioxide in air. Mixtures of carbon dioxide and oxygen also had only a slight effect on δ-toxin titers (Table X). In contrast with these data, Murphy and Haque (1967) found that production of δ-toxin on heart infusion agar plates was optimal at a concentration of 10% carbon dioxide in air. Differences between hemolytic titers in air alone and in the optimal carbon dioxide-air mixture were not large, however.

Yoshida (1963) achieved satisfactory production of δ-toxin in the dialyzable CCY medium of Gladstone and van Heyningen (1957). One liter of CCY medium was placed in a 3-liter conical flask and mechanically gyrated at 37°C for 18–24 hours. Aeration had to be carefully controlled if optimal titers were to be obtained. The inability of earlier investigators to produce the toxin in fluid media was probably due to their failure to appreciate the necessity for adequate aeration of the broth cultures. According to Yoshida, formation of δ-toxin in CCY medium proceeded without lag in association with bacterial growth, reaching a plateau after 18–24 hours. Using strain 146P, Murphy and Haque (1967) obtained similar results on heart infusion agar plates with the cultivation technique of Birch-Hirschfeld (1933). Toxin production reached a maximum after incubation for 20 hours.
6. Beta- and Delta-Toxins of S. aureus

**TABLE X**

<table>
<thead>
<tr>
<th>Carbon dioxide concentration (%)</th>
<th>Growth (O.D. 650 μμ)</th>
<th>Hemolytic titer (HU/ml)</th>
<th>Growth (O.D. 650 μμ)</th>
<th>Hemolytic titer (HU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.50</td>
<td>100 50</td>
<td>0.41</td>
<td>160 40</td>
</tr>
<tr>
<td>25</td>
<td>0.49</td>
<td>200 40</td>
<td>0.32</td>
<td>200 40</td>
</tr>
<tr>
<td>35</td>
<td>0.51</td>
<td>400 40</td>
<td>0.42</td>
<td>400 50</td>
</tr>
<tr>
<td>50</td>
<td>0.67</td>
<td>2560 80</td>
<td>0.53</td>
<td>640 80</td>
</tr>
<tr>
<td>75</td>
<td>0.54</td>
<td>1590 40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Figures are the means of three experiments. α-Toxin titers were obtained with rabbit erythrocytes and those of δ-toxin with human erythrocytes. After Wiseman (1963).*

2. Purification

Several investigators have attempted to isolate the δ-toxin. Marks and Vaughan (1950) employed overnight extraction of cultures with ethanol at −4°C. After centrifugation, the ethanol was evaporated in a stream of air. Further overnight extraction with diethyl ether left a residue which contained the δ-toxin with an average yield of about 25%. The partially purified preparation was nondialyzable, could not be Seitz filtered without loss of activity, and suffered no change of titer after heating at 65° or 100°C up to 2 hours. In those strains of *S. aureus* producing both β- and δ-toxins, Marks and Vaughan also reported that the two could be separated by adsorption of the δ-toxin onto alumina at pH 8.0.

Jackson and Little (1958b) claimed that δ-toxin could be separated from α-toxin by heating at 60°C for 10–15 minutes. This treatment led to a sharp drop in δ-toxin hemolytic activity, which suggested the existence of two toxins, one heat labile and the other heat stable. Recovery of heat-stable δ-toxin was effected by precipitation of freeze-dried crude material with ethanol at pH 4.0 and at a temperature of −5° to −20°C. The precipitated toxin was further extracted with 75% ethanol in water resulting in a yield of 40% of the original toxin. Traces of α-toxin still remained, however.

Yoshida (1963) developed a procedure for the crystallization of α-toxin which satisfied two criteria of homogeneity—one peak was observed in the ultracentrifuge and one line of precipitation was obtained in Ouchterlonly plates in the presence of antisera. Nevertheless, the crystalline toxin was later shown to be contaminated with ribonuclease and β-toxin (Gladstone and Yoshida, 1967). As a result, one cannot accept their molecular weight and amino acid determinations.
The method of Wiseman and Caird (1968) results in a 30-fold increase in specific activity when crude δ-toxin is compared to purified material. Culture supernatant fluid was dialyzed against acetate buffer at pH 4.0. The active substance found in the precipitate after centrifugation was redissolved and treated with hydroxyapatite at pH 7.0 with elution of toxin being accomplished by addition of 2 M sodium chloride. Further purification was achieved on a column of diethylaminoethyl (DEAE) cellulose at pH 7.0. The hemolytic activity was not retained in the column under these conditions and appeared in the first of several protein peaks eluted in presence of a 0-0.5 M sodium chloride gradient. Although the toxin appeared to be pure when examined in agar gel diffusion plates using an antiserum to crude material prepared in rabbits, other tests of homogeneity were not carried out. Since this report was published, prolonged incubation has revealed the presence of an additional precipitation line. The purification procedure has therefore been modified to include precipitation with 30-45% saturated ammonium sulfate followed by chromatography on DEAE-cellulose. The active effluent was recycled once through the DEAE column in contrast with a single passage in the earlier procedure. Toxin (at a concentration of 6 mg/ml) obtained with the modified method gives one line of precipitation when examined by immunoelectrophoresis and agar gel diffusion techniques. One N-terminal amino acid residue is seen when an acid hydrolyzate of dinitrophenyl-toxin is chromatographed. Tentatively we have assigned the N-terminal position in δ-toxin to proline; its occurrence in this position is perhaps unusual.

C. Characteristics

I. Hemolytic Spectrum

Williams and Harper (1947) observed that the δ-toxin possessed a wider hemolytic spectrum than α- or β-toxins, being lytic for erythrocytes of rabbit, sheep, man, monkey, horse, rat, mouse, and guinea pig. Marks and Vaughan (1950) claimed that the toxin could be recognized by its lysis of human and horse red cells at 37°C and noted that it potentiated lysis of human and sheep erythrocytes by β-toxin. Gladstone (1966) stated that all erythrocyte species that have been tested are susceptible in varying degree to the action of the toxin, though the differential susceptibility is not as apparent as with α- and β-toxins. This is clear from Table XI in which Wiseman (unpublished data) has compared the sensitivities of β- and δ-toxins to various erythrocyte species. Thus, δ-toxin lyases all spe-
6. BETA- AND DELTA-TOXINS OF S. AUREUS

TABLE XI
Hemolytic Spectrum of Staphylococcal Toxins

<table>
<thead>
<tr>
<th>Red cells</th>
<th>Sensitivity of red cells (HU/ml) to</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \beta )-Toxin</td>
<td>( \delta )-Toxin</td>
</tr>
<tr>
<td></td>
<td>R-I</td>
<td>252F</td>
</tr>
<tr>
<td>Man</td>
<td>512</td>
<td>128</td>
</tr>
<tr>
<td>Monkey</td>
<td>&lt;16</td>
<td>-</td>
</tr>
<tr>
<td>Rabbit</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>Sheep</td>
<td>2048</td>
<td>512</td>
</tr>
<tr>
<td>Ox</td>
<td>512</td>
<td>512</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Horse</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>

*Figures are the means of two determinations. Titrations of the two toxins were not carried out simultaneously, but erythrocyte sensitivity spectra for each toxin are reproducible. After Wiseman (unpublished data).

Table XI shows the hemolytic spectrum of staphylococcal toxins, indicating sensitivity of red cells to different toxins. The table lists several species and their sensitivity levels to \( \beta \)-toxin and \( \delta \)-toxin.

Species, whereas monkey, ox, and guinea pig cells are resistant to \( \beta \)-toxin. However, there has never been any clear agreement among investigators regarding the hemolytic spectra of staphylococcal toxins, a fact mediated by (1) variability of erythrocyte sensitivity to the toxins in a single animal, (2) differences in toxin concentration and technique of titration used in various laboratories, and (3) the use of impure preparations.

