ABSTRACT OF THESIS

Name *of Candidate* .... Gordon Marcy Wiseman ....

Address ....

Degree .... D.Sc. .... *Date* .... April 18, 1974 ....

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 phosphatidyl-inositol 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

Table of Contents

Table of Contents

List of Tables

List of Figures

Acknowledgements

Acknowledgements

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 phosphatidyl-inositol 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.

Preface

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 exocellular 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 antisera 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. Antisera 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 exis-
tence 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 CO_2 and air. The method depends upon
a different rate of diffusion through the agar and a dif-
ferent 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 characteris-
tic pattern of lysis on sheep and rabbit blood agar and by
the degree to which it was neutralized on these agars by
commercial antisera. 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. Histi-
dine 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 CO_2, 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 lysin formation intra-cellularly 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 lysin. 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[1]) 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[2]; 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

[1,2]Manuscript in Appendix.

Table I

Purification of Staphylococcal Alpha Hemolysin

Reference	Strain	Procedures[1]	Sp. Act. (HU/mg protein or nitrogen)
Madoff and Weinstein (1962)	Wood 46	D,C,A,B	4.2×10^4
Kumar et al. (1962)	"	A,A	?
Bernheimer and Schwartz (1963)	"	D,A,A,E	1.9×10^4
Goshi et al. (1963)	?	D,D,B,B	8×10^4
Lominski et al. (1963)	Wood 46	D,C,B,D,B	1.2×10^5
Jackson (1963a)	" 209-60	D,D,B	?
Robinson and Thatcher (1963)	?	D,D,B,A	?
Cooper et al. (1966)	Wood 46	D,C,A,B,B	1.2×10^6
Coulter (1966)	"	D,C,A	10^4
Arbuthnott et al. (1967)	"	D,A,A,E,F	2×10^4
Fackrell (1973) cited by Wiseman et al. (1974)	"	D,D,C,D,B	1.3×10^5

[1]A=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   SUPERNATANT
|
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   SUPERNATANT
|
buffer, dialysis
|
ION EXCHANGE
|
PURIFIED ALPHA LYSIN

Fig. 1.   Purification Scheme for Staphylococcal Alpha Toxin.

Table II

Amino Acid Analyses of Staphylococcal Alpha Hemolysin

	Bernheimer and Schwartz[1]	Coulter[2]	Fackrell and Wiseman[2,3]	Six and Harshman[2] (A)	(B)
methionine	10	4	2	6	6
aspartic acid	44	50	20	40	43
threonine	23	23	10	22	23
serine	22	20	10	19	19
glutamic acid	21	22	16	19	20
proline	7	10	14	8	9
glycine	23	24	28	20	24
alanine	12	14	12	11	11
valine	12	16	8	13	14
isoleucine	13	16	6	13	14
leucine	15	14	8	13	14
tyrosine	9	10	6	9	10
phenylalanine	10	10	4	8	8
lysine	23	26	12	21	23
histidine	4	4	4	4	4
arginine	10	8	6	8	8
ammonia	71	very high	very high	?	?
total residues	329	271	166	234	250

[1]Residues were calculated for a molecular weight of 44,000 as determined by the Archibald method.
[2]Histidine residues set at 4.
[3]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 in-
active 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 pre-
pared 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 electrophoreti-
cally 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 interconver-
sion suggested by the Arbuthnott-Bernheimer groups is not
clear. Since all four of Wadstrom's components were hemo-
lytically 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 similari-
ties 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 <u>Staphylococcus</u> <u>aureus</u> Separated by Isoelectric Focusing

McNiven et al.		Wadstrom	
Component	pI	Component	pI
Alpha A[1]	8.55±0.12	Alpha I[2]	8.5
Alpha B	9.15±0.07	Alpha IV	9.2
Alpha C	7.36±0.03	Alpha II	7.0
Alpha D	6.28±0.11	Alpha III	5.0

[1]Alpha A ⇄ Alpha B
[2]Alpha I ⇄ Alpha IV

Table IV

Some Characteristics of Staphylococcal Alpha Hemolysin

Reference	Strain	Sed. Coeff. (S_{20w})	Mol. Wt.	pI	N-terminus
Kumar et al. (1962)	Wood 46	1.45	$1\text{-}1.5 \times 10^4$	-	-
Bernheimer and Schwartz (1963)	"	3.0^a 12.0^b	4.4×10^4	-	-
Lominski et al. (1963)	"	3.1	-	-	-
Cooper et al. (1966)	"	2.8	-	-	-
Coulter (1966)	"	2.8	2.1×10^4	-	histidine arginine
Arbuthnott et al. (1967)	"	3.0	-	-	-
Wadstrom (1968)	Wood 46 V 8, M 18	-	-	alpha I $8.0\text{-}8.7^a$	-
Wiseman and Caird (1970)	Wood 46	-	-	-	histidine
Forlani et al. (1971)	?	2.8	3.3×10^4	-	-
McNiven et al. (1972)	Wood 46	-	3.6×10^4	alpha A 8.55^a	-
Six and Harshman (1973a,b)	"	(A) 3.0 (B) 3.0	2.8×10^4 2.6×10^4	7.2 8.4	alanine alanine
Fackrell and Wiseman (1974c)	"	1.4^c	4.5×10^4	8.5	histidine

[a] Major component.
[b] Inactive aggregate.
[c] Unstable. After standing several days, $S_{20w} = 2.8$.

on the interaction of alpha toxin with artificial membranes, the latter are far removed in character from natural membranes.

Wiseman and Caird (1970, 1972[1]) 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.

---

[1]Published papers in Appendix.

Table V

Toxin-Induced Liberation of Non-esterified Fatty Acids from Selected Substrates[1]

Substrate	Toxin	Fatty acid (milliequivalents)
phospholipids (rabbit erythrocytes)	alpha	0
"	phospholipase A (snake venom)	16×10^{-4}
phosphatidyl-choline	alpha	0
"	phospholipase A (snake venom)	8×10^{-4}

[1]Wiseman (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[1]

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

[1]Wiseman (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:

$$(1) \quad I^1 + P^2 \rightleftarrows IP \rightleftarrows T^3 + P$$
$$(2) \quad T + S^4 \rightleftarrows TS \rightarrow lysis + T$$
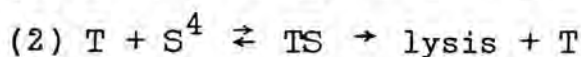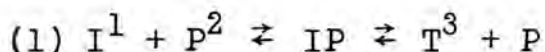
An analysis of toxin-treated ghosts further revealed that protein content was reduced (Table VII).

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[1]

Assay	mg/ml[2]	
	Untreated membranes	Membranes treated with alpha hemolysin
Phospholipid	1.19	1.20
Total lipid	1.45	1.50
Cholesterol	0.33	0.34
Total carbohydrate	0.26	0.27
Hexosamine	0.14	0.14
Sialic acid	0.12	0.12
Pentose (as ribose)	0.07	0.06
Nitrogen	0.50	0.39
Nitrogen as protein[3]	3.10	2.42
Protein[4]	1.84	1.66

[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 phosphatidyl-
choline 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 ± 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[1]). As with alpha toxin, incubation of cultures in the presence of CO_2 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

---

[1] Publication in Appendix, pp. 239-243.

Table VIII

Purification of Staphylococcal Beta Hemolysin

Reference	Strain	Procedures[1]	Sp. Act. (HU/mg protein or nitrogen)
Robinson et al. (1958)	L16	D,B,D,A	?
Jackson (1963b)	J32a	D,D,B	?
Chesbro et al. (1965)	UNH-Donita	B	?
Wiseman and Caird (1967)	R-1, 252F	B,D,C	?
Maheswaran et al. (1967)	T19	D,C,B,B	7×10^4
Gow and Robinson (1969)	MB534	D,C,B,E	5×10^5
Haque and Baldwin (1969)	681	D,B	10^7
Wadstrom and Mollby (1971a)	R-1	B,A[2],C	3×10^{11}

[1]A=electrophoresis, B=ion exchange chromatography, C=gel filtration (molecular sieve), D=precipitation, E=gradient fractionation.
[2]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 $pI = 9.5 \pm 0.1$. 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 IX

Staphylococcal Hemolysins
Amino Acid Composition*

Amino Acid	Alpha	Beta	Gamma	Delta
Ammonia	High	441.6	141.4	218.5
Aspartic Acid	121.2	135.1	142.0	164.1
Threonine	56.9	53.8	68.0	119.9
Serine	65.1	98.7	77.4	96.3
Glutamic Acid	93.0	116.9	102.4	66.4
Proline	81.2	-	50.2	14.0
Glycine	170.1	121.2	125.0	112.4
Alanine	75.8	78.2	63.2	75.2
Valine	50.1	57.8	51.0	76.0
Cysteine	-	12.2	-	-
Methionine	12.1	-	3.5	-
Isoleucine	40.4	52.5	44.4	102.7
Leucine	46.0	58.8	57.8	61.2
Tyrosine	33.5	6.3	30.6	9.3
Phenylalanine	28.3	41.4	35.8	53.5
Lysine	70.9	98.0	78.4	24.3
Histidine	20.0	24.8	27.2	3.5
Arginine	34.5	39.6	38.6	21.4

*Residues/1,000 amino acid residues (after Fackrell 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 $\pm$ 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[1]; 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 poly-saccharides 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

[1]Publication in Appendix.

Table X

Some Characteristics of Staphylococcal Beta Hemolysin

Reference	Strain	Sed. Coeff. (S_{20w})	Mol. Wt.	pI	Enzyme activity
Mollby and Wadstrom (1970)	R-1	-	-	9.5 ± 0.1	?
Gow and Robinson (1969)	MB534	1.7	-	-	?
Chesbro and Kucic (1971)	UNH-15	-	15,500[1]	-	?
Chesbro et al. (1965)	UNH-Donita	-	59,000[2]	8.6-8.9	carbohydrase
Wadstrom and Mollby (1971a)	R-1	-	38,000[3]	9.4 ± 0.1	phospho-lipase
Fackrell and Wiseman (1974c)	R-1	1.8	26,000[3] 16,110[4]	9.5	phospho-lipase

[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 sensiti-
vity 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 under-
taken, 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 con-
centrations 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 $-N^+(CH_3)_3$ 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, 1968[1]; 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

[1]Publication 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 intra-
peritoneal 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 μg. Wadstrom and Mollby (1971b,1972)
reported that the LD_{50} dose of beta lysin was in the range
of 10-100 μg for mice, rabbits and guinea pigs, and in
chicken embryos, the dose was 0.25-10 μg. Their view is
that the toxin on a weight basis is as toxic as alpha toxin.
However, their results were not precisely reported as μg/kg
body weight. Arbuthnott (1970) and Fackrell and Wiseman

(1974c) have reported that the LD_{50} of alpha toxin is
0.68-1 $LD_{50}/\mu g$ 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 discrepan-
cies 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 <u>S. aureus</u>. 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 anti-hemolysins.

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

Reference	Strain	Procedures[1]	Sp. Act. (HU/mg protein or nitrogen)
Jackson and Little (1958b)	1363,2426, 2428,2429	H,D,G	3200
Yoshida (1963)	Foggie	(1)H,D,G,G,B,B (2)H,B,B	120 400
Caird and Wiseman (1970)	E-delta	D,D,B,B	12,000
Kreger et al. (1971)	Wood 46M[6]	B	400^2 200^3
Heatley (1971)	186x[7]	H,D,G,G,G,D	250-300
Kapral and Miller (1971)	PG114	F,D	400^4 200^5
Kantor et al. (1972)	Wood 46M	B	75

[1]A=electrophoresis, B=ion exchange chromatography, C=gel filtration, D=precipitation, E=gradient fractionation, F=Diaflo ultrafiltration, G=extraction with organic solvent, H=heating.

[2],[4]Insoluble delta lysin.
[3],[5]Soluble delta lysin.
[6]Derived from Wood 46 by UV irradiation.
[7]Derived from Newman by subculture.

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 $\pm$ 0.3 and 5.0 $\pm$ 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 $\pm$ 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 XII

Some Characteristics of Staphylococcal Delta Hemolysin

Reference	Delta lysin conc.	pI	Mol. Wt.	Sed. Coeff. (S_{20w})
Yoshida (1963)	0.4%	-	68,320[1] 74,000[2]	6.1
Hallander (1963)	?	-	>200,000[3]	-
Mollby and Wadstrom (1970)	?	9.6±0.2	-	-
Maheswaran and Lindorfer (1970)	?	(I) 3.32 (II) 3.75 (III) 8.45	-	-
Caird and Wiseman (1970)	6.0 mg/ml	-	>200,000[4]	2.8 9.8
Kreger et al. (1971)	0.6%[7]	(I) 9.5±0.3 (II) 5.0±0.2	-	1.9 4.9 11.9
Kantor et al. (1972)	?	(I) 4.65 (II) 6.70 (III) 9.00	103,000[5] 195,000[6]	6.04
Heatley (1971)	6.0 mg/ml	-	-	4.9

[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 electro-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[1]) 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.

---

[1]Paper 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
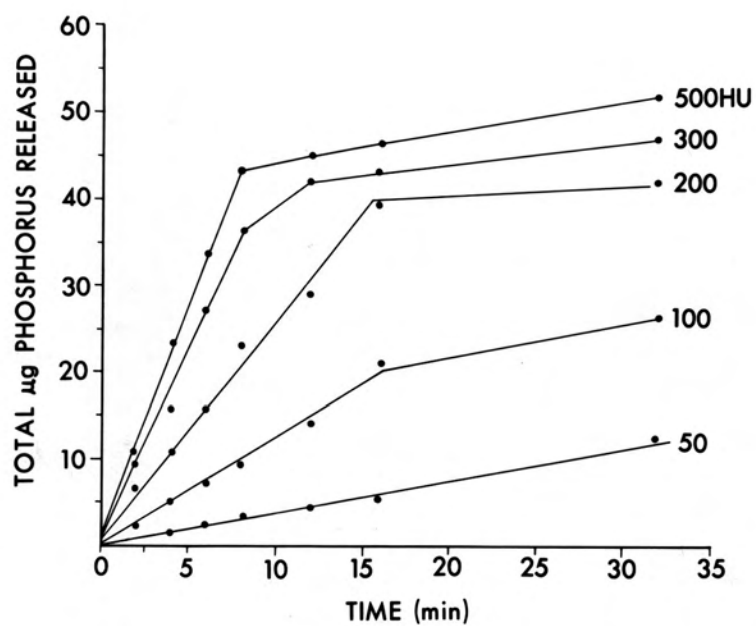
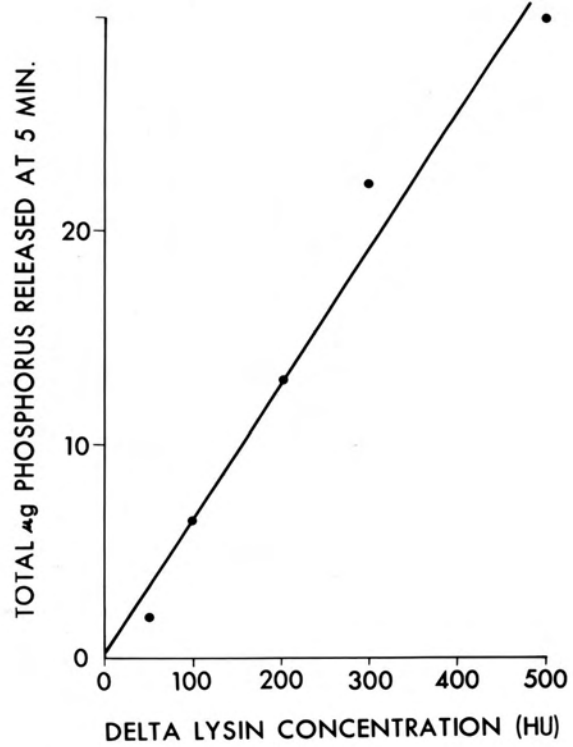FIG. 2   DEGRADATION OF PHOSPHATIDYLINOSITOL BY DELTA LYSIN.

FIG. 3   RATE OF DEGRADATION OF PHOSPHATIDYLINOSITOL
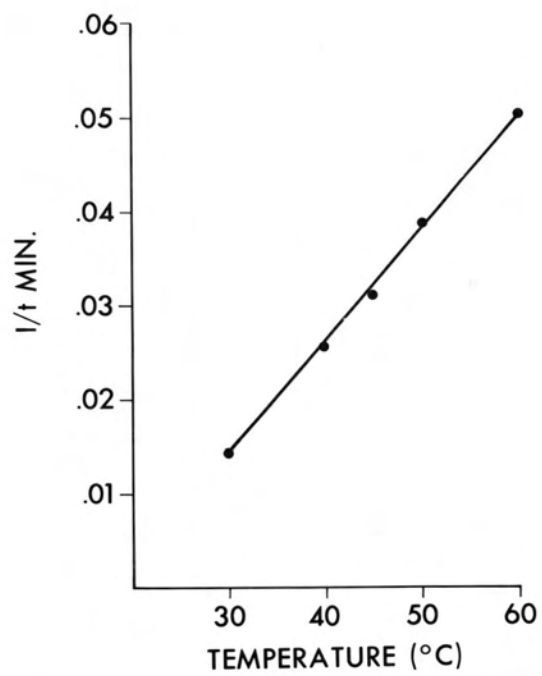BY DELTA LYSIN.

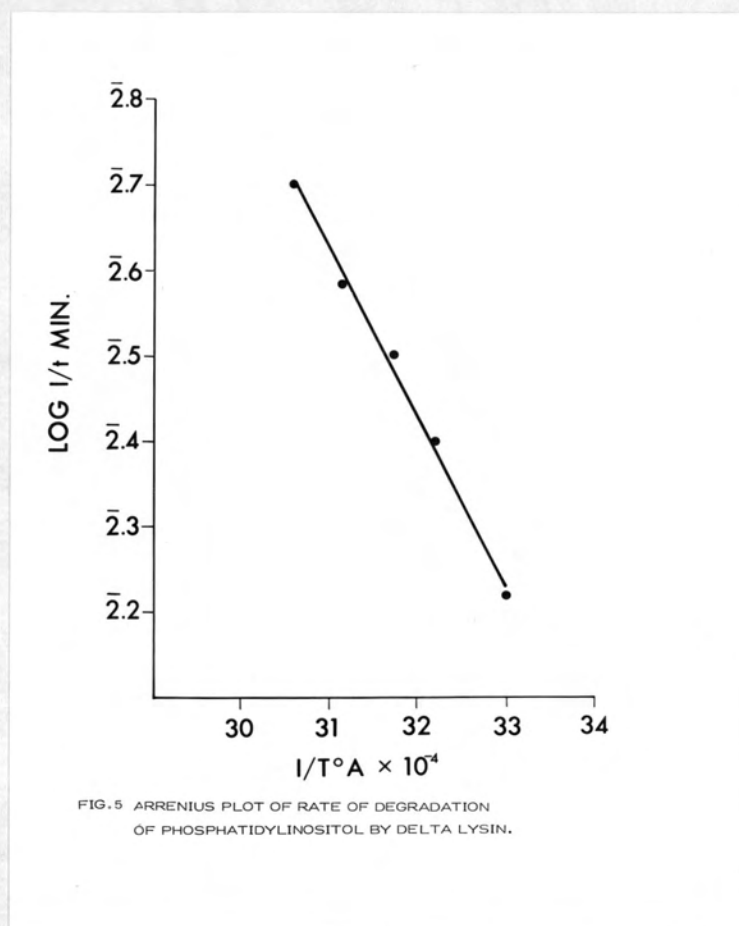FIG. 4   EFFECT OF TEMPERATURE ON RATE OF DEGRADATION
OF PHOSPHATIDYLINOSITOL BY DELTA LYSIN.

FIG. 5 ARRENIUS PLOT OF RATE OF DEGRADATION
OF PHOSPHATIDYLINOSITOL BY DELTA LYSIN.

useful to remember that interaction of proteins and phos-
pholipids is a common phenomenon. On the other hand, the
view has been put forward that the delta lysin is a phospho-
lipase 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) con-
firmed Kapral's (1967,1972) observation that hemolytic
activity of delta hemolysin was inhibited by several phos-
pholipids 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

Erythro-cytes	HU/mg protein[1]	% total phospholipid[2]		
		phosphatidyl-ethanolamine	phosphatidyl-serine	phosphatidyl-inositol
sheep	2929	26.2	14.1	2.9
rat	1700	21.5	10.8	3.5
rabbit	1644	31.9	12.2	1.6
cat	1500	22.2	13.2	7.4
dog	882	22.4	15.4	2.2
guinea pig	803	24.6	16.8	2.4

[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

Species	Beta lysin titre (HU/ml)	% Sphingomyelin[1]		
		Wiseman and Caird	Dawson	Nelson
Sheep	1810	50.5	49.0	51.0
Ox	1280	45.0	44.0	46.2
Man	256	21.6	-	-
Rabbit	160	19.7	-	19.0
Goat	113	38.0	40.0	45.9
Horse	< 20	15.0	23.0	13.5
Guinea pig	< 20	16.6	-	11.1
Monkey	< 20	11.4	-	-

[1]From 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[1]). 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

---

[1] Paper 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 dermo-
necrosis 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_{50} 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_{50} 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 conta-
minant was present in concentrations sufficient to kill the
animals. As shown in the Table, Fackrell and Wiseman have
determined that the LD_{50} 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_{50}
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

| Reference | Animal | Dose (mg/kg)[3] | |
		Lethal	Dermone-crotic[4]
Kreger et al. (1971)	mouse	110 (MLD)	-
	rabbit	30 (MLD)	0.5-1.0
	guinea pig	-	"
Kreger et al. (1971)[1]	rabbit	-	$<$1-1
	guinea pig	-	"
Gladstone (1966)[2]	mouse	>10 (LD_{50})	-
	rabbit	-	0.005-0.5
Wadstrom and Mollby (1972)	rabbit	5000 (LD_{50})	-
Fackrell and Wiseman (1974c)	mouse	4 (LD_{50})	0.1
	guinea pig	4 "	0.1

[1]Delta lysin supplied by Dr. Kapral (Infect. Immun. 4, 541, 1971).
[2]Supplied by Dr. Yoshida (Biochim. Biophys. Acta 71, 544, 1963).
[3]Calculated on the basis of 25 g. mouse, 1 kg. rabbit, average weights.
[4]These 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 $alpha_2$, 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[1]). 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% CO_2 and oxygen. Mollby and Wadstrom did not aerate the liquid cultures apart from that obtained

---

[1]Paper 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% CO_2 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

Reference	Strain	Procedure[1]	Sp. Act. (HU/mg protein)
Guyonnet et al. (1968)	5R	B	1000-2500
Mollby and Wadstrom (1971)	"	B,A	?
Fackrell and Wiseman (1974b)	"	I,C,D,F,D	10^5

[1]B=ion exchange chromatography, A=electrofocusing, I=ultra-filtration, 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 x 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 intes-
tines 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 (μ) values of 1600 and 4800
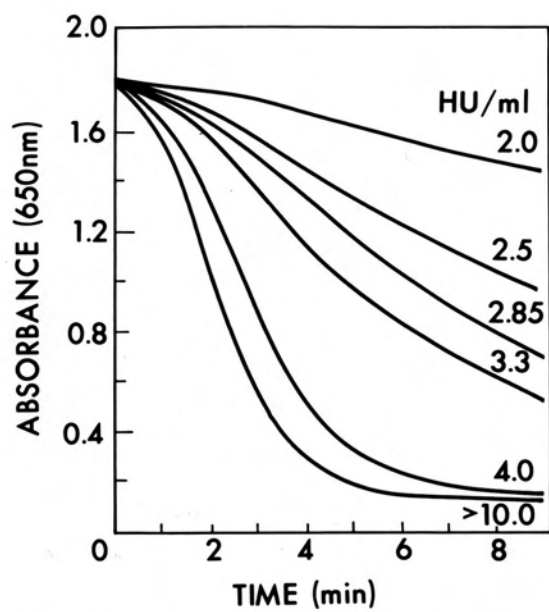
FIG. 6. HEMOLYSIS OF RABBIT ERYTHROCYTES IN THE PRESENCE OF
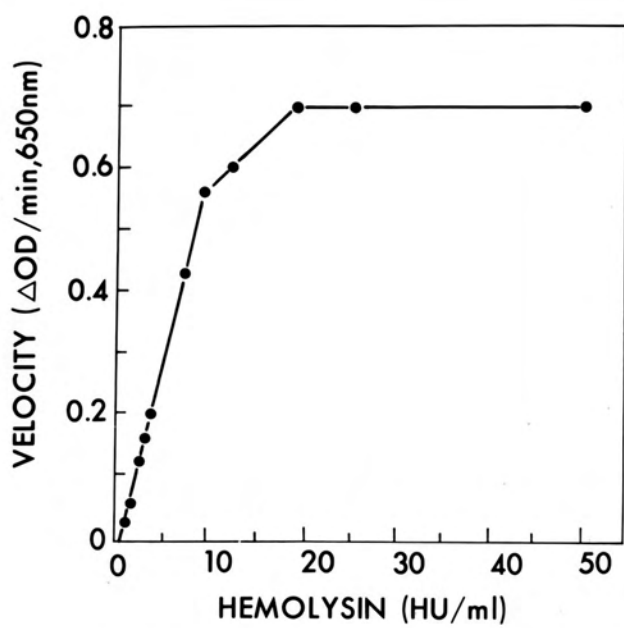GAMMA LYSIN. LYSIN CONCENTRATION (HU/ml) VERSUS TIME.

FIG. 7. HEMOLYSIS OF RABBIT ERYTHROCYTES IN THE PRESENCE OF GAMMA
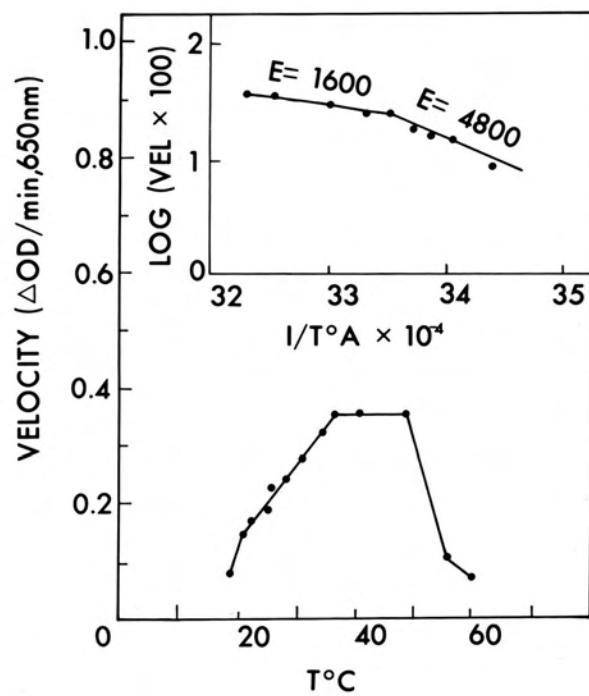LYSIN. REACTION VELOCITY VERSUS LYSIN CONCENTRATION.

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[1]) 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

---

[1]Paper in Appendix.

FIG. 9 . EFFECT OF pH ON GAMMA LYSIN–RED CELL INTERACTION.
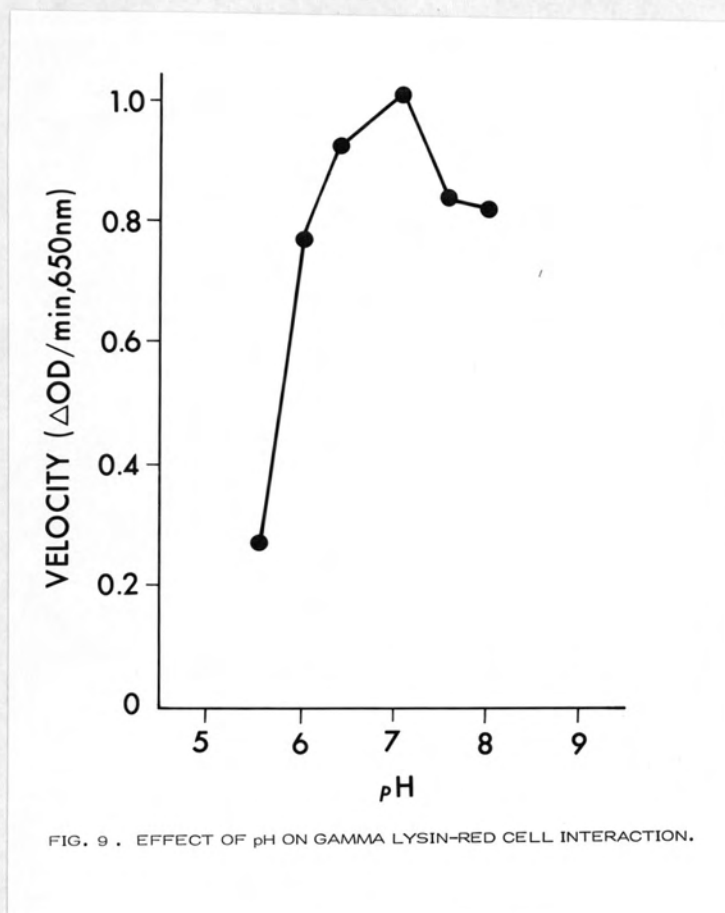
or not phospholipids are in fact attacked by gamma lysin, it certainly had no effect on phosphatidylserine, phosphatidylethanolamine, sphingomyelin or phosphatidylinositol. These observations rule out contamination of the gamma lysin with beta and delta lysins. Furthermore, neither TAME nor Azocoll were 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

Arbuthnott, J.P. 1970. Microbial toxins. Vol. III, Chapter 5. N.Y. Academic Press.

Arbuthnott, J.P., Freer, J.H., and Bernheimer, A.W. 1967. Physical states of staphylococcal alpha toxin. J. Bact. 94, 1170-1177.

Arbuthnott, J.P., Freer, J.H., and Billcliffe, B. 1973. Lipid-induced polymerization of staphylococcal alpha toxin. J. Gen. Microbiol. 75, 309-319.

Bernheimer, A.W. 1970. Microbial toxins. Vol. I, Chapter 5. N.Y. Academic Press.

Bernheimer, A.W., and Schwartz, L.L. 1963. Isolation and composition of staphylococcal alpha toxin. J. Gen. Microbiol. 30, 455-468.

Bernheimer, A.W., Kim, K.S., Remsen, C.C., Antanavage, J., and Watson, S.W. 1972. Factors affecting interaction of staphylococcal alpha toxin with membranes. Infect. Immun. 6, 636-642.