2. EFFECT OF INHIBITORS AND ACTIVATORS

Jackson and Little (1958a) found that a variety of substances would inhibit the hemolytic activity of \( \delta \)-toxin. On the one hand, erythrocyte ghosts and hemoglobin reduced activity, and on the other, various proteins were inhibitory. Rabbit and human serums, globulins, albumin, and gelatin were all capable of reducing hemolytic activity; \( \gamma \)-globulin at a concentration of 1% was the most effective. Gladstone and Yoshida (1967) found that fibrinogen, \( \alpha \)- and \( \beta \)-globulins, and mucoproteins markedly inhibited action of the toxin, while partial inhibition was achieved with \( \gamma \)-globulin and albumin (Table XII).

Wiseman and Caird (1968) also noted that \( \delta \)-toxin of strains Newman and E-delta was strongly inhibited by 0.1% rabbit and human serums and to a lesser extent by 0.1% human \( \gamma \)-globulin, bovine albumin, and gelatin. They found that there was some inhibition by cholesterol at a concentration of 2 mg/ml, an observation in keeping with that of Gladstone and van Heyningen (1957) but which Gladstone and Yoshida (1967) were not able to confirm at a concentration of 10 mg/ml.

In contrast with \( \beta \)-toxin, metal cations have not been shown to enhance the activity of \( \delta \)-toxin, nor was EDTA or citrate found to inhibit hemo-
TABLE XII

Effect of Various Plasma Protein Fractions and Lecithin on Hemolysis by Purified δ-Toxin

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Final concentration (%)</th>
<th>Hemolysis (%)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction I (fibrinogen)</td>
<td>0.1</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>25</td>
<td>65</td>
</tr>
<tr>
<td>Fraction II (γ-globulin)</td>
<td>0.5</td>
<td>31</td>
<td>55</td>
</tr>
<tr>
<td>Fraction III (β-globulin, lipoprotein)</td>
<td>0.1</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>20</td>
<td>71</td>
</tr>
<tr>
<td>Fraction IV (α-globulin, lipoprotein)</td>
<td>0.1</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>17</td>
<td>75</td>
</tr>
<tr>
<td>Fraction V (albumin)</td>
<td>0.5</td>
<td>62</td>
<td>10</td>
</tr>
<tr>
<td>Fraction VI (mucoprotein)</td>
<td>0.1</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>Human serum</td>
<td>0.2</td>
<td>5</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>24</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>Rabbit serum (normal)</td>
<td>0.2</td>
<td>9</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>18</td>
<td>75</td>
</tr>
<tr>
<td>Lecithin (animal)</td>
<td>0.2</td>
<td>72</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>65</td>
<td>7</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>70</td>
<td>—</td>
</tr>
</tbody>
</table>

*aAfter Gladstone and Yoshida (1967).*

lytic activity (Jackson and Little, 1958a). These observations were confirmed by Wiseman and Caird (unpublished data) who were unable to enhance δ-toxin hemolytic titers over a range of $10^{-2}$–$10^{-6} M$ Mg$^{2+}$ and Ca$^{2+}$.

D. Mode of Action of Delta-Toxin and Its Relationship to Pathogenicity of *S. aureus*

1. Is the Toxin an Enzyme?

Until recently, nothing was known of the chemical nature and mode of action of the δ-toxin. Several investigators (Jackson and Little, 1958a; Marks and Vaughan, 1950) have reported that δ-toxin was stable to heating, although Yoshida (1963) and Wiseman and Caird (unpublished) have found that highly purified preparations are thermolabile and have noted that such preparations are sensitive to the action of trypsin and chymotrypsin. That the toxin has a high molecular weight is indicated by the fact that it is nondialyzable and will pass out in the void volume of a column of Sephadex G-200 according to Hallander (1963) and to Wiseman and Caird.
Kapral (1967) reported that the hemolytic activity of δ-toxin was "neutralized" by phospholipids. The activity appeared to be adsorbed to the phospholipids from which it could be recovered by removal of the phosphate radical by nonhemolytic Bacillus cereus phospholipase. The nature of the reaction between δ-toxin and phospholipids was not ascertained.

Wiseman and Caird (1968), using a highly purified preparation, have shown that δ-toxin releases acid-soluble phosphorus from erythrocytes in direct proportion to their hemolytic sensitivity (Fig. 2). Thus, the evidence is strong that hemolytic and phosphorus-liberating activity are identical. They further showed that the toxin attacks phosphatidylinositol, releasing 19.5 μg phosphorus after 60 minutes of incubation (Table XIII). About 2 μg of phosphorus was released from phosphatidylserine and a trace from phosphatidyl-choline. Sphingomyelin was not hydrolyzed by δ-toxin in contrast with its susceptibility to β-toxin. It was also noted that the rate of release of acid-soluble phosphorus from phosphatidylinositol was linear with respect to time, indicating that the reaction follows first-order kinetics. The reaction rate of δ-toxin is directly propor-

![Fig. 2. The hemolytic sensitivity in hemolytic units per milliliter of all red cell species tested was plotted against the amount of acid-soluble phosphorus released from these species in the presence of δ-toxin. The linear correlation between the two phenomena is strong evidence that hemolysis is due to release of the organic phosphorus from a substrate containing it. Key: 1 = human; 2 = guinea pig; 3 = rabbit; 4 = bovine; 5 = goat; 6 = sheep; 7 = horse.](image-url)
TABLE XIII
SPECIFICITY OF THE ACTION OF δ-TOXIN ON PHOSPHOLIPIDS

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Incubation time (minutes)</th>
<th>Phosphorus released(^{\text{a}}) (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingomyelin</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>30</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.7</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>30</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>2.3</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>30</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>19.5</td>
</tr>
<tr>
<td>Toxin control</td>
<td>60</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^{a}\)Figures are the differences between phosphorus released in the presence and absence of toxin. Toxin control is toxin without phospholipids.

tional to temperature between 20 and 56°C, whereas Doery et al. (1965) and Maheswaran and Lindorfer (1967) both found that maximum activity of β-toxin was in the region of 40°C.

2. WHAT KIND OF ENZYME IS THE DELTA-TOXIN?

Judging from the fact that the phosphorus released by δ-toxin from phosphatidylinositol is acid soluble, it is suggested without further proof that the enzyme is a phospholipase C-like β-toxin. The reaction may be as follows:

\[
\text{Phosphatidylinositol} \rightarrow \text{inositol phosphate + diglyceride}
\]

It is not yet known whether the presence of a second or third atom of phosphorus on the inositol moiety would change the specificity of the δ-toxin. Furthermore, this work requires confirmation with highly purified substrate.

3. ANTIGENICITY AND IN VIVO MODE OF ACTION—RELATIONSHIP OF DELTA-TOXIN TO PATHOGENICITY

It has not been possible to demonstrate conclusively the antigenicity of δ-toxin preparations in view of the fact that all normal serums and serum components so far tested have inhibited activity at relatively low concentrations (Table XII; Section C,2). Gladstone and Yoshida (1967), furthermore, noted that the zone of hemolysis did not correspond with the line of precipitation when δ-toxin was incubated with commercial α-antitoxin in a blood agar plate. Wiseman and Caird (1968) have observed that highly purified δ-toxin, when incubated with a rabbit-produced antiserum to crude δ-toxin, gives one strong line of precipitation in a double diffusion plate. Although prolonged incubation did yield an additional line, modification of the purification procedure (Section B,2) yielded a preparation.
considered to be homogeneous on the basis of several criteria. This material consistently gave one line of precipitation in double diffusion plates and in immunoelectrophoresis and disk electrophoresis experiments. In their opinion, then, it is very unlikely that the $\delta$-toxin is not antigenic.