Birch-Hirschfeld, L. 1934. Ueber die wirksamkeit der extracts von auf zellophanagar gezüchteten staphylokokken. Z. Immunforsch. 81, 260-285.

Bonventre, P.F. 1970. Microbial toxins. Vol. I, Chapter 2. N.Y. Academic Press.

Bramann, J., and Norlin, G. 1951. A factor in yeast dialysate of significance in the formation of Staphylococcus toxin. Acta Path. Microbiol. Scand. 29, 127-131.

Buckelew, A.R., and Colacicco, G. 1971. Lipid monolayers. Interactions with staphylococcal alpha-toxin. Biochim. Biophys. Acta 233, 7-16.

Burnet, F.M. 1930. The production of staphylococcal toxin. J. Path. Bact. 33, 1-16.

Caird, J.D., and Wiseman, G.M. 1970. Purification of the delta toxin of Staphylococcus aureus. Can. J. Microbiol. 16, 703-708.

Camejo, G., Colacicco, G., and Rapport, W.M. 1968. Lipid monolayers: interactions with the apoprotein of high density plasma lipoprotein. J. Lipid Res. 9, 562-569.

Chesbro, W.R., and Kucic, V. 1971. Beta hemolysin: a persistent impurity in preparations of staphylococcal nuclease and enterotoxin. Appl. Microbiol. 22, 233-241.

Chesbro, W.R., Heydrick, F.P., Martineau, R., and Perkins, G.N. 1965. Purification of staphylococcal beta hemolysin and its action on staphylococcal and streptococcal cell walls. J. Bact. 89, 378-389.

Christie, R., and North, E.A. 1941. Observations on staphylococcal beta toxin and its production by strains isolated from staphylococcal lesions in man. Aust. J. Exp. Biol. Med. Sci. 19, 323-327.

Cooper, L.Z., Madoff, M.A., and Weinstein, L. 1966. Heat stability and species range of purified staphylococcal alpha toxin. J. Bact. 91, 1686-1692.

Coulter, J.R. 1966. Production, purification, and composition of staphylococcal alpha toxin. J. Bact. 92, 1655-1662.

Coulter, J.R., and Mukherjee, T.M. 1971. Electron microscopic localization of alpha toxin within the staphylococcal cell by ferritin-labelled antibody. Infect. Immun. 4, 650-655.

Dalen, A.B. 1973a. Production of staphylococcal alpha toxin in a defined medium and identification of a stimulating factor from yeast extract. J. Gen. Microbiol. 74, 53-60.

Dalen, A.B. 1973b. The induction of staphylococcal alpha toxin by histidine. J. Gen. Microbiol. 74, 61-69.

de Christmas, M.J. 1888. Recherches experimentales sur la suppuration. Ann. Inst. Pasteur 2, 469-478.

Dodge, J.T., Mitchell, C., and Hanahan, D.J. 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Arch. Biochem. Biophys. 100, 119-130.

Doery, H.M., and North, E.A. 1961. The interaction of staphylococcal toxin and ganglioside. I. Inactivation of the lethal effect of staphylococcal toxin in mice. Aust. J. Exp. Biol. Med. Sci. 39, 333-344.

Doery, H.M., Magnusson, B.J., Cheyne, I.M., and Gulasekharam, J. 1963. A phospholipase in staphylococcal toxin which hydrolyzes sphingomyelin. Nature 198, 1091-1092.

Doery, H.M., Magnusson, B.J., Gulasekharam, J., and Pearson, J.E. 1965. The properties of phospholipase enzymes in staphylococcal toxins. J. Gen. Microbiol. 40, 283-296.

Dolman, C.E., and Wilson, R.J. 1940. The kitten test for _Staphylococcus_ enterotoxin. Can. Publ. Hlth. J. <u>31</u>, 68-71.

Duncan, J.L., and Cho, G.J. 1971. Production of staphylococcal alpha toxin. I. Relationship between cell growth and toxin formation. Infect. Immun. <u>6</u>, 456-461.

Duncan, J.L., and Cho, G.J. 1972. Production of staphylococcal alpha toxin. II. Glucose repression of toxin formation. Infect. Immun. <u>6</u>, 689-694.

Elek, S.D. 1959. _Staphylococcus pyogenes_ and its relation to disease. Edinburgh. Livingstone.

Elek, S.D., and Levy, E. 1950. Distribution of hemolysins in pathogenic and non-pathogenic staphylococci. J. Path. Bact. <u>62</u>, 541-554.

Fackrell, H.B. 1973. Studies with gamma hemolysin of _Staphylococcus aureus_. Ph.D. thesis. University of Manitoba.

Fackrell, H.B., and Wiseman, G.M. 1974a. Immunogenicity of the delta hemolysin of _Staphylococcus aureus_. J. Med. Microbiol. In press.

Fackrell, H.B., and Wiseman, G.M. 1974b. Production and purification of the gamma hemolysin of _Staphylococcus aureus_ "Smith 5R". J. Gen. Microbiol. In press.

Fackrell, H.B., and Wiseman, G.M. 1974c. Properties of the gamma hemolysin of _Staphylococcus aureus_ "Smith 5R". J. Gen. Microbiol. In press.

Fackrell, H.B., and Wiseman, G.M. 1974d. Mode of action of the gamma hemolysin of _Staphylococcus aureus_ "Smith 5R". J. Gen. Microbiol. In press.

Forlani, L., Bernheimer, A.W., and Chiancone. E. 1971. Ultracentrifugal analysis of staphylococcal alpha toxin. J. Bact. <u>106</u>, 138-142.

Freer, J.H., Arbuthnott, J.P., and Bernheimer, A.W. 1968. Interaction of staphylococcal alpha toxin with artificial and natural membranes. J. Bact. <u>95</u>, 1153-1168.

Freer, J.H., Arbuthnott, J.P., and Billcliffe, B. 1973. Effects of staphylococcal alpha toxin on the structure of erythrocyte membranes: a biochemical and freeze-etching study. J. Gen. Microbiol. <u>75</u>, 321-332.

Fritsche, D. 1970. Untersuchungen zur funktion des beta-hämolysins von _Staphylococcus aureus_. Zbl. Bakt. I. Abt. Orig. <u>213</u>, 348-355.

Galanti, B., Paradisi, F., Mancini, A., and Guisti, G. 1968. An early effect of Staphylococcus alpha-toxin on cells growing in culture: ATP levels in normal and intoxicated cells. Path. Microbiol. 32, 277-284.

Ganczarski, A. 1962. Lodz. Towarz. Nauk. Wydzial IV, Nr. 44, 1-84.

Gladstone, G.P. 1938. The production of staphylococcal alpha hemolysin in a chemically-defined medium. Brit. J. Exp. Path. 19, 208-226.

Gladstone, G.P. 1966. Staphylococcal hemolysins. Postepy Mikrobiol. 5, 145-160.

Gladstone, G.P., and Van Heyningen, W.E. 1957. Staphylococcal leucocidins. Brit. J. Exp. Path. 38, 123-137.

Gladstone, G.P., and Yoshida, A. 1967. The cytopathic action of purified staphylococcal delta hemolysin. Brit. J. Exp. Path. 48, 11-19.

Glenny, A.T., and Stevens, N.F. 1935. Staphylococcus toxins and antitoxins. J. Path. Bact. 40, 201-210.

Goshi, K., Cluff, L.E., and Norman, P.S. 1963. Studies on the pathogenesis of staphylococcal infection. V. Purification and characterization of staphylococcal alpha hemolysin. Bull Johns Hopkins Hosp. 112, 15-30.

Gow, J.A., and Robinson, J. 1969. Properties of purified staphylococcal beta hemolysin. J. Bact. 97, 1026-1032.

Guyonnet, F., and Plommet, M. 1970. Hémolysine gamma de Staphylococcus aureus: purification et propriétés. Ann. Inst. Pasteur 118, 19-33.

Guyonnet, F., Plommet, M., and Bouillanne, C. 1968. Purification de l'hémolysine gamma de Staphylococcus aureus. C.R. Acad. Sc. (Paris) 267, 1180-1182.

Hallander, H.O. 1963. Fractionation of staphylococcal toxins by gel-filtration. Acta Path. Microbiol. Scand. 59, 543-552.

Hallander, H.O. 1968. Characterization and partial purification of staphylococcal delta-lysin. Acta Path. Microbiol. Scand. 72, 586-600.

Hallander, H.O., and Bengtsson, S. 1967. Studies on the cell toxicity and species specificity of purified staphylococcal toxins. Acta Path. Microbiol. Scand. 70, 107-119.

Haque, R. 1963. Purification and properties of staphylo-
coccal beta hemolysin. Ph.D. thesis. Ohio State University.

Haque, R., and Baldwin, J.N. 1964. Purification and
properties of staphylococcal beta-hemolysin. I. Production
of beta-hemolysin. J. Bact. 88, 1304-1309.

Haque, R., and Baldwin, J.N. 1969. Purification and
properties of staphylococcal beta hemolysin. II. Purifi-
cation of beta hemolysin. J. Bact. 100, 751-759.

Heatley, N.G. 1971. A new method for the preparation and
some properties of staphylococcal delta hemolysin. J. Gen.
Microbiol. 69, 269-278.

Heydrick, F.P., and Chesbro, W.R. 1962. Purification and
properties of staphylococcal beta toxin. Bact. Proc. p. 63.

Hoffman, E.M., and Streitfeld, M.M. 1965. The antibiotic
activity associated with preparations of delta hemolysin of
Staphylococcus aureus. Can. J. Microbiol. 11, 203-211.

Jackson, A.W. 1962. Staphylococcal gamma lysin and its
differentiation from delta lysin. Abstracted in VIIIth
International Congress for Microbiology Abstracts.

Jackson, A.W. 1963a. Purification of staphylococcal-alpha-
lysin. Can. J. Biochem. Physiol. 41, 219-229.

Jackson, A.W. 1963b. Purification of staphylococcal beta-
lysin. Can. J. Biochem. Physiol. 41, 755-761.

Jackson, A.W., and Little, R.M. 1956. The delta-toxin of
Micrococcus pyogenes var. aureus. Bact. Proc. p. 88.

Jackson, A.W., and Little, R.M. 1957. Leucocidal effect
of staphylococcal delta lysin. Can. J. Microbiol. 3, 101-
102.

Jackson, A.W., and Little, R.M. 1958a. Staphylococcal
toxins. II. Factors affecting hemolysis by delta lysin.
Can. J. Microbiol. 4, 435-444.

Jackson, A.W., and Little, R.M. 1958b. Staphylococcal
toxins. III. Partial purification and some properties of
delta lysin. Can. J. Microbiol. 4, 453-461.

Jeljaszewicz, J., Szmigielski, S., Korbecki, M., and Zak,
C. 1965. Histochemical demonstration of changes in enzyma-
tic activity of KB cells produced by staphylococcal alpha
and beta hemolysins. J. Infect. Dis. 115, 421-428.

Kantor, H.S., Temples, B., and Shaw, W.V. 1972. Staphylo-
coccal delta hemolysin: purification and characterization.
Arch. Biochem. Biophys. 151, 142-156.

Kapral, F.A. 1967. Neutralization of Staphylococcus aureus
delta hemolysin by phospholipids. Bact. Proc. p. 64.

Kapral, F.A. 1972. Inhibition of Staphylococcus aureus
delta hemolysin by phospholipids. Proc. Soc. Exp. Biol.
Med. 141, 519-521.

Kapral, F.A., and Miller, M.M. 1971. Product of Staphylo-
coccus aureus responsible for the scalded skin syndrome.
Infect. Immun. 4, 541-545.

Kayser, A. 1968. L'Hemolysine staphylococcique delta.
Action sur les érythrocytes et les stromas, sur les sphéro-
plasts bacteriens et sur le jaune d'oeuf. Bull. Soc. Chim.
Biol. 50, 1797-1808.

Klainer, A.S., Chang, T.W., and Weinstein, L. 1972. Effects
of purified staphylococcal alpha toxin on the ultrastructure
of human and rabbit erythrocytes. Infect. Immun. 5, 808-813.

Klebs, E. 1872. Beiträge zur pathologischen anatomie der
schusswunde. Leipzig. pp. 104-122.

Korbecki, M., and Jeljaszewicz, J. 1965. Action of staphy-
lococcal toxins in cell cultures. J. Infect. Dis. 115, 205-
213.

Kraus, R., and Clairmont, P. 1900. Uber hämolysin und
antihämolysine. Wein. Klin. Wschr. 13, 49-56.

Kreger, A.S., and Bernheimer, A.W. 1971. Disruption of
bacterial protoplasts and spheroplasts by staphylococcal
delta hemolysin. Infect. Immun. 3, 603-605.

Kreger, A.S., Kim, K.-S., Zaboretsky, F., and Bernheimer,
A.W. 1971. Purification and properties of staphylococcal
delta hemolysin. Infect. Immun. 3, 449-465.

Kumar, S., and Lindorfer, R.K. 1962. The characterization
of staphylococcal toxins. I. The electrophoretic migration
of the alpha hemolytic, dermonecrotic, lethal, and leucocidal
activities of crude toxin. J. Exp. Med. 115, 1095-1106.

Kumar, S., Locken, K., Kenyon, A., and Lindorfer, R.K. 1962.
The characterization of staphylococcal toxins. II. The
isolation and characterization of a homogeneous staphylo-
coccal protein possessing alpha hemolytic, dermonecrotic,
lethal and leucocidal activities. J. Exp. Med. 115, 1107-
1115.

Leboeuf-Trudeau, T., de Repentigny, J., Frenette, R.M., and Sonea, S. 1967. Inhibitory effects of tryptophan analogues on growth and alpha toxin activity of Staphylococcus aureus. Bact. Proc. p. 64.

Lominski, I., Arbuthnott, J.P., and Spence, J.B. 1963. Purification of Staphylococcus alpha-toxin. J. Path. Bact. 86, 258-262.

MacFarlane, M.G., and Knight, B.C.J.G. 1941. The biochemistry of bacterial toxins. I. The lecithinase activity of Clostridium welchii toxins. Biochem. J. 35, 884-902.

Madoff, M.A., and Weinstein, L. 1962. Purification of staphylococcal alpha-hemolysin. J. Bact. 83, 914-918.

Madoff, M.A., Artenstein, M.S., and Weinstein, L. 1963. Studies of the biologic activity of purified staphylococcal alpha-toxin: II. The effect of alpha-toxin on Ehrlich ascites carcinoma cells. Yale J. Biol. Med. 35, 382-389.

Maheswaran, S.K., and Lindorfer, R.K. 1967. Staphylococcal beta hemolysin. II. Phospholipase C activity of purified beta hemolysin. J. Bact. 94, 1313-1319.

Maheswaran, S.K., and Lindorfer, R.K. 1970. Purification and partial characterization of staphylococcal delta hemolysin. Bact. Proc. p. 78.

Maheswaran, S.K., Smith, K.L., and Lindorfer, R.K. 1967. Staphylococcal beta hemolysin. I. Purification of beta hemolysin. J. Bact. 94, 300-305.

Maniar, A.C., Westlake, C., and Warner, P. 1967. Prevalence of staphylococcal anti-delta lysin in man. Can. J. Microbiol. 13, 925-929.

Marks, J. 1951. The standardization of staphylococcal alpha antitoxin with special reference to anomalous hemolysins including delta lysin. J. Hyg. (London) 49, 52-56.

Marks, J., and Vaughan, A.C.T. 1950. Staphylococcal delta hemolysin. J. Path. Bact. 62, 597-615.

Martin, R.G., and Ames, B.N. 1961. Measurement of sedimentation coefficients of enzymes in the preparative ultracentrifuge. J. Biol. Chem. 236, 1372-1379.

McNiven, A.C., and Arbuthnott, J.P. 1971. Cell-associated alpha toxin from Staphylococcus aureus. J. Med. Microbiol. 5, 123-127.

McNiven, A.C., Owen, P., and Arbuthnott, J.P. 1972. Multiple forms of staphylococcal alpha toxin. J. Med. Microbiol. 5, 113-122.

Meduski, J.W., and Hochstein, P. 1972. Hot-cold hemolysis: the role of positively charged membrane phospholipids. Experientia 15, 565-566.

Mollby, R., and Wadstrom, T. 1970. Protides of the biological fluids. pp. 465-469. Oxford. Pergamon Press.

Mollby, R., and Wadstrom, T. 1971. Separation of gamma hemolysin from Staphylococcus aureus Smith 5R. Infect. Immun. 3, 633-635.

Morgan, F.G., and Graydon, J.J. 1936. Toxins of the Staphylococcus, with especial reference to the estimation of potency. J. Path. Bect. 43, 385-401.

Murphy, R.A., and Haque, R. 1967. Purification and properties of staphylococcal delta hemolysin. I. Production of delta hemolysin. J. Bact. 94, 1327-1333.

Nelis, P. 1935. Contribution a l'étude de la toxine staphylococcique et de ses dérivés (antitoxine et anatoxine staphylococcique). Bull. Acad. Med. Belg. 15, 539-568.

Nelson, G.J. 1967. Lipid composition of erythrocytes in various mammalian species. Biochim. Biophys. Acta 144, 221-232.

Noll, H. 1966. Chain initiation and control of protein synthesis. Science 151, 1241-1245.

Novak, E., Seifert, J., and Raskova, H. 1970. Effect of staphylococcal alpha toxin on mitochondrial Mg^{2+} (Na^+, K^+) activated adenosine triphosphatase. Toxicon 8, 261-270.

Novak, E., Seifert, J., Buchar, E., and Raskova, H. 1971a. Effect of staphylococcal alpha toxin on the phosphorylation of ADP by rat liver mitochondria-I. Inhibition of phosphorylation by a thermo-stable factor. Toxicon 9, 211-218.

Novak, E., Seifert, J., Buchar, E., and Raskova, H. 1971b. Effect of staphylococcal alpha toxin on the phosphorylation of ADP by rat liver mitochondria-II. Effect of staphylococcal alpha toxin upon electron transport. Toxicon 9, 361-366.

Parker, J.T., Hopkins, J.G., and Gunther, A. 1926. Further studies on the production of Staphylococcus aureus toxin. Proc. Soc. Exp. Biol. (N.Y.) 23, 344-346.

Rahal, J.J. 1972. Comparative effects of purified staphylococcal alpha and delta toxins on mitochondrial metabolism. J. Infect. Dis. 126, 96-103.

Rahal, J.J., Plaut, M.E., Rosen, H., and Weinstein, L. 1967. Purified staphylococcal alpha toxin: effect on epithelial ion transport. Science 155, 1118-1120.

Robinson, J., and Thatcher, F.S. 1963. Studies with staphylococcal toxins. VII. Separation of a proteolytic enzyme from alpha hemolysin. Can. J. Microbiol. 9, 697-702.

Robinson, J., Thatcher, F.S., and Gagnon, J. 1958. Studies with staphylococcal toxins. IV. The purification and metallic requirements of specific hemolysins. Can. J. Microbiol. 4, 345-361.

Rose, H.G., and Oklander, M. 1965. Improved procedure for the extraction of lipids from human erythrocytes. J. Lipid Res. 6, 428-431.

Samánek, M., and Zajic, F. 1965. Proc. 2nd Intern. Pharmacol. Mtg. Prague, 1963. Vol. 9, p. 199. Oxford, Pergamon Press.

Six, H.R., and Harshman, S. 1973a. Purification and properties of two forms of staphylococcal alpha toxin. Biochem. 12, 2672-2676.

Six, H.R., and Harshman, S. 1973b. Physical and chemical studies on staphylococcal alpha toxins A and B. Biochem. 12, 2677-2682.

Sizer, I.W. 1943. Effects of temperature on enzyme kinetics. Advan. Enzymol. 3, 35-62.

Smith, M.L., and Price, S.A. 1938. Staphylococcus gamma hemolysin. J. Path. Bact. 47, 379-393.

Sonea, S., de Repentigny, J., and Frappier, A. 1965. A reversible metabolic control of virulence characteristics of a toxinogenic Staphylococcus aureus strain. Can. J. Microbiol. 11, 383-394.

Speth, V., Wallach, D.F.H., Weidekamm, E., and Knüfermann, H. 1972. Micromorphologic consequences following perturbation of erythrocyte membranes by trypsin, phospholipase A, lysolecithin, sodium dodecyl sulphate and saponin: a correlated freeze-etching study. Biochim. Biophys. Acta 255, 386-394.

Svihovek, J., Masek, K., and Jiricka, Z. 1965. Proc. 2nd Intern. Pharmacol. Mtg. Prague, 1963. Vol. 9, p. 193, Oxford. Pergamon Press.

Symington, D.A., and Arbuthnott, J.P. 1969. Effects of Staphylococcus alpha-toxin and streptolysin S on the oxidation of succinate by ascites tumour cells. J. Med. Microbiol. 2, 495-505.

Thal, A., and Egner, W. 1954. Local effect of staphylococcal toxin. Studies on blood vessels with particular reference to phenomenon of dermonecrosis. Arch. Path. (Chicago) 57, 392-404.

Thal, A., and Egner, W. 1961. Site of action of Staphylococcus alpha toxin. J. Exp. Med. 113, 67-82.

Thelestam, M., Mollby, R., and Wadstrom, T. 1973. Effects of staphylococcal alpha-, beta-, delta-, and gamma-hemolysins on human diploid fibroblasts and HeLa cells: evaluation of a new quantitative assay for measuring cell damage. Infect. Immun. 8, 938-946.

Turgeon, P., Grimard, S., and de Repentigny, J. 1966. Inhibitions produites par la 8-azaguanine chez une souche toxinogène de Staphylococcus aureus. Can. J. Microbiol. 12, 588-590.

Van Heyningen, W.E., and Mellanby, J. 1971. Microbial toxins. Vol. IIA, Chapter 2. N.Y. Academic Press.

Van de Velde, H. 1894. Étude sur le mecanisme de la virulence du Staphylocoque pyogene. Cellule 10, 401-460.

Wadstrom, T. 1968. Studies on extracellular proteins from Staphylococcus aureus. IV. Separation of alpha toxin by isoelectric focusing. Biochim. Biophys. Acta 168, 228-242.

Wadstrom, T., and Mollby, R. 1971a. Studies on extracellular proteins from Staphylococcus aureus. VI. Production and purification of beta hemolysin in large scale. Biochim. Biophys. Acta 242, 288-307.

Wadstrom, T., and Mollby, R. 1971b. Studies on extracellular proteins from Staphylococcus aureus. VII. Studies on beta hemolysin. Biochim. Biophys. Acta 220, 308-320.

Wadstrom, T., and Mollby, R. 1972. Some biological properties of purified staphylococcal hemolysins. Toxicon 10, 511-519.

Walbum, L.E. 1921a. L'action de divers sels metaliques sur la production de staphylolysine. C.R. Soc. Biol. (Paris) 85, 376-377.

Walbum, L.E. 1921b. Action de la staphylolysine sur les globules de chevre. C.R. Soc. Biol. (Paris) 85, 1205-1206.

Walbum, L.E. 1922. Studien über die bildung der bakteriellen toxine.-I. Mitteilung. Staphylolysin. Biochem. Z. 129, 367-443.

Weissman, G., Sessa, G., and Bernheimer, A.W. 1966. Staphylococcal alpha toxin: effects on artificial lipid spherules. Science 154, 772-774.

Wiegershausen, B. 1960. Über einige pharmakologische eigenschaften von staphylolysin. Arch. Exp. Path. Pharm. 238, 144-145.

Wiegershausen, B. 1962. On the pharmacology of staphylococcal toxin (Wood 46). V. The effect of Staphylococcus toxin on the isolated heart, auricles and vessels of various species. Acta Biol. Med. Ger. 9, 517.

Williams, R.E.O., and Harper, G.J. 1947. Staphylococcal hemolysins on sheep-blood agar with evidence for a fourth hemolysin. J. Path. Bact. 59, 69-78.

Wiseman, G.M. 1961. The interrelationships of some diffusible products of Staphylococcus aureus. M.Sc. thesis, University of Manitoba.

Wiseman, G.M. 1965a. Some characteristics of the beta-hemolysin of Staphylococcus aureus. J. Path. Bact. 89, 187-207.

Wiseman, G.M. 1965b. Factors affecting the sensitization of sheep erythrocytes to staphylococcal beta lysin. Can. J. Microbiol. 11, 463-471.

Wiseman, G.M. 1968. The nature of staphylococcal beta hemolysin. II. Effect on mammalian cells. Can. J. Microbiol. 14, 179-181.

Wiseman, G.M. 1970. Microbial toxins. Vol. III, Chapter 6. N.Y. Academic Press.

Wiseman, G.M., and Caird, J.D. 1967. The nature of staphylococcal beta hemolysin. I. Mode of action. Can. J. Microbiol. 13, 369-376.

Wiseman, G.M., and Caird, J.D. 1968. Phospholipase activity of the delta hemolysin of Staphylococcus aureus. Proc. Soc. Exp. Biol. Med. 128, 428-430.

Wiseman, G.M., and Caird, J.D. 1970. Mode of action of the alpha toxin of <u>Staphylococcus</u> <u>aureus</u>. Can. J. Microbiol. <u>16</u>, 47-50.

Wiseman, G.M., and Caird, J.D. 1972. Further observations on the mode of action of the alpha toxin of <u>Staphylococcus</u> <u>aureus</u> "Wood-46". Can. J. Microbiol. <u>18</u>, 987-992.

Wiseman, G.M., Caird, J.D., and Fackrell, H.B. 1974. Trypsin-mediated activation of the alpha hemolysin of <u>Staphylococcus</u> <u>aureus</u> "Wood-46". J. Med. Microbiol. In Press.

Yoshida, A. 1963. Staphylococcal delta hemolysin. I. Purification and chemical properties. Biochim. Biophys. Acta <u>71</u>, 544-553.

Appendix

IMMUNOGENICITY OF THE DELTA HEMOLYSIN OF

STAPHYLOCOCCUS AUREUS

H.B. FACKRELL[1] and G.M. WISEMAN

Department of Medical Microbiology
University of Manitoba
Winnipeg, Canada

[1]Present address: Department of Biology, University
of Calgary, Calgary, Alberta.

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 Fack-

rell, 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 $50^{\circ}C$.

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 there-
after. Two days after the last injection, the animals

were bled from the heart and the serum was stored at $-20^{O}C$.

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.

REFERENCES

CAIRD, J.D., and WISEMAN, G.M. 1970. Purification of
the delta toxin of Staphylococcus aureus. Can. J.
Microbiol., 16, 703.

CAMPBELL, D.H., GARVEY, S., CREMER, E., and SUSSDORF,
H. 1970. Methods in Immunology. 2nd Edition. W. A.
Benjamin, New York.

DONAHUE, J.A. 1969. Antistaphylococcal hemolysins
and delta lysin inhibitor in adult human serum.
Can. J. Microbiol., 15, 957.

FACKRELL, H.B., and WISEMAN, G.M. 1974. Production
and purification of the gamma hemolysin of Staphylo-
coccus aureus "Smith 5R". J. Gen. Microbiol., in
press.

GLADSTONE, G.P., and YOSHIDA, A. 1967. The cytopathic
action of purified staphylococcal delta hemolysin.
Brit. J. Exp. Path., 48, 11.

GOMORI, G. 1955. Methods in Enzymology. Vol. I,
Chapter 16. Academic Press, New York.

HALLANDER, H.O. 1963. Fractionation of staphylococcal
toxins by gel filtration. Acta Path. Microbiol. Scand.,
59, 543.

KANTOR, H.S., TEMPLES, B., and SHAW, W.V.   1972.

   Staphylococcal delta hemolysin: purification and

   characterization. Arch. Biochem. Biophys., 151, 142.

KREGER, A.S., KIM, K.S., ZABORETSKY, F., and SHAW, V.

   1971.   Purification and properties of staphylococcal

   delta hemolysin. Infect. Immun., 3, 449.

MARKHAM, R.   1942.   A steam distillation apparatus

   suitable for micro-Kjeldahl analysis. Biochem. J.,

   36, 790.

WISEMAN, G.M., and CAIRD, J.D.   1967.   The nature of

   staphylococcal beta hemolysin.   I.   Mode of action.

   Can. J. Microbiol., 13, 369.

WISEMAN, G.M., and CAIRD, J.D.   1972.   Further observa-

   tions on the mode of action of the alpha toxin of

   Staphylococcus aureus "Wood 46".   Can. J. Microbiol.,

   18, 987.

WISEMAN, G. M., CAIRD, J. D., and FACKRELL, H. B.   1974.

Trypsin-mediated activation of the alpha hemolysin of

Staphylococcus aureus "Wood 46." J. Med. Microbiol. In

press.

Yoshida, A.   1963.   Staphylococcal delta hemolysin.   I.

Purification and chemical properties. Biochim. Biophys.

Acta, 71, 544.