Nothing is known of the in vivo activity of $\delta$-toxin. It has been suggested by various authors that $\delta$-toxin is of no consequence in the pathogenesis of staphylococcal disease since its action is inhibited in vitro by normal serum components. Gladstone (1966) has however shown that the toxin is necrotic when injected intracutaneously into rabbits in large doses, but is not lethal for laboratory mice.

Elek and Levy (Table XI; Section I,F) have shown that in $S.\text{aureus}$ strains from human sources, the association of $\delta$-toxin production with disease is very nearly as high as that of coagulase. In addition, about 86% of coagulase-positive strains from animal sources produced $\delta$-toxin. Joiris (1952) has also noted the close association of $\delta$-toxin production with strains isolated from lesions in man. As with other staphylococcal toxins, it is nevertheless hard to ascribe a particular role to $\delta$-toxin in the pathogenesis of staphylococcal disease.

Recently, Hoffmann and Streitfeld (1965) found that partially purified preparations of $\delta$-toxin inhibited growth of several species of gram-positive bacteria including $S.\text{aureus}$; this suggests that the toxin might function as a selective growth agent for the strains producing it. In view of the widespread prevalence of antibacterial agents, this observation awaits confirmation with highly purified material.

REFERENCES


Mode of action of the alpha toxin of *Staphylococcus aureus*

G. M. Wiseman and J. D. Caird

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Mode of action of the alpha toxin of *Staphylococcus aureus*

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Rabbit erythrocytes treated with the alpha toxin of *Staphylococcus aureus*, strain "Wood-46", liberate substances which contain nitrogen, absorb at 280 μm, and react with Folin phenol reagent. The susceptibility of different erythrocyte species to alpha toxin is correlated with (a) the quantity of reaction products released by toxin from the cells and (b) the degree of natural proteolytic activity possessed by the cells. Alpha toxin was, however, without effect upon albumin, fibrinogen, casein, and hemoglobin even when these proteins had been denatured with urea. In view of the evidence, it is suggested that the toxin is secreted by the *Staphylococcus* as an inactive protease which must be activated by another protease. The degree of activity of this protease in various red cell species would explain their differential sensitivity to alpha toxin.

**Introduction**

The alpha toxin of *Staphylococcus aureus* strain "Wood-46" destroys the structural and functional integrity of many species of cells by a process which remains unknown. Bernheimer and Schwartz (2) and Robinson and Thatcher (10) showed that their toxin preparations would not degrade casein. Coulter (4), who used egg phosphatidylcholine as substrate, could not demonstrate phospholipase activity. We have also been unable to do so in our laboratory. In addition to the various physiological disturbances caused by the toxin (1, 5, 9), another activity identified with it has been that reported by Weissmann et al. (13). These investigators stated that the toxin induces release of previously sequestered anions or glucose from artificial phospholipid spherules. How far this observation applies to normal membranes is questionable.

In the present study we have found that alpha toxin liberates from red cell membranes, substances which absorb at 280 μm (millimicrons) or at 750 μm in the presence of Folin phenol reagent. Hemolytic sensitivity of various erythrocyte species to toxin is also directly correlated with the release of these substances by toxin. In 1953, Morrison and Neurath (8) showed that human erythrocytes possessed proteolytic activity. We have also shown that the proteolytic activity of the red cell species used in this study is correlated with their hemolytic sensitivity to alpha toxin.

**Materials and Methods**

Toxin production and purification methods were those of Marquardt (unpublished work). Hemolytic titrations were performed as described by Wiseman (14). Protein degradation products were assayed according to Lowry et al. (7) or, as nitrogen, by a microKjeldahl technique. Optical density determinations were made with the Unicam SP-800B double-beam recording spectrophotometer (Pye Unicam, Cambridge, England). Proteolytic activity of toxin and membrane preparations was demonstrated by the techniques of Morrison and Neurath (8). Sanger's method (12) was applied to N-terminal analysis of the toxin.

Sheep and horse erythrocytes were obtained from National Biological Laboratories, Winnipeg. Guinea pig red cells were obtained from animals kept in the department and rabbit cells either from National Biological or the department's animals. All were collected and stored in Alsever's solution (3) until used.

Lipid-free membrane protein was prepared from rabbit red cells by the method of Rosenberg and Guidotti (11), in which the material is extracted for 48 h continuously with ether in a Soxhlet apparatus. The yield of lipid-free membrane protein from 300 ml of packed rabbit cells was about 0.1 g dry weight. The dried product gave the following analysis: protein 82%, carbohydrate as glucose 1%, and phosphorus 0.7%. Cholesterol values were negligible. The lipid-free protein was only partly soluble in water. Suspension of the protein in 2% (w/v) sodium dodecyl sulphate in water effected complete solution. The presence of the detergent at this concentration did not affect the activity of alpha toxin or the natural proteolytic activity of the lipid-free preparation.

The experimental design used was that given in Table I. Intact erythrocytes (8), a KCNS extract of erythrocytes, or ether-extracted cells (11) were used as sources of erythrocyte proteolytic enzyme. Other proteins were also substituted for the urea-denatured hemoglobin when called for. After the reagents had been mixed, the tubes were placed in a water bath at 37 °C. At suitable time intervals, 2-ml portions were withdrawn and immediately added to 5 ml of 5% (w/v) trichloroacetic acid in water. The contents of the tubes stood for 2 h on the bench. The precipitate which formed was removed by filtration through Whatman No. 2 filter paper. Assays for hydrolys products were carried out on the filtrates.

There was the possibility that nucleotides were released from the red cell either by the addition of urea and
TABLE I
Experimental design

<table>
<thead>
<tr>
<th>Reagents added</th>
<th>Volume (ml) in tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Denatured hemoglobin</td>
<td>5.0</td>
</tr>
<tr>
<td>Erythrocyte proteolytic enzyme*</td>
<td>1.5</td>
</tr>
<tr>
<td>Alpha toxin†</td>
<td>1.0</td>
</tr>
<tr>
<td>0.01 M phosphate saline buffer pH 7.0</td>
<td>7.5</td>
</tr>
</tbody>
</table>

*Erythrocyte proteolytic enzyme was present as intact erythrocytes, a KCNS extract, or ether-extracted cells.
†Toxin titer was 1024-4096 hemolytic units/ml with rabbit erythrocytes. Variation of titers over this range did not alter results appreciably. In some experiments, alpha antitoxin (Wellcome CPP97/63A) or heated toxin were used as additional controls.

TABLE II
Relationship of erythrocyte sensitivity to alpha toxin with their natural proteolytic activity

<table>
<thead>
<tr>
<th>Erythrocyte species</th>
<th>mg N/ml released after 5 min*</th>
<th>From hemoglobin by red cells</th>
<th>From erythrocytes by toxin</th>
<th>Hemolytic sensitivity (HU/ml)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>2.93</td>
<td>1.75</td>
<td>4096</td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td>1.50</td>
<td>0.47</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>Guinea pig</td>
<td>1.32</td>
<td>0.49</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>Horse</td>
<td>0.84</td>
<td>0.14</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Man</td>
<td>0.64</td>
<td>0.06</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

* Determined in the presence of 6.5 M urea. Values are the difference between control and test. Detection of hydrolysis products by absorption at 280 mp or at 750 mp with the Lowry protein reagent gave similar results.
† The same volume of toxin (0.2 ml) was used in titration of each red cell species.

trichloroacetic acid or of toxin itself. We did, in fact, note that trichloroacetic acid precipitation of erythrocytes resulted in the appearance of substances which absorbed principally at 260 mp. However, the presence of nucleotides or nucleosides in our test systems and their contribution to the optical density determinations at 280 mp was compensated for by the use of appropriate controls in the reference beam of our spectrophotometer. Thus the optical densities observed were the difference between control and test systems. In the case of nitrogen determinations, control values were subtracted from the test values.