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,
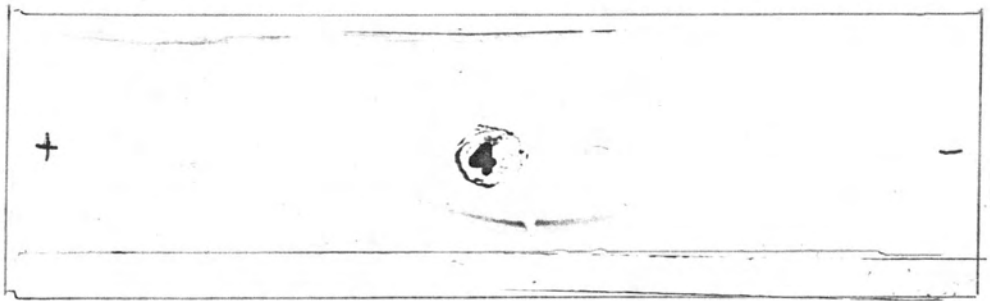   (2) Kreger's delta lysin, (3) gamma lysin,
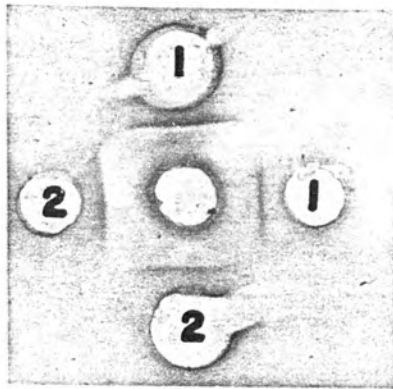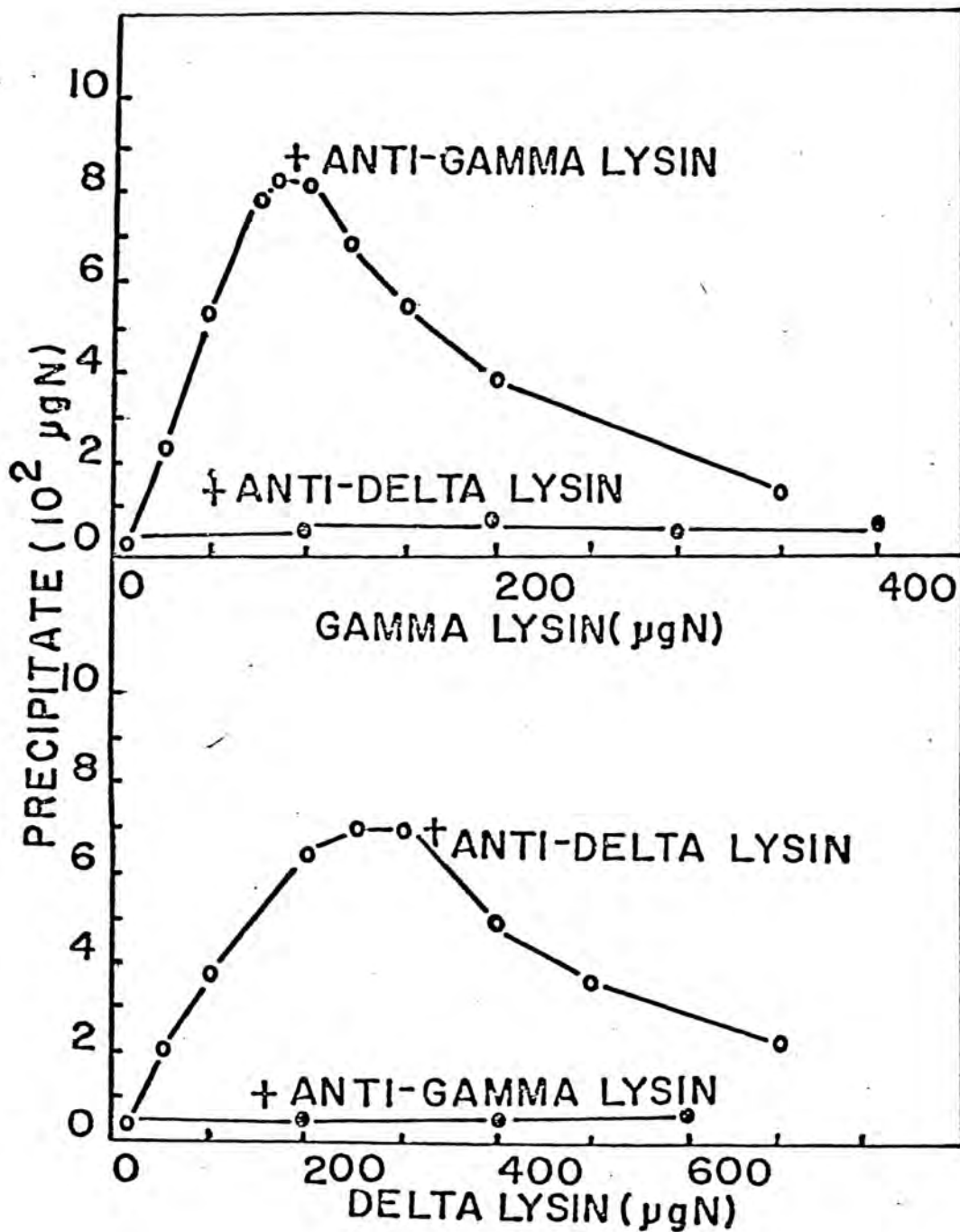   and (4) beta lysin.
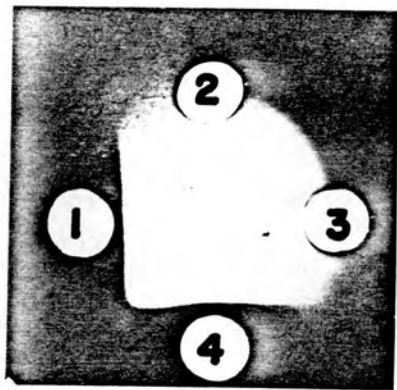
+   −
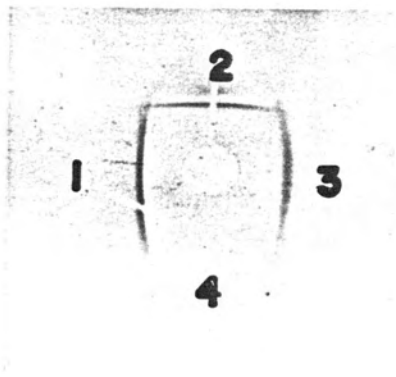
Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

TRYPSIN-MEDIATED ACTIVATION OF THE ALPHA HEMOLYSIN

OF <u>STAPHYLOCOCCUS</u> <u>AUREUS</u> "WOOD 46"

G.M. WISEMAN, J.D. CAIRD and H.B. FACKRELL[1]

University of Manitoba

Department of Medical Microbiology

Winnipeg, Canada

[1]Present address: Department of Biology, University of Calgary, Calgary, Alberta.

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 (Fackrell, 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°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 carboxy-methylcellulose (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 immunologically 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 spectrophotometer 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 tempera-
ture.

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% NH_4OH (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 um (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 deter-
mination was based on unfiltered protoxin, since filtra-
tion removed much of the hemolytic activity. Chromato-
grams 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_{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 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 pro-
teases 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 re-
flect 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 sus-
pensions. 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 choles-terol 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 lysolecithin 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 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.

REFERENCES

ARBUTHNOTT, J.P. 1970. Staphylococcal alpha toxin. In Microbial toxins, Vol. III. Academic Press, New York, p. 189.

ARBUTHNOTT, J.P., FREER, J.H., and BILLCLIFFE, BRONWEN. 1973. Lipid-induced polymerization of staphylococcal alpha toxin. J. Gen. Microbiol., 75, 309.

BERNHEIMER, A.W., and SCHWARTZ, L.L. 1963. Isolation and composition of staphylococcal alpha toxin. J. Gen. Microbiol., 30, 455.

BUCKELEW, A.R., and COLACICCO, G. 1971. Lipid mono-layers. Interactions with staphylococcal alpha-toxin. Biochim. Biophys. Acta, 233, 7.

CAMEJO, G., COLACICCO, G., and RAPPORT, W.M. 1968. Lipid monolayers: interactions with the apoprotein of high density plasma lipoprotein. J. Lipid Res., 9, 562.

CAMPBELL, D.H., GARVEY, J.S., CREMER, N.E., and SUSSDORF, D.H. 1970. Methods in Immunology. Benjamin, Inc., N.Y., p. 216.

DIXON, M., and WEBB, E.C. 1964. Enzymes. Longmans, London, p. 528.

DODGE, J.T., MITCHELL, C., and HANAHAN, D.J. 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Arch. Biochem, Biophys., 100, 119.

FACKRELL, H.B. 1973. Studies with gamma hemolysin of _Staphylococcus aureus_. Ph.D. Thesis, Manitoba.

FACKRELL, H.B., and WISEMAN, G.M. 1974. Properties of the gamma hemolysin of _Staphylococcus aureus_ "Smith 5R". In press.

FRAENKEL-CONRAT, H., HARRIS, J.I., and LEVY, A.L. 1955. Recent developments in techniques for terminal and sequence studies in peptides and proteins. _Methods Biochem. Anal._, 2, 359.

FREER, J.H., ARBUTHNOTT, J.P., and BERNHEIMER, A.W. 1968. Interaction of staphylococcal alpha toxin with artificial and natural membranes. _J. Bacteriol._, 95, 1153.

FREER, J.H., ARBUTHNOTT, J.P., and BILLCLIFFE, BRONWEN. 1973. Effects of staphylococcal alpha toxin on the structure of erythrocyte membranes: a biochemical and freeze-etching study. _J. Gen. Microbiol._, 75, 321.

GOW, J.A. 1968. Purification and properties of staphylococcal alpha and beta hemolysins. M.Sc. Thesis, University of Western Ontario.

GOW, J.A., and ROBINSON, J. 1969. Properties of purified staphylococcal beta hemolysin. _J. Bacteriol._, 97, 1026.

HALLANDER, H.O. 1963. Fractionation of staphylococcal toxins by gel filtration. _Acta Path. Microbiol. Scand._, 59, 543.

HERSKOVITS, T.T.   1967.   In Methods in Enzymology,
Vol. XI.   Academic Press, N.Y., p.771.

HUMMEL, B.C.W.   1959.   A modified spectrophotometric
determination of chymotrypsin, trypsin and thrombin.
Can. J. Biochem. Physiol., 37, 1393.

PATAKI, G.   1966.   Techniques of thin-layer chromato-
graphy in amino acid and peptide chemistry.   de
Gruyter, Berlin.

ROBINSON, J., THATCHER, F.S., and MONTFORD, J.   1960.
Studies with staphylococcal toxins.   V.   Possible
identification of alpha hemolysin with a proteolytic
enzyme.   Can. J. Microbiol., 6, 183.

WEISSMANN, G., SESSA, G., and BERNHEIMER, A.W.   1966.
Staphylococcal alpha toxin: effects on artificial
lipid spherules.   Science, 154, 772.

WISEMAN, G.M., and CAIRD, J.D.   1970.   Mode of action
of the alpha toxin of Staphylococcus aureus.
Can. J. Microbiol., 16, 47.

WISEMAN, G.M., and CAIRD, J.D.   1972.   Further observa-
tions on the mode of action of the alpha toxin of
Staphylococcus aureus "Wood 46".   Can. J. Microbiol.,
18, 987.

WITTLER, R.G., and PILLEMER, L.   1948.   The immuno-
chemistry of toxins and toxoids.   V.   The solubility
of staphylococcal toxin in methanol-water mixtures
under controlled conditions of pH, ionic strength
and temperature.   J. Biol. Chem., 174, 23.

TABLE I

SUMMARY OF DATA FOR PURIFICATION OF ALPHA HEMOLYSIN

STEP	TOTAL HEMOLYSIN (HU)*	RECOVERY (%)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (HU/mg)	PURIFICATION (fold)
Crude lysin	20,235,000	100	49,962	405	1
Methanol precipitate	11,347,200	56	6,304	1,800	4
Ammonium sulphate precipitation	7,375,500	36	1,755	4,200	10
Sephadex G-75	8,118,000	40	335	24,200	60
Ammonium sulphate precipitation	8,200,00	41	185	44,200	109
CM cellulose	7,790,00	39	62.3	125,000	308

*Hemolytic units.

TABLE II

EFFECT OF TRYPSIN ON ALPHA PROTOXIN OF
STAPHYLOCOCCUS AUREUS

Test	Hemolytic activity (HU/ml)	Proteolytic activity (O.D. 520 nm)
Trypsin control[1]	<10	1.64
Trypsin + STI[2]	<10	0.06
STI control	<10	0.04
Toxin control[3]	747	0.02
Toxin + trypsin		
30 s exposure	730	0.88
1 min. "	678	0.88
5 min. "	526	0.57
15 min. "	170	0.26
30 min. "	53	0.20
60 min. "	21	0.21

[1]Trypsin concentration = 200 μg/ml.

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

[3]Protoxin is by definition nonhemolytic, but becomes
hemolytic in the presence of red cells because of their
content of activating protease.

TABLE III

EXPERIMENTAL DESIGN

Additions	Volume (ml) of reagents added to tube numbers			
	1	2	3	4
Alpha protoxin*	2.0	2.0	2.0	0
CMC slurry**	0	2.0	0	0
CMC-trypsin complex***	0	0	2.0	2.0
Buffer****	3.0	1.0	1.0	3.0

*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

Samples from tube	Hemolytic titres (HU/ml) of		
	Unfiltered toxin	Filtered toxin	% recovery
1*	901**	45	5
2	671	34	5
3	531	341	65
4	< 16	< 16	–

*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 posi-
tions 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 iso-
leucine. 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 rechroma-
tographed.