The absorption spectrum of the toxin over the range 200-400 mp was determined at the final concentration used in the tests. No absorption at 280 mp was observed at that dilution although hemolytic activity was high in all experiments.

Results

The hemolytic spectrum of our Wood 46 alpha toxin preparations agreed with that given for the toxin by Elek (6), which is surprising in view of the known variability of erythrocytes.

Culture supernatant fluids were lethal for mice and dermonecrotic for rabbits. We performed N-terminal analyses of the purified toxin and confirmed that histidine is the N-terminal residue, but no other amino acids were detected in our chromatograms.

It is evident in Table II that the hemolytic sensitivity of the five species of red cells to alpha toxin is correlated with the toxin's ability to liberate nitrogen from the cells. It is also of interest to note that the natural proteolytic activity (that is, their action on hemoglobin)
associated with these erythrocyte species is correlated with their sensitivity to alpha toxin. In addition to its action on intact cells, the alpha toxin released reaction products from lipid-free membrane protein prepared from rabbit erythrocytes (Fig. 1).

We found that the toxin had no effect upon either denatured or undenatured hemoglobin, fibrinogen, albumin, or casein whereas membrane-bound proteases of rabbit red cells degraded all of these proteins. In Fig. 2 it is evident that alpha toxin did not attack hemoglobin alone, but incubation of toxin and KCNS extract (erythrocyte-free proteolytic enzyme) together without hemoglobin released low levels of nitrogen. When hemoglobin was added to the toxin–KCNS system, a large increase in activity was observed as reflected in the amount of nitrogen liberated as compared to the other combinations.

Discussion

We agree with Coulter's observation that histidine is an N-terminal group of alpha toxin. However, we could not confirm the presence of arginine as an additional N-terminus. Coulter found that disulphide bond reduction did not yield two peptide chains. Treatment in this way caused the disappearance of histidine but not arginine as the N-terminus. It is difficult, for this reason, to reconcile our results with Coulter's work.

Our investigation showed that nitrogen is liberated from erythrocyte species by alpha toxin in direct relationship to their hemolytic sensitivity. In parallel with this observation, substances which absorb at 280 μ or at 750 μ in the presence of Lowry protein reagent have also been detected. These observations are in keeping with the appearance of peptides as the major reaction product of proteolytic activity. We suggest that hemolysis and the liberation of these reaction products are caused by the same event. It appears probable that the alpha toxin is a protease, but it is noteworthy that it has no action on hemoglobin or other proteins tested.

The fact that natural proteolytic activity of the red cell species tested is correlated with their sensitivity to toxin suggests that, in some way, the toxin depends upon the proteases for its activity. The toxin might expose a substrate in the erythrocyte membrane, which is then degraded by the membrane-bound proteases. It has occurred to us, however, that the toxin is perhaps secreted by the Staphylococcus as an inactive precursor. This precursor is activated only by a particular erythrocyte protease and its presence or absence would explain the sensitivity of a given red cell species to the toxin. Evidence that our hypothesis is correct is reinforced by the fact that the liberation of reaction products from hemoglobin by the red cell proteases is enhanced when alpha toxin is present. Alpha toxin alone has no effect on hemoglobin, but when activated by membrane-bound enzymes, it seems that the hemoglobin is attacked by the toxin. This would account for the observed enhancement. It might be argued that the products of the reaction were not the result of the toxin's action upon red cells but rather the result of the red cell proteases' action upon the protein toxin. This is unlikely since toxin at the
concentrations used in all experiments did not absorb at 280 μm, as we have already pointed out.

Acknowledgments

We are indebted to Dr. S. K. Maheswaran of the Department of Veterinary Microbiology and Public Health, University of Minnesota, St. Paul, Minn. 55101, who kindly provided us with the details of Dr. W. Marquardt’s unpublished methods. These methods may be obtained from either Dr. Marquardt or Dr. Maheswaran.

Our studies were supported by the Medical Research Council of Canada. Dr. Maheswaran held a Medical Research Council Fellowship in our laboratory in 1968-69.

Purification of the delta toxin of
*Staphylococcus aureus*

J. D. Caird and G. M. Wiseman

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Purification of the delta toxin of *Staphylococcus aureus*

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Received February 23, 1970


An improved procedure for the purification of the delta toxin of *Staphylococcus aureus* strain E-delta has been devised which relies upon precipitation at pH 4.0 and further treatment with ammonium sulphate. A final step consists of passage twice through a column of DEAE-cellulose. Toxin obtained by this method appeared to be homogeneous on the basis of immunodiffusion and electrophoresis studies. However, two peaks with sedimentation coefficients of 2.8 S and 9.8 S were observed when toxin was examined in the ultracentrifuge. Proline was identified as the N-terminal amino acid. No other N-terminal amino acids were detected in the purified toxin.

**Introduction**

The existence of a hemolysin of *Staphylococcus aureus* characterized by its ability to lyse human erythrocytes was first demonstrated by Williams and Harper (14). These workers investigated the relationship between test tube hemolysin titrations and hemolysis on blood agar plates, and observed a third hemolysin which was not neutralized by antitoxin to alpha and beta toxins. This third hemolysin was called the delta toxin.

Earlier attempts at purification of the delta toxin were those of Marks and Vaughan (9) and Jackson and Little (7), both of whom partially purified their preparations by alcohol extraction. Jackson and Little achieved an eightfold purification of the toxin. More recently, Yoshida (18) obtained a 20-fold increase in specific activity by chromatography of toxin on columns of calcium phosphate gel and triethylaminoethyl cellulose. The delta toxin purified in this manner was crystallized from distilled water. Yoshida reported that the product obtained had a molecular weight of 68 000 with a sedimentation coefficient of 6.1 S. However, Gladstone and Yoshida (6) later found that Yoshida's crystalline delta toxin was contaminated with ribonuclease and beta toxin, and we must therefore question the validity of the original data.

In an earlier study we isolated toxin contaminated with at least one other antigen (17), and we made that procedure the basis of the improved method which is presented in the present paper. It was also important to confirm with purer material our initial observation that delta toxin degraded phosphatidylinositol.

**Materials and Methods**

The "E-delta" strain of *S. aureus* was used for the production of delta toxin, and its origin and methods of toxin production are described elsewhere (7). Buffer solutions were prepared according to Cruickshank (4). Protein estimations were performed according to the method of Lowry et al. (8).

Assays of the hemolytic activity of delta toxin were based on the technique of Wiseman (16), except that human erythrocytes were used. We obtained outdated group 0 cells from the Red Cross Blood Transfusion Service in Winnipeg.

Sedimentation analysis of toxin was performed at 20 °C in the Spinco model E ultracentrifuge, which was equipped with a Schlieren optical system. The samples were sedimented in a synthetic boundary cell at 60 000 rev/min.

Immunodiffusion analyses were based on the methods of Ouchterlony (12) as modified by Wiseman (15). Immuno-electrophoresis was performed on 2.5 x 7.5 cm glass microscope slides which were layered with 3 ml of

**TABLE I**

Effect of pH on activity of delta toxin

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Precipitation*</th>
<th>Precipitate</th>
<th>Supernatant Fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>4.0</td>
<td>+++</td>
<td>2560</td>
<td>&lt;20</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>+++</td>
<td>2560</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>+++</td>
<td>1280</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>+++</td>
<td>1280</td>
<td>640</td>
</tr>
<tr>
<td>Phosphate</td>
<td>6.0</td>
<td>+++</td>
<td>640</td>
<td>1280</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>+++</td>
<td>640</td>
<td>1280</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>+++</td>
<td>160</td>
<td>1280</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>+</td>
<td>160</td>
<td>1280</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>8.0</td>
<td>+</td>
<td>80</td>
<td>2560</td>
</tr>
<tr>
<td></td>
<td>8.5</td>
<td>+</td>
<td>80</td>
<td>2560</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>&lt;20</td>
<td></td>
<td>2560</td>
</tr>
</tbody>
</table>

*Precipitation is +++ (heavy), ++ (moderate), + (slight).*
†Values are the mean of two experiments.
agar medium. A 0.1-ml volume of toxin (6 mg/ml protein) was placed in the well, which measured 2 mm in diameter, and a current of 10 mA/cm was applied for 150 min. The antiserum well was cut after electrophoresis was complete. Agar composition, washing, and staining techniques were those described in (15) above.

A Canalco trial kit (Canal Industrial Corp., Bethesda, Md.) was used in disc electrophoresis experiments. For this procedure, polyacrylamide gel of standard pore size was prepared (11). A sample of protein (60 µg) was applied to the column, stacked at pH 8.9, and run at pH 9.5 for 40 min. Bands were stained with 0.2% amido black dissolved in 7.5% acetic acid, and destaining was effected in an electric field in the presence of 7.5% acetic acid in water.

The N-terminal amino acid analysis of delta toxin was performed according to the technique of Fraenkel-Conrat et al. (5). The dinitrophenyl (DNP) amino acids were chromatographed on Whatman No. 4 paper in the presence of Blackburn and Lowther's r-amyl alcohol solvent system (2).

Results

The Effect of pH on Toxin Activity

Fifty-milliliter volumes of crude delta toxin were dialyzed at 4°C for 48 h against 0.05 M buffers over the pH range of 4.0-9.0. It was found (Table I) that the toxin completely pre-

<table>
<thead>
<tr>
<th>% Ammonium Sulphate</th>
<th>Hemolytic Activity, HU/ml</th>
<th>Protein, µg/ml</th>
<th>Volume, ml</th>
<th>% Recovery</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1024</td>
<td>770</td>
<td>100</td>
<td>100</td>
<td>1.3</td>
</tr>
<tr>
<td>15</td>
<td>256</td>
<td>610</td>
<td>8</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>30</td>
<td>256</td>
<td>455</td>
<td>15</td>
<td>4</td>
<td>0.6</td>
</tr>
<tr>
<td>45</td>
<td>2048</td>
<td>425</td>
<td>26</td>
<td>52</td>
<td>4.9</td>
</tr>
<tr>
<td>60</td>
<td>256</td>
<td>570</td>
<td>15</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>75</td>
<td>64</td>
<td>440</td>
<td>5</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>90</td>
<td>16</td>
<td>360</td>
<td>5</td>
<td>1</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Fig. 1. Chromatography of delta toxin on DEAE-cellulose. The upper graph shows the first passage of toxin through the column. The lower graph shows the second passage of toxin through the same column. Open circles are hemolytic Units (HU/ml) of toxin and solid circles represent optical density (O.D.) at 280 µm.
Concentration of 0.05 M precipitated without loss of activity at a pH of 4.0–4.5 with little or no hemolytic activity remaining in the supernatant fluid after centrifugation. Activity in the supernatant increased with increasing pH until precipitation was negligible at pH values above 8.0. Thus, as an initial purification step, crude toxin was dialyzed against 0.05 M acetate buffer at pH 4.0 for 48 h.

**Crude toxin**

0.05 M acetate dialysis, pH 4.0

**Precipitate dissolved in 0.05 M Tris-HCl, pH 9.0**

0.05 M phosphate dialysis, pH 7.0

**Ammonium sulphate precipitation**

0.05 M phosphate dialysis, pH 7.0

**First DEAE column passage**

Concentration and 0.05 M phosphate dialysis, pH 7.0

**Second DEAE column passage**

Distilled water dialysis and lyophilization

Scheme I. Purification scheme for delta toxin.

**Ammonium Sulphate as a Toxin Precipitant**

Delta toxin precipitated at pH 4.0 was completely dissolved in 0.05 M Tris-HCl buffer, pH 9.0, and dialyzed against 0.05 M phosphate buffer at pH 7.0 for 24 h. Residual precipitate after 24-h dialysis against the phosphate buffer was negligible and loss of activity was less than that observed after 48 h of dialysis as indicated in Table I. Solid ammonium sulphate was added at 4 °C to the dialyzed toxin to a final concentration of 90% saturation with 15% increments. After the salt had dissolved, the solution stood for 1 h at 4 °C. The solution was then centrifuged at 20000 g for 30 min and further salt was added to the clear supernatant fluid. The precipitate which resulted from each salt concentration was dialyzed against 0.05 M phosphate buffer at pH 7.0.

Results presented in Table II indicate that maximum recovery with the greatest increase in specific activity was effected at 45% concentrations of ammonium sulphate. Acetate-precipitated toxin was thus redissolved and then further precipitated at this concentration of the salt as a second step.

**Column Chromatography of the Delta Toxin**

A jacketed column (3 × 40 cm) was packed with Mann DEAE-cellulose and equilibrated with 0.05 M phosphate buffer at pH 7.0 for 24 h. Delta toxin partially purified by acetate and ammonium sulphate precipitation was dialyzed against this buffer for 24 h and then applied to the column as a solution which contained 10–20 mg of total protein. Elution was accomplished with the phosphate buffer. As observed in Fig. I, hemolytic activity passed unadsorbed through the column under these conditions, accompanied by several other inactive protein peaks. The tubes which contained hemolytic activity were

---

**TABLE III**

Efficiency of the purification scheme for delta toxin

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume, ml</th>
<th>Hemolytic activity, HU/ml*</th>
<th>Protein, µg/ml</th>
<th>% recovery</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude toxin</td>
<td>1000</td>
<td>512</td>
<td>1400</td>
<td>100</td>
<td>0.4</td>
</tr>
<tr>
<td>Acetate precipitate</td>
<td>100</td>
<td>2560</td>
<td>2170</td>
<td>50</td>
<td>1.2</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation</td>
<td>100</td>
<td>2048</td>
<td>425</td>
<td>40</td>
<td>4.8</td>
</tr>
<tr>
<td>DEAE first</td>
<td>50</td>
<td>2048</td>
<td>179</td>
<td>20</td>
<td>11.4</td>
</tr>
<tr>
<td>DEAE second</td>
<td>40</td>
<td>2048</td>
<td>176</td>
<td>16</td>
<td>11.6</td>
</tr>
</tbody>
</table>

*Hemolysin titrations and protein values were the means of two determinations.
Fig. 2. Immunodiffusion of delta toxin. Number one well contains crude delta toxin from the E-delta strain. Number two well contains purified toxin and number three, rabbit-produced antiserum.

Fig. 3. Immunoelectrophoresis of purified delta toxin. Toxin was contained in upper well and antiserum in lower trough.

Fig. 4. Disc electrophoresis of purified delta toxin on polyacrylamide gel column. Toxin runs at pH 9.5.

Fig. 5. Sedimentation pattern of purified delta toxin after 12 min at 60 000 rev/min in 0.01 M phosphate buffer, pH 7.0, in Spinco model E ultracentrifuge.
In addition, one strongly stained band of protein was observed after disc electrophoresis of the toxin (Fig. 4). It should be noted that it was possible to apply only 60 μg total protein to the column. However, the technique is very sensitive.

In contrast with the foregoing, two sedimentation bands were observed when 6 mg/ml of toxin was examined in the ultracentrifuge, as shown in Fig. 5. The sedimentation coefficients were calculated to be 2.8 S and 9.8 S.