Figure 3. Plot of 1/v against 1/s (Lineweaver-Burk)
for activated alpha toxin and trypsin.
V_{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.
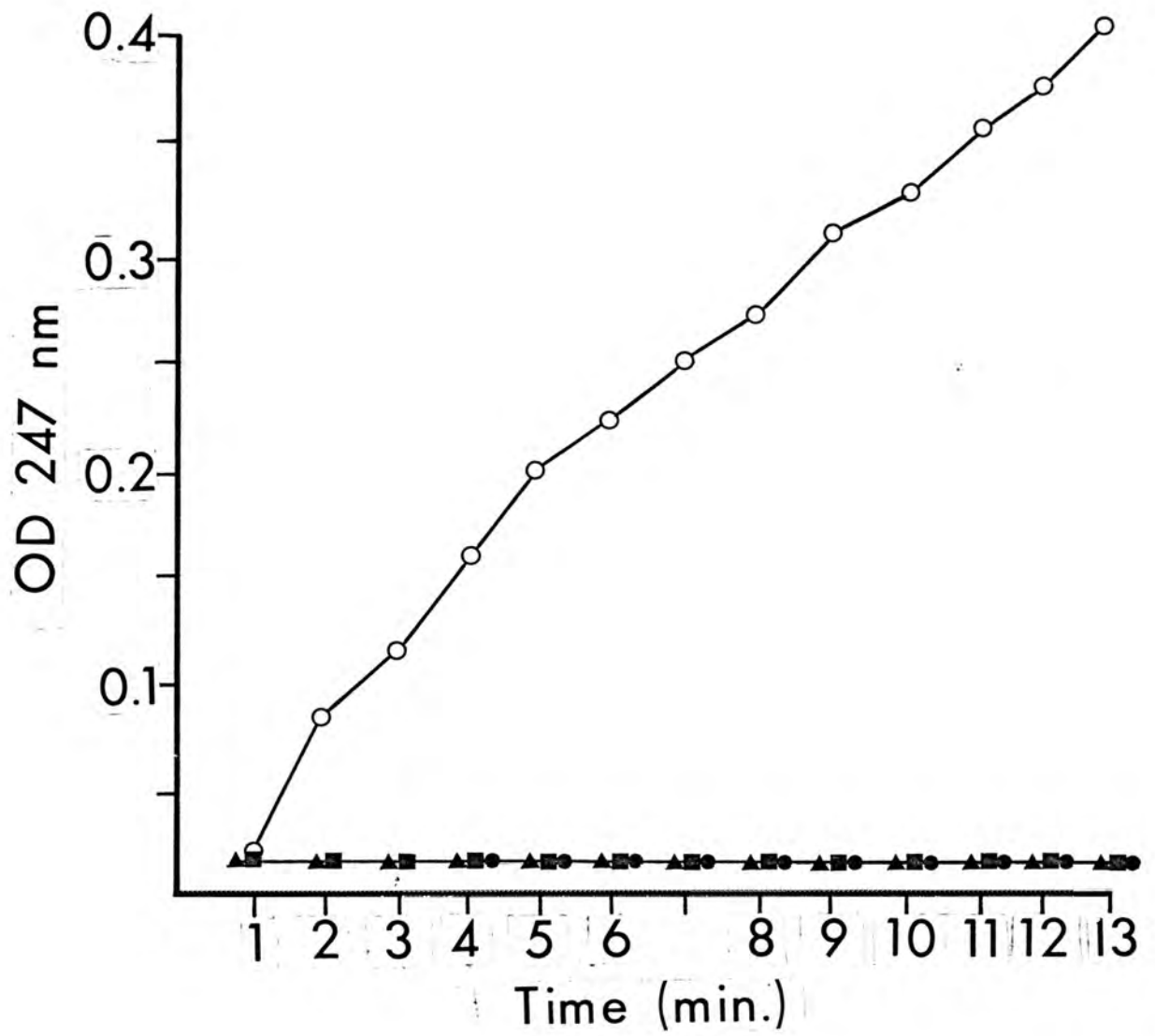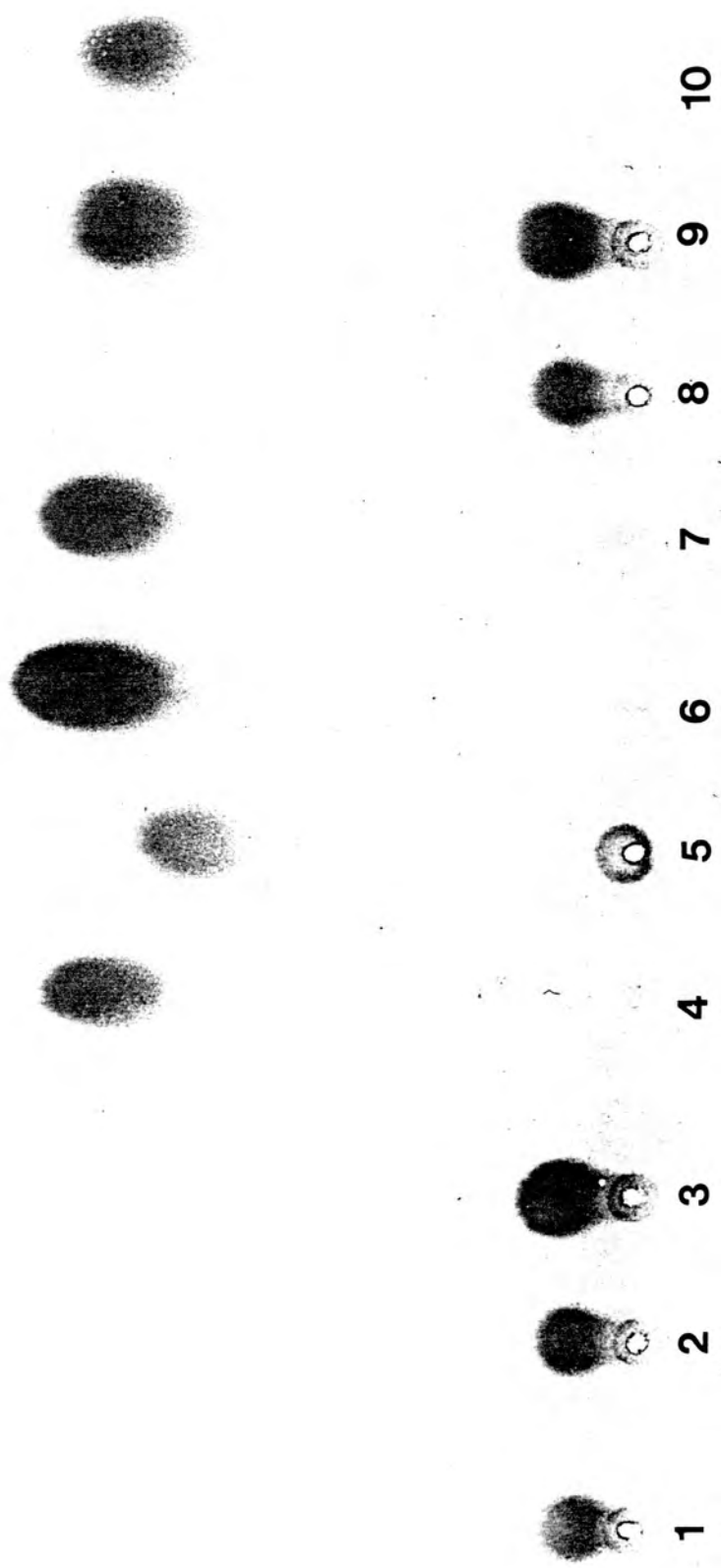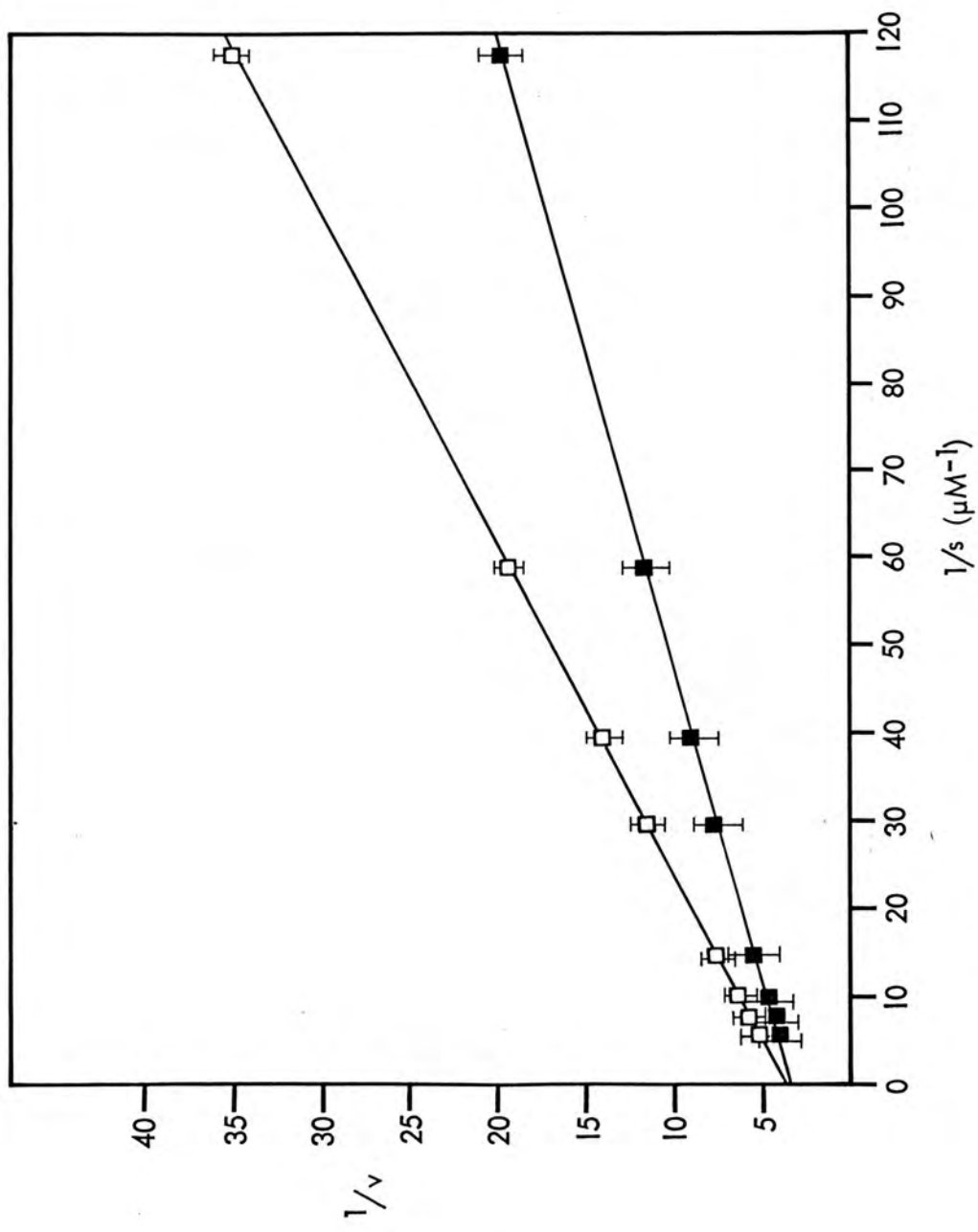
Fig. 1

Fig. 2

Fig. 3

PRODUCTION AND PURIFICATION OF THE GAMMA HEMOLYSIN

OF STAPHYLOCOCCUS AUREUS "SMITH 5R"

H.B. FACKRELL* and G.M. WISEMAN

Department of Medical Microbiology

University of Manitoba

Winnipeg, Canada

Running Head: STAPHYLOCOCCAL GAMMA LYSIN

*Present Address: Department of Biology, University of
Calgary, Calgary, Alberta.

SUMMARY

The gamma hemolysin of S. aureus Smith 5R was produced on Dolman-Wilson agar overlain with cellophane. Maximal yields of crude lysin 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 lysin was purified 2700-fold with 75% recovery by ultrafiltration, gel filtration and ammonium sulphate fractionation. The specific activity of the lysin 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 lysin 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; Fackrell, 1973).

Production and purification of the gamma lysin have been described by Plommet & Bouillane (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 cm^2 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 Fackrell, 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^OC.
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 pro-
duction (Fig. 4). Maximal hemolysin production and growth
occurred at 37^OC but a shift of 3^OC from this temperature
resulted in a significant decrease in the amount of hemolysin
found in the supernatant. No hemolysin was detected at 25^OC
or 50^OC although slight growth occurred at 25^OC.

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^OC 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^OC. 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 μg 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, electro-focusing 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 pro-
duction 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 investi-
gation 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

REFERENCES

BIRCH-HIRSCHFELD, L. (1934). Ueber die wirksamkeit der extrakte von auf zellophanagar gezuchteten Staphylokokken. Zeitschrift fur Immunitaetsforschung 81, 260-285.

CAMPBELL, D.H., GARVEY, J.S., CREMER, N.E. & SUSSDORF, D.H. (1970). Methods in Immunology. 2nd Edition, New York: Benjamin.

DOLMAN, C.E. & WILSON, R.J. (1940). The kitten test for Staphylococcus enterotoxin. Canadian Public Health Journal 31, 68-71.

ELEK, S.D. (1959). Staphylococcus pyogenes and its Relation to Disease. Edinburgh: Livingstone.

FACKRELL, H.B. (1973). Studies with gamma hemolysin of Staphylococcus aureus. Ph.D. Thesis. University of Manitoba, Winnipeg.

FACKRELL, H.B. & WISEMAN, G.M. (1974). Properties of the gamma hemolysin of Staphylococcus aureus "Smith 5R". Journal of General Microbiology. In press.

FACKRELL, H.B., WISEMAN, G.M. & CAIRD, J.D. (1974). Purification of the alpha hemolysin of Staphylococcus aureus "Wood 46". Journal of Medical Microbiology. In Press.

GOMORI, G. (1955). Methods in Enzymology. Vol. I, Chapter 16. New York: Academic Press.

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

JACKSON, A.W. (1962). Staphylococcal gamma lysin and its differentiation from delta lysin. VIIIth International Congress for Microbiology Abstracts.

MARKHAM, R. (1942). A steam distillation apparatus suitable for micro-Kjeldahl analysis. Biochemical Journal 36, 790-791.

MARKS, J. (1951). The standardization of staphylococcal alpha-antitoxin, with special reference to anomalous hemolysins including delta-lysin. Journal of Hygiene 49, 52-66.

MOLLBY, R. & WADSTROM, T. (1971). Separation of gamma hemolysin from Staphylococcus aureus Smith 5R. Infection and Immunity 3, 633-635.

PLOMMET, M. & BOUILLANNE, C. (1966). Production des hemolysines staphylococciques delta et gamma. Annales de Biologie Animale, Biochimie, Biophysique 6, 529-532.

SMITH, M.L. & PRICE, S.A. (1938). Staphylococcus gamma hemolysin. Journal of Pathology and Bacteriology 47, 379-393.

WISEMAN, G.M. (1970). Microbial Toxins. Vol. III, pp. 237-263. New York: Academic Press.

WISEMAN, G.M. & CAIRD, J.D. (1972). Further observations on the mode of action of the alpha toxin of Staphylococcus aureus "Wood 46". Canadian Journal of Microbiology 18, 987-992.

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

Medium	Growth (O.D. 650 nm)	Hemolysin Titre (HU/ml)**
Liquid Medium		
Dolman-Wilson	0.51	78
Gladstone's	0.11	0
Stolp & Petzold	0.24	13
Brain Heart Infusion	0.48	23
Semi-Solid Medium*		
Dolman-Wilson	0.55	0
Gladstone's	0.35	0
Stolp & Petzold	0.30	0
Brain Heart Infusion	0.45	0
Solid Medium*		
Dolman-Wilson	0.63	0
Gladstone's	0.59	0
Stolp & Petzold	0.52	0
Brain Heart Infusion	0.40	0

*Media subjected to freezing and thawing.
**Hemolytic Units/ml.

Table 2. Gamma hemolysin production on different media*

Medium	Growth (O.D. 650 nm)	Hemolysin Titre (HU/ml±S$\bar{x}$)**
Dolman-Wilson	0.810	453±21
Gladstone's	0.850	441±27
Brain Heart Infusion	0.650	263±14
Veal Infusion	0.710	205±20
Liver Infusion	0.790	152±10
Stolp & Petzold	0.516	136±9
Blood Agar	0.610	86±6
Trypticase Soy	0.492	30±2
Nutrient Agar	0.320	15±1
Casamino Acids	0.116	6±1

*Overlain with cellophane.
**Hemolytic Units/ml.

Table 3. Ultrafiltration of gamma hemolysin

Membrane		Total Hemolysin (HU) *	Total Protein (mg)	Specific Activity (HU/mg)	Purification (fold)
None		125,000	90	1,400	1.0
UM-10	retained	125,000	64	2,000	1.4
	passed	0	15	–	–
PM-30	retained	125,000	62	2,000	1.4
	passed	0	14	–	–
XM-50	retained	125,000	31	4,000	2.9
	passed	0	56	–	–
XM-100	retained	35,000	17	2,000	1.4
	passed	90,000	66	1,400	0.0

*Hemolytic Units.

Table 4. Ammonium sulphate fractionation of gamma hemolysin

| Ammonium sulphate (% saturation)* | Precipitate | | Specific Activity (HU/mg) | Purification (fold) |
	Total Hemolysin (HU)	Total Protein (mg)		
15	0	0.205	–	–
30	2,200	0.325	6,769	3.1
45	3,500	0.480	7,291	3.4
60	4,300	0.510	8,431	3.9
75	4,400	1.100	4,000	1.9
90	4,300	1.790	2,402	1.1
100	4,500	2.010	2,238	1.0
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

NaCl (M)	Supernatant		Precipitate	
	Hemolysin (HU/ml)	Protein (mg/ml)	Hemolysin (HU/ml)	Protein (mg/ml)
0 (distilled water)	0	0.045	1,300	0.305
0 (phosphate buffer, 0.01M)	270	0.104	990	0.245
0.01 "	320	0.130	880	0.225
0.03 "	470	0.135	795	0.230
0.06 "	520	0.140	630	0.218
0.10 "	640	0.150	510	0.205
0.30 "	970	0.154	275	0.200
0.50 "	1,060	0.160	145	0.190
0.60 "	1,240	0.174	70	0.180
1.00 "	1,300	0.220	0	0.140
2.00 "	1,300	0.230	0	0.125

Table 6. <u>Purification of gamma hemolysin: Summary of data</u>

Purification Step	Total Hemolysin (HU)	Total Protein (mg)	Recovery %	Specific Activity (HU/mg)	Purification (fold)
Crude lysin	151,900	3,844.0	100	40	1.0
Ultrafiltration	148,750	1,214.0	98	123	3.1
Gel Filtration	128,000	198.9	84	643	16.3
$(NH_4)_2SO_4$ Fractionation	107,250	41.7	70	2,572	64.0
Dialysis	115,500	15.8	76	7,333	185.6
NaCl Extraction	115,500	9.8	76	11,737	297.0
$(NH_4)_2SO_4$ Fractionation	112,450	1.1	74	108,125	2,736.0

CAPTIONS TO FIGURES

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

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

Figure 3   The effect of pH on production of gamma lysin by the Smith 5R strain. Hemolysin titres •———•; growth o———o. pH4-6, succinate buffer; pH6-8 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.

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

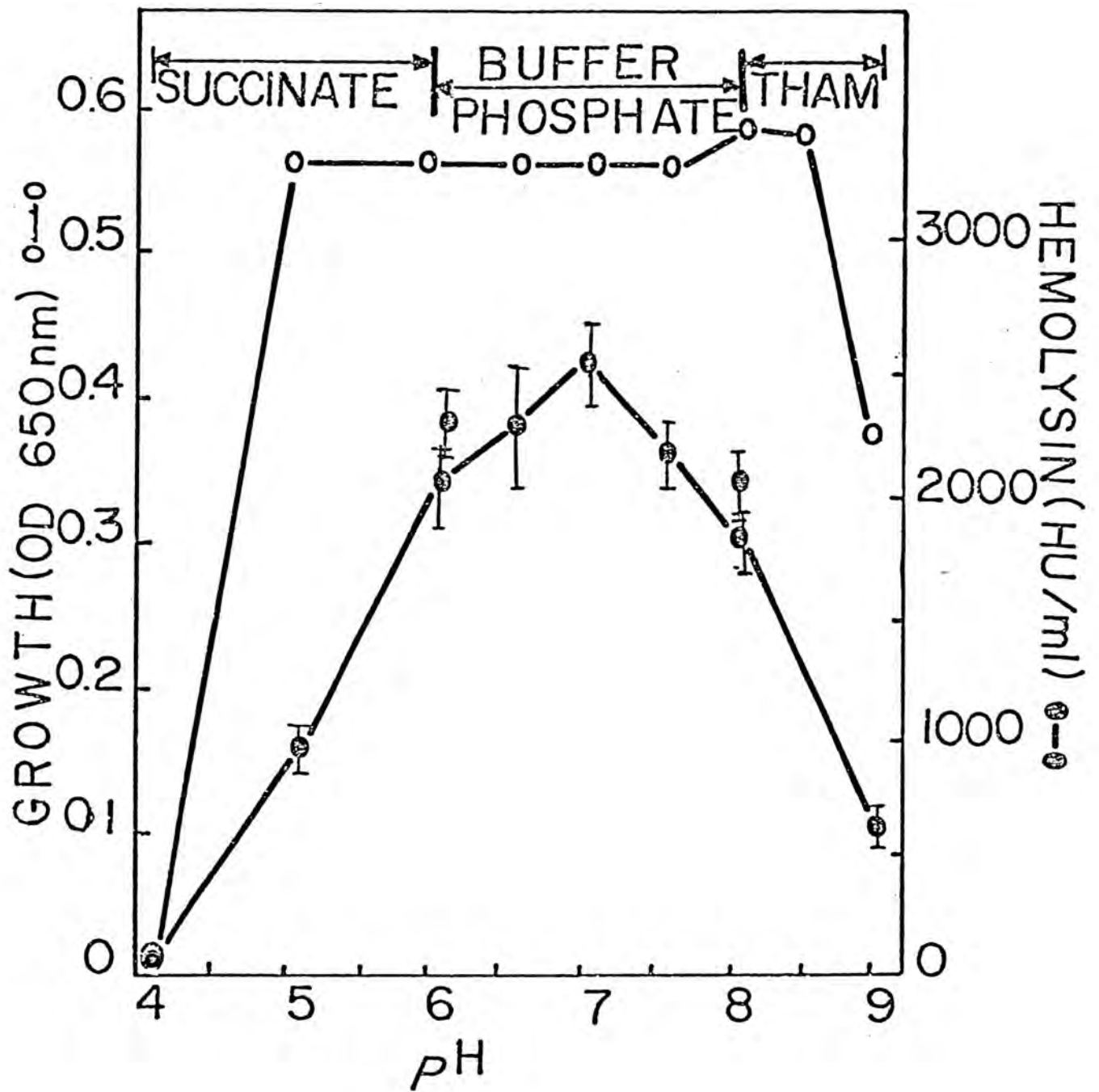Figure 8   Immunoelectrophoresis of purified gamma lysin. Purified lysin is contained in the well, anti-gamma IgG in the trough.
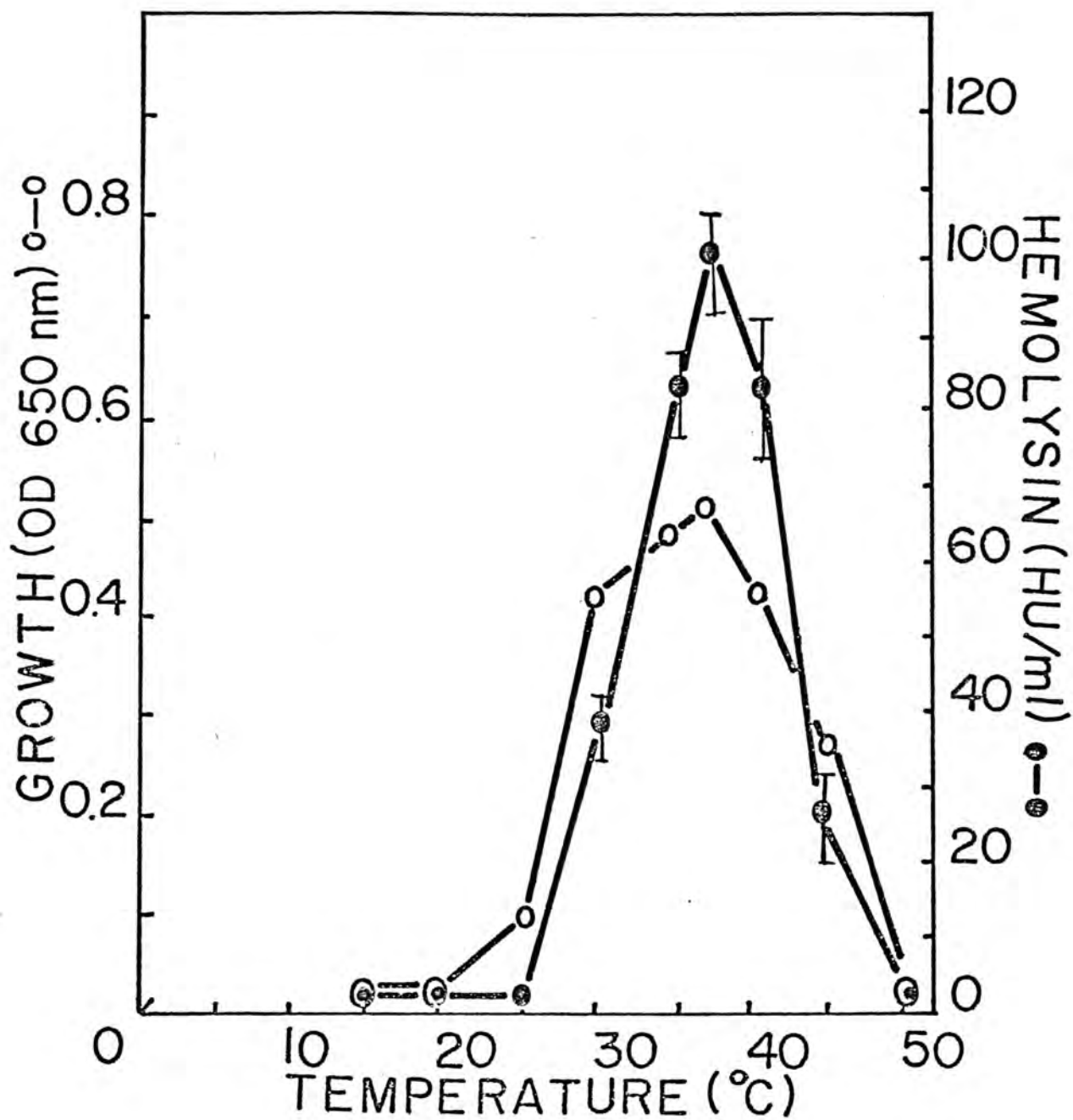
Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