Of particular interest was the N-terminal analysis of delta toxin. Figure 6 shows that only one amino acid was identified as the N-terminus. This appeared to be DNP-proline. The spot which migrated with the solvent front was free dinitrophenol. The only other amino acid which possessed an \( R_f \) value close to that of proline and the unknown was DNP-phenylalanine.

**Discussion**

The purification scheme recently published by Wiseman and Caird (17) yielded toxin which gave a single line of precipitation in immunodiffusion analyses, but subsequent tests detected the presence of an additional antigen. Furthermore, this procedure required the adsorption of toxin to hydroxylapatite and it was observed that specific activities obtained with this step were not constant. The present procedure avoids the use of hydroxylapatite and results in a delta toxin preparation which seems homogeneous on the basis of several criteria.

The question of purity is always a difficult one to deal with since one can never be certain that the methods used are sensitive enough to detect all protein present. All of our tests were made on a toxin solution which could not be further concentrated without precipitation, yet the evidence indicated that only one species of protein was probably present. It is, however, difficult to reconcile our findings in the ultracentrifuge with those of immunodiffusion, disc electrophoresis, and N-terminal analyses. It has occurred to us that the toxin may be unstable in the ultracentrifuge with the result that two molecular fragments are produced. It should be noted that staphylococcal alpha toxin has been reported to exist at least in three physical forms (1).

With regard to our observation that proline is the probable N-terminal amino acid of the delta toxin, it should be mentioned that Noll (10) has never detected the presence of proline, arginine,
and histidine as N-terminal groups in his series of proteins. This led Coulter (3) to suggest that the tendency of staphylococcal alpha toxin to associate might in some way be related to the presence of histidine as the N-terminus of that toxin. We might therefore speculate that delta toxin also forms aggregates, although there is little evidence other than the ultracentrifugal data to support this.

The question of the toxin’s antigenicity is still unsettled. Unfortunately we failed to provide direct evidence for its immunogenicity since the hemolytic activity of the delta toxin is inhibited by normal serum and some other proteins as well as by antiserum (6, 17). Gladstone and Yoshida (6) were unable to obtain a line of precipitation which corresponded with the edge of a zone of hemolysis caused by the toxin, an observation which we confirmed. If indeed the toxin is non-antigenic, and we consider it unlikely, then the single protein we have observed in our immunodiffusion analyses is a contaminant. Nevertheless we must point out that disc electrophoresis and N-terminal analysis indicate that only one species of protein is present unless the delta toxin is not a protein. There is no evidence to support this possibility (17).

We confirmed (unpublished data) that delta toxin from strain E-delta, purified by the procedure reported here, releases acid-soluble phosphate from phosphatidylinositol as reported earlier (17).

Some of the newer techniques of separation science, for example electrophoresing, when applied to delta toxin will probably detect multiple forms of toxin which differ in weight and charge but which are beyond the resolution of other methods. This has recently been observed with the alpha toxin (13). Perhaps classical delta toxin of S. aureus might be defined as that toxin which lyser human erythrocytes to a greater degree than those of other species, which is inhibited by 0.1% solutions of bovine albumin and gelatin (6, 17) and which degrades phosphatidylinositol with the liberation of acid-soluble phosphates (17).

Acknowledgments

Our studies were supported by the Medical Research Council, Ottawa, Canada. One of us (G. M. W.) is a Medical Research Council Scholar.

Further observations on the mode of action of the alpha toxin of *Staphylococcus aureus* “Wood-46”

G. M. Wiseman and J. D. Caird

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Pages 987–992
Further observations on the mode of action of the alpha toxin of 
Staphylococcus aureus "Wood-46"1

G. M. WISEMAN AND J. D. CAIRD
Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba
Accepted March 13, 1972.

WISEMAN, G. M., and J. D. CAIRD. 1972. Further observations on the mode of action of the alpha toxin of 

Further evidence has been obtained which supports the view that alpha toxin from Staphylococcus 
aureus "Wood 46" is a protease which requires activation by erythrocyte membrane proteases. Rabbit 
erythrocyte antiprotease prepared in mice inhibited degradation of rabbit ghosts by the toxin. Supernatant 
fluid of toxin-treated ghosts incubated with EDTA and then passed through a column of Sephadex 
G-75 yielded a fraction which was hemolytic and proteolytic. These activities were both neutralized by 
alpha antitoxin prepared in rabbits, but not by control sera.

It was also observed that alpha antitoxin inhibited the proteolytic activity of rabbit ghosts not exposed 
to toxin, in contrast with control sera. Inhibitory ability was removed from the antitoxin by adsorption 
with a heavy suspension of ghosts, and this treatment destroyed the antitoxin's capacity to neutralize 
hemolytic activity of the alpha toxin.

WISEMAN, G. M., et J. D. CAIRD. 1972. Further observations on the mode of action of the alpha toxin of 
Staphylococcus aureus (souche "Wood 46") est une protéase qui doit être activée par des protéases de 
de membrane érythrocytaire. Un sérum antiprotéase érythrocytaire de lapin, obtenu de souris, empêche 
de lyser des membranes érythrocytaires de lapin. Le surnageant de membranes traitées par la toxine montre, 
après incubation en présence de EDTA et filtration par une colonne de Sephadex G-75, une fraction 
émolétyque et protéolytique. Ces propriétés sont neutralisées par l'antitoxine alpha préparée avec des 
lapins, mais non pas par des sérum de contrôle.

En outre, il a été observé que l'antitoxine alpha inhibe l'activité protéolytique de membranes érythro-
cytaires de lapin qui n'étaient pas exposées préalablement à la toxine, contrairement aux sérum de 
déglutant. Après contact avec une suspension concentrée de membranes érythrocytaires, l'antitoxine perd 
son activité inhibitrice. Ce traitement détruit aussi la capacité de l'antitoxine de neutraliser l'activité 
érythrocytaire de la toxine alpha.

Introduction

It was recently suggested (7) that alpha toxin 
from the Wood-46 strain of Staphylococcus 
aureus is an inactive protease activated by 
enzymes of the erythrocyte membrane. The 
erythrocyte hemolytic spectrum of the toxin was 
explained on the basis of a quantitative varia-
tion in membrane proteolytic activity from 
species to species.

This study presents additional evidence in 
support of the earlier work.

Materials and Methods

Production and purification of toxin, sources, and 
storage of erythrocytes were based on methods described 
in Wiseman and Caird (7). Erythrocyte ghosts were pre-
pared according to the methods of Morrison and Neurath 
(5), and were used at a minimum concentration of about 
60 µg N/ml. This ensured that maximal amounts of toxin 
would be activated by the ghosts. Nitrogen was assayed 
by the microKjeldahl technique of Markham (4).

1Received October 18, 1971.

Assay of Proteolytic Activity

Azocoll (Calbiochem, Los Angeles, Calif.) was made 
up to a concentration of 5 mg/ml in distilled water. 
Two milliliters of test solution were added to 5 ml of 
the azocoll suspension, incubated at 37°C for 30 min, 
and filtered through Whatman No. 1 filter paper. The 
liberated color was read at 520 nm in a Unicam SP 800B 
spectrophotometer.

Assay of Hemolytic Activity

Hemolytic titrations with rabbit erythrocytes were 
set up as described (6). However, the series of tubes 
which contained the 50% visual end point was cen-
trifuged at 2000 × g and 1 ml of supernatant fluid was 
diluted 1:3 with distilled water. The optical density 
(O.D.) of these was read at 541 nm in the spectrophotom-
eter and plotted against the loga dilution of toxin. The 
50% end point was obtained by comparison with data 
taken from a standard curve prepared with distilled 
water.

Preparation of Antiserum

Antiprotease

Twenty albino CF1 male mice (Quebec Breeding 
Farms, St. Eustache, Que.) weighing about 25 g each 
received on the 1st day by intraperitoneal injection 
0.1 ml rabbit-ghost suspension which contained 450 µg 
N/ml. At two subsequent 21-day intervals, the mice
received a further 0.1-ml injection. One week after the last injection, the mice were sacrificed and their blood pooled. Serum was separated from the blood and was stored at −20°C.

**Antitoxin**

Antibody to Wood-46 alpha toxin was prepared in 2-month-old New Zealand white rabbits obtained from the Canadian Research Animal Farm, Bradford, Ont. The rabbits were injected subcutaneously with doses of toxin increasing from 0.1 to 1.0 ml (2560 hemolytic units (HU)/ml) at 2-week intervals over a period of 3 months. One week after the last injection, the rabbits were bled from the heart and the blood was treated in the same manner as that from mice. One milliliter of antitoxin neutralized 7946 HU of toxin.

**Results**

**Relationship of Hemolytic Sensitivity to Toxin Adsorption**

One milliliter of alpha toxin and 1 ml of ghost suspension were incubated together at 37°C in a water bath for 1 h. The suspension of ghosts and toxin was centrifuged and the supernatant was titrated against rabbit erythrocytes. The toxin alone was also titrated against the five erythrocyte species shown in Table 1.

We found that adsorption of toxin to erythrocyte ghost species increased with lytic susceptibility of the intact erythrocytes. A small amount of toxin (32 HU/ml) was not adsorbed to the rabbit ghosts. We also observed (data not shown) that toxin adsorption to rabbit ghosts was directly correlated with ghost concentration up to about 65 μg N/ml. At higher concentrations, no further toxin was adsorbed.

**Effect of EDTA on Ghost-Toxin Supernatant**

A suspension of rabbit erythrocyte ghosts was centrifuged at 10 000 × g for 15 min. The sedimented ghosts were resuspended in 20 ml of toxin solution containing 62 000 HU/ml and incubated 60 min at 37°C, centrifuged at 10 000 × g for 15 min, and the supernatant made 0.01 M with respect to disodium EDTA. Further incubation of this supernatant material at 37°C for 1 h was followed by dialysis at 4°C against three changes of 0.05 M phosphate buffer, pH 7.0, for 24 h. Hemolytic titrations were performed after each step.

Three times as much hemolytic activity was recovered from the supernatant fluid of toxin-treated ghosts in the presence of EDTA when compared to toxin-ghost supernatant fluid, as Table 2 shows. Removal of EDTA by dialysis of the fluid against phosphate-buffered saline abolished most of the observed enhancement. The toxin itself was unaffected by EDTA, or by dialysis.

Toxin-ghost supernatant fluid containing EDTA is the material subjected to gel filtration on Sephadex G-75 in a later experiment.

**Effect of Incubation upon Ghost Proteolytic Activity**

Enhancement of toxin titers in toxin-ghost supernatant fluid treated with EDTA suggested that the toxin interacted with a soluble component of the ghosts. This was investigated by placing a suspension of rabbit erythrocyte ghosts in a water bath at 37°C. Two-milliliter quantities were withdrawn at time intervals ranging from 30 s to 30 min, and centrifuged at 2500 × g for 5 min. The collected supernatant fluid was assayed for proteolytic activity. As shown in Fig. 1, proteolytic activity was found in the supernatant fluid in increasing amounts as incubation progressed.

**Behavior of Toxin-Ghost Supernatant Fluid on Sephadex Columns**

A column of Sephadex G-75 (Pharmacia, Montreal, Que.) measuring 2.5 × 90 cm was

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**TABLE 1**

Recovery of alpha toxin from ghost supernatant fluid

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Total hemolytic activity, HU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial toxin control</td>
<td>62 000</td>
</tr>
<tr>
<td>Initial toxin control + EDTA</td>
<td>62 000</td>
</tr>
<tr>
<td>Toxin-ghost supernatant</td>
<td>35 320</td>
</tr>
<tr>
<td>Supernatant + EDTA</td>
<td>114 000</td>
</tr>
<tr>
<td>Dialyzed supernatant - EDTA solution</td>
<td>52 500</td>
</tr>
</tbody>
</table>

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prepar and stabilized with 0.05 M phosphate buffer at pH 7.0. Fifteen milliliters of sample (EDTA toxin-ghost supernatant) were applied to the column at 4°C and 5-ml fractions were collected in a Buchler Fractomat II (Canlab). The void volume of this column measured with blue dextran (Pharmacia) was 125 ml.

Results shown in Fig. 2 indicate that no proteolytic activity was associated with the toxin control nor was hemolytic activity associated with the ghost supernatant fluid when these were passed separately through the column. When ghost supernatant fluid containing toxin was passed through the column, a fraction was obtained which contained both hemolytic and proteolytic activity. This was neutralized by alpha antitoxin (see Table 3). Also present was another fraction which was proteolytic but non-hemolytic. This peak was displaced to the right of the ghost protease control.

**Effect of Antighost Sera on Ghost Proteolytic Activity**

An antiserum against rabbit ghosts prepared in mice and mouse serum controls was mixed in a series of test tubes with an equal volume of rabbit ghosts and incubated at 37°C for 15 min. Alpha toxin to a final concentration of 9 HU/ml or buffered saline was then added to some of the tubes and all were assayed for proteolytic activity at various time intervals up to 30 min. The final concentration of sera in the solutions was 1.6% v/v.

Antiserum prepared in mice against rabbit ghosts inhibited ghost enzymatic activity and abolished the increased proteolytic activity

**TABLE 3**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Hemolysis, HU/ml</th>
<th>Proteolytic activity at 30 min, O.D. 520 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxin</td>
<td>454</td>
<td>0</td>
</tr>
<tr>
<td>Toxin + control serum</td>
<td>501</td>
<td>0</td>
</tr>
<tr>
<td>Toxin + antitoxin</td>
<td>74</td>
<td>0</td>
</tr>
<tr>
<td>Toxin + adsorbed antitoxin*</td>
<td>454</td>
<td>0</td>
</tr>
<tr>
<td>Ghosts</td>
<td>&lt;4</td>
<td>0.075</td>
</tr>
<tr>
<td>Ghosts + control serum</td>
<td>&lt;4</td>
<td>0.080</td>
</tr>
<tr>
<td>Ghosts + antitoxin</td>
<td>&lt;4</td>
<td>0.020</td>
</tr>
<tr>
<td>Ghosts + adsorbed antitoxin*</td>
<td>&lt;4</td>
<td>0.080</td>
</tr>
<tr>
<td>Ghosts + toxin</td>
<td>354</td>
<td>0.100</td>
</tr>
<tr>
<td>Ghosts + toxin + control serum</td>
<td>436</td>
<td>0.120</td>
</tr>
<tr>
<td>Ghosts + toxin + antitoxin</td>
<td>&lt;4</td>
<td>0.012</td>
</tr>
<tr>
<td>Column toxin†</td>
<td>380</td>
<td>0.100</td>
</tr>
<tr>
<td>Column toxin + control serum</td>
<td>400</td>
<td>0.100</td>
</tr>
<tr>
<td>Column toxin + antitoxin</td>
<td>&lt;4</td>
<td>0.010</td>
</tr>
</tbody>
</table>

*Wood 46 alpha antitoxin was adsorbed 3 times with rabbit ghosts at a concentration of 1.09 mg N/ml. Ghosts were removed from the antitoxin before it was added to fresh ghosts.

†Column toxin is the hemolytic and proteolytic fraction obtained by passing EDTA toxin-ghost supernatant fluid through Sephadex G-75.
observed when toxin was present (Fig. 3). The mouse serum control slightly elevated ghost proteolytic activity, but almost completely prevented the enhanced liberation of color observed when ghosts are incubated in the presence of toxin. Incubation of ghosts at 100°C for 5 min also destroyed proteolytic activity, and consequently there was no color enhancement when toxin was added. The sera did not agglutinate the rabbit ghosts.

**Hemolysis in the Presence of Antiserum to Ghosts**

Rabbit erythrocytes, 1.5 ml of a 2% suspension, were incubated 15 min at 37°C with 0.1 ml of the antiserum to rabbit ghosts prepared in mice. Alpha toxin to a final concentration of 9 HU/ml was added to the red cell-serum mixture and the O.D. at 650 nm was measured at 1-min intervals in the spectrophotometer.

As shown in Fig. 4, the addition of antiserum caused the prelytic phase to increase in length by nearly 6 min when compared to the toxin control. The presence of mouse control serum also increased this phase but not to the same extent as antiserum. Apart from the effect of the sera on the prelytic phase, rates of hemolysis (i.e. slope) were similar to that of toxin alone.

**The Effect of Alpha Antitoxin on Hemolysis and Proteolytic Activity of Toxin-Ghost Supernatant Fluid**

Rabbit ghosts and alpha toxin to a final concentration of 454 HU/ml were incubated together at 37°C for 20 min. This permitted activation of the toxin by the ghosts. Antitoxin, control serum, or buffer was then added and incubation proceeded for a further 30 min. The tubes were plunged into ice water and centrifuged at 4°C. Proteolytic and hemolytic assays were performed on the supernatant fluid.

Hemolytic and proteolytic activities of ghost supernatant fluids containing toxin were both inhibited when alpha antitoxin prepared in rabbits was added after 20 min as Table 3 shows. Control sera had little effect upon the activities of toxin and ghosts incubated separately, apart from slight enhancement of hemolysis. However,
incubation of ghosts alone with antitoxin reduced their proteolytic activity about fourfold. Consequently, the antitoxin was adsorbed 3 times with normal rabbit ghosts at a concentration of 1.09 mg N/ml. Remarkably, the adsorbed antitoxin no longer inhibited ghost proteolytic activity and it could not neutralize the hemolytic activity of the alpha toxin.

Similar observations were made with the fraction obtained from the Sephadex G-75 column which contained proteolytic and hemolytic activities as indicated in the table. In addition, antitoxin inhibited the proteolytic activity of the nonhemolytic protease peak which came off the column after the protease control.

Discussion

The immunofluorescence studies of Klainer et al. (3) have shown that toxin is detected on the surface of rabbit erythrocytes during the period of maximal hemolysis. They found that the same concentration of toxin added to human red cells produced no fluorescence unless toxin concentration was increased tenfold. Our findings also indicate that erythrocyte sensitivity to toxin is correlated with their ability to adsorb toxin.

Rabbit ghost suspensions incubated without toxin showed that the supernatant fluid contained proteolytic activity in increasing amounts as incubation progressed. Thus, proteolytic enzyme is loosely bound to the erythrocyte membranes, as little activity was observed in the first few minutes of incubation. It may be that when toxin is incubated with ghosts, it combines with protease to form a "complex" which can be dissociated by EDTA. Bernheimer and Schwartz (1) found that their alpha-2 fraction of toxin obtained by zone electrophoresis showed an 18-fold increase in hemolytic activity when dialyzed against borate-EDTA and precipitated by ammonium sulfate. They suggested that the alpha-2 preparation consisted of inactive toxin capable of activation. Our view is that the interaction of toxin with a component of the supernatant fluid (probably protease) confers proteolytic activity upon the toxin molecule. Additional evidence comes from passage of toxin-ghost supernatant fluid through Sephadex G-75. The activated toxin molecule has a lower molecular weight since its peak of activity followed that of the unactivated material. We feel that the non-hemolytic protease peak may be the protease activator which also is changed since its activity is displaced to the right of the ghost protease control. The status of the "complex" that appears to be dissociated by EDTA is unclear but it might not be identified in column fractions because of the equilibrium that would exist between activator and toxin. On the other hand, it cannot be entirely ruled out that toxin-ghost interaction releases a membrane protease with an elution volume identical with that of activated toxin. This does seem unlikely when one considers that the proteolytic activity associated with the activated toxin is neutralized by alpha antitoxin.

Rabbit ghost antiprotease prepared in mice inhibited the liberation of color from azocoll when toxin and erythrocytes were incubated together, as would be expected if the toxin required activation by membrane proteases. However, this could be due to mechanical blocking of toxin receptor sites by antibody on the membrane surface.

It might be said that the inability of antiprotease mouse serum to prevent hemolysis of rabbit erythrocytes by alpha toxin argues against the role of membrane proteases as activators (Fig. 4). However, antibodies to enzymes do not always inhibit activity. There are numerous examples of enzyme-antienzyme flocculates which remain fully active (2).

In summary, Wood-46 alpha toxin probably combines with activator (erythrocyte protease) in the supernatant fluid and on the ghost membrane surface. The "complex" formed does not irreversibly inactivate the toxin. The reaction sequence may be as follows:

\[ \text{I}^1 + \text{P}^2 \rightleftharpoons \text{I}^3 \rightleftharpoons \text{T}^4 + \text{P} \]

\[ \text{T} + \text{S}^5 \rightleftharpoons \text{T} \text{S}^6 \rightarrow \text{lysis} + \text{T} \]

The evidence supporting this scheme is based on a number of observations, (a) sensitivity of red cell species to toxin is directly related to their level of proteolytic activity as cited elsewhere as: $\text{I}^1 = \text{inactive toxin precursor}$, $\text{P} = \text{erythrocyte protease}$, $\text{I}^3 = \text{toxin-protease complex}$, $\text{T} = \text{active toxin}$, $\text{S} = \text{substrate (protein)}$, $\text{T} \text{S}^6 = \text{toxin-substrate complex}$.
(7), and (b) the action of membrane proteases on suitable substrates is enhanced in the presence of alpha toxin which alone has no proteolytic activity. (c) antiprotease inhibits this enhancement, (d) heat destroys the proteolytic activity of ghosts and prevents the adsorption of toxin, (e) EDTA increases hemolytic titers when added to toxin-ghost supernatants, suggesting that toxin forms a dissociable "complex" with an unidentified substance, presumably protease, (f) hemolytic and proteolytic activities coincide after toxin-ghost supernatant fluid is passed through Sephadex G-75, and (g) the hemolytic and proteolytic activities of toxin-ghost supernatant fluid are both inhibited by alpha antitoxin. This observation is true provided that toxin and ghosts are preincubated before antitoxin is added, and is evidence that activation of the toxin by protease does not affect its neutralization by antitoxin.

We are left to discuss the inhibition of rabbit-ghost proteolytic activity by alpha antitoxin and its abolition by adsorption of the antitoxin with ghosts. The toxin has to be activated when it is injected into the rabbit, and it appears that antibodies are formed against the activated toxin and against the protease activator. It is conceivable that toxin activation produces an altered membrane protease which elicits the formation of antibodies against the natural protease as well as itself. Antibody could also be formed against the toxin-activator "complex" although one does not know how long this state exists. If this reasoning is correct, it should follow that an alpha antitoxin will contain antibody to ghost proteolytic activity. We have shown this and have removed the serum's inhibitory capacity by pretreatment with ghosts. Furthermore, alpha antitoxin adsorbed with ghosts should not neutralize hemolytic activity of the toxin and this observation has in fact been made.

Acknowledgments

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Addendum

Observations relating to the Wood-46 alpha toxin have now been confirmed with alpha toxin from S. aureus strains G-143 and NCTC 9715.