CRUDE GAMMA LYSIN

DISC GEL ELECTROPHORESIS

POSITIVE   NEGATIVE

PURIFIED GAMMA LYSIN

DISTANCE (cm.)
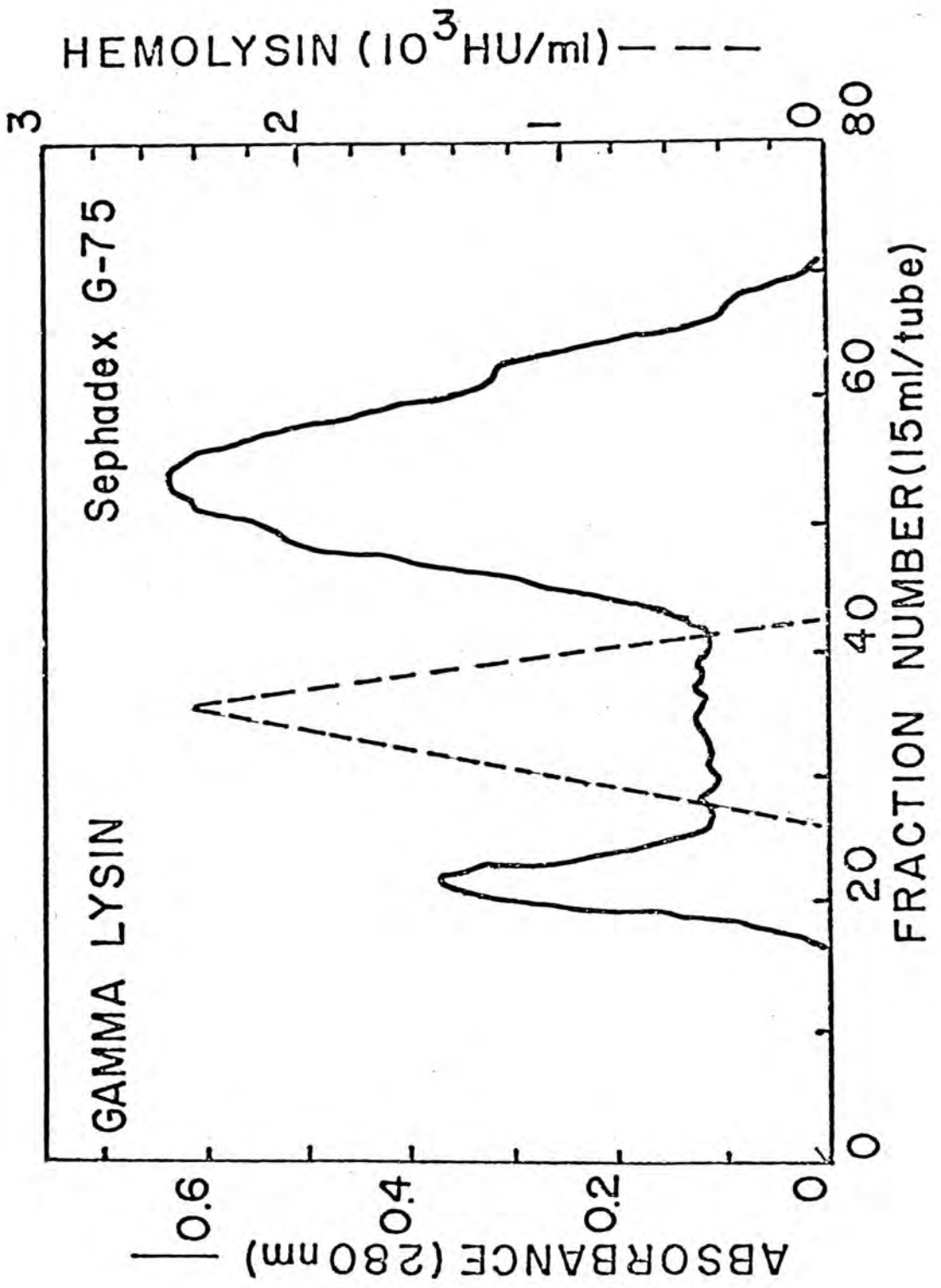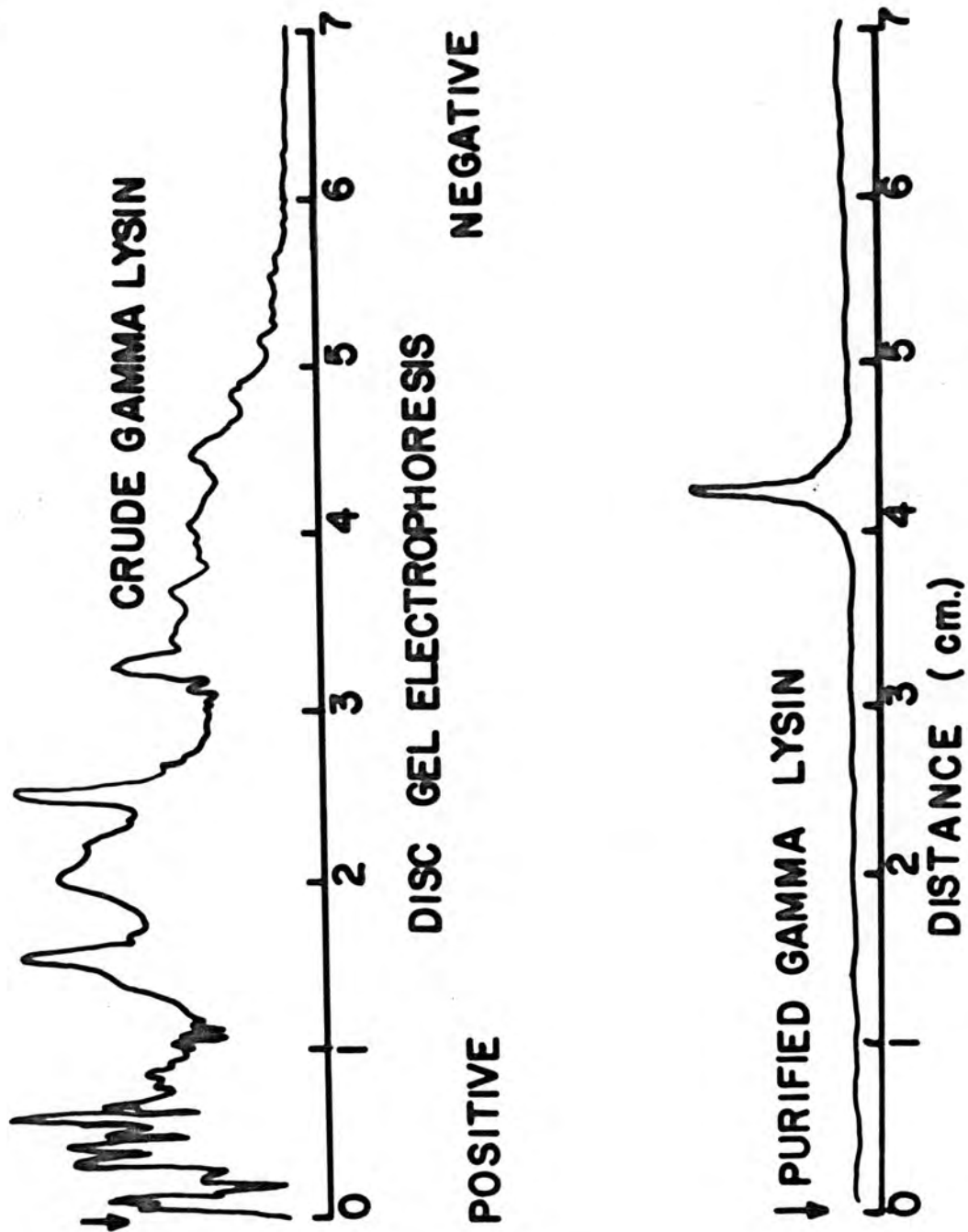
Fig. 7
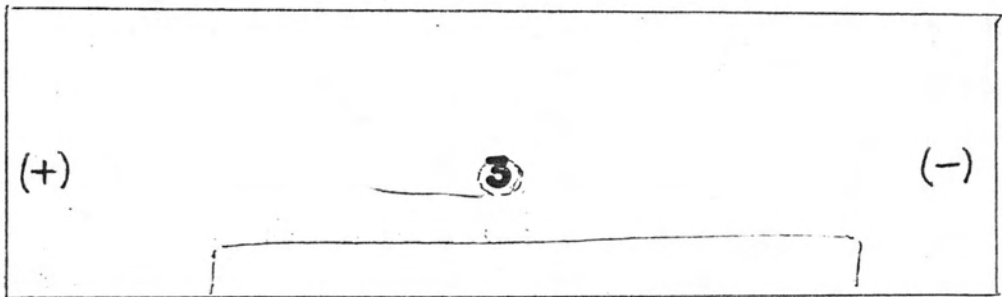
(+)   ⑤   (−)
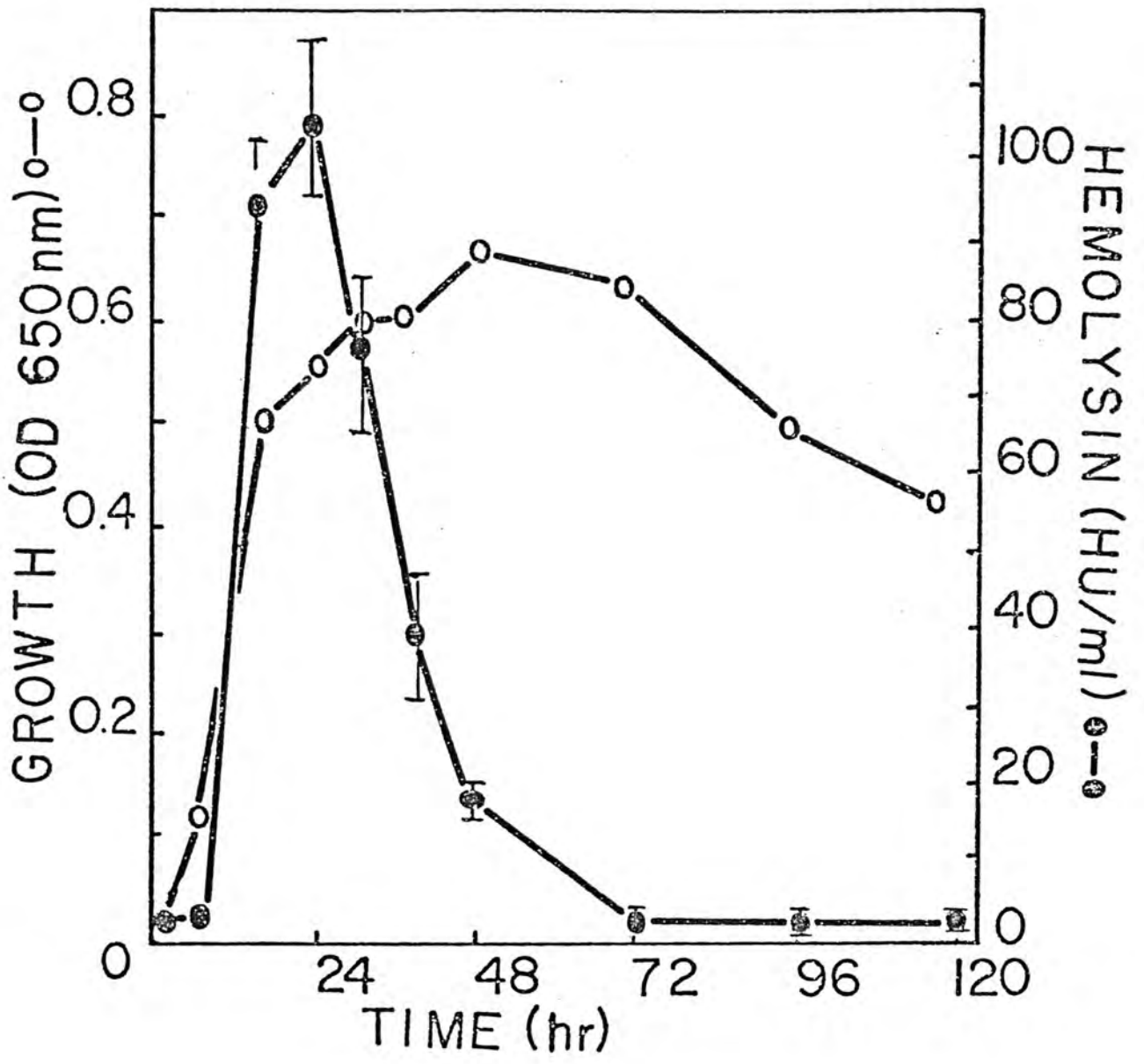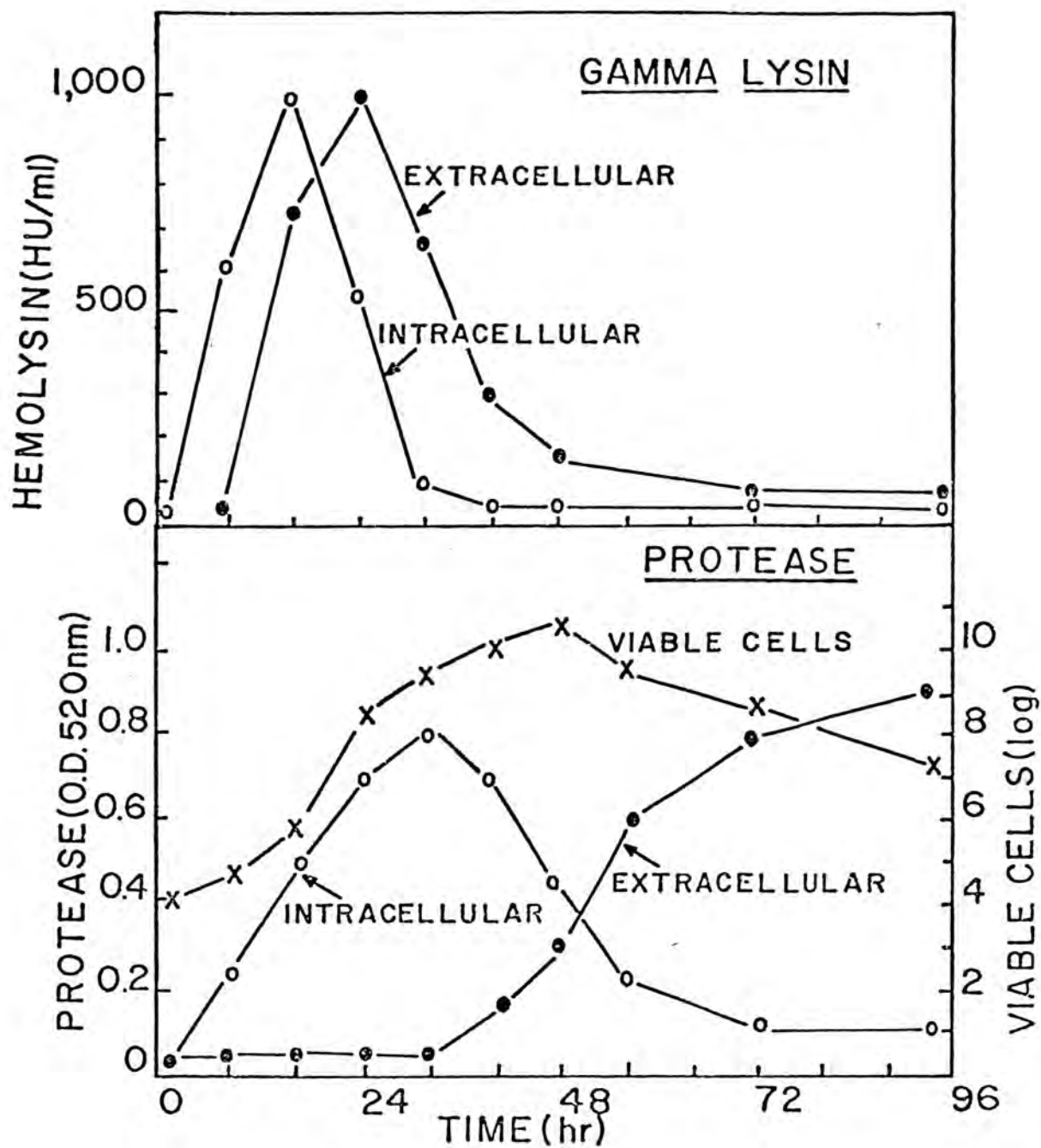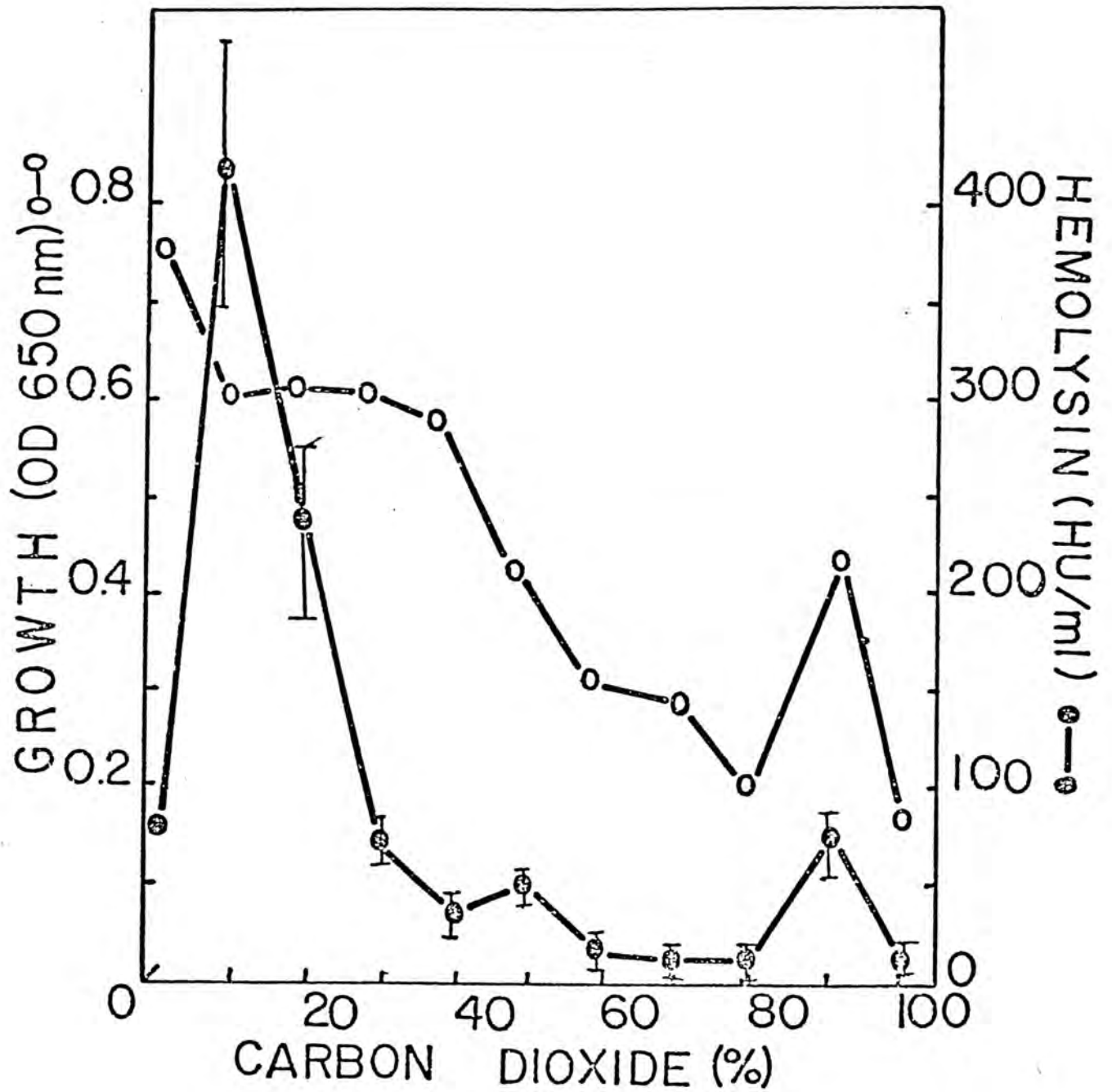
Fig. 8

PROPERTIES OF THE GAMMA HEMOLYSIN OF
STAPHYLOCOCCUS AUREUS "SMITH 5R"

H.B. Fackrell[1] and G.M. Wiseman

Department of Medical Microbiology

University of Manitoba, Winnipeg, Canada

[1]Present address: Department of Biology, University of Calgary, Calgary, Alberta.

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 37OC 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 ultra-centrifuge 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 (Fackrell 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 con-
centrations 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 re-
ceived 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, re-
sembles 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 LD_{50} dose of delta lysin for mice was 125 mg although such a large LD_{50} 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 LD_{50} 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 S_{20w} 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 iso-electric 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 character-ized 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 intra-cardially. Gamma lysin also lysed human leucocytes and destroyed C6 (human lymphoblast) cells.

Acknowledgments

ANDREWS, P. (1964). Estimation of the molecular weights of proteins by Sephadex gel filtration. Biochemical Journal 91: 222-233.

BERNHEIMER, A.W. (1964). Staphylococcal alpha toxin. Annals of the New York Academy of Science 128: 112-123.

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

BIRCH-HIRSCHFELD, L. (1934). Ueber die wirksamkeit der extrakte vonauf zellophanagar gezuchteten staphylokokken. Zeitschrift fur Immunitaetsforschung 81: 260-285.

CAIRD, J.D., & WISEMAN, G.M. (1970). Purification of the delta toxin of Staphylococcus aureus. Canadian Journal of Microbiology 16: 703-708.

CAMPBELL, D.H., GARVEY, S., CREMER, E., & SUSSDORF, H. (1970). Methods in Immunology. 2nd ed. New York. Benjamin.

CHESBRO, W., & KUCIC, V. (1971). Beta hemolysin: a persistent impurity in preparations of staphylococcal nuclease and enterotoxin. Journal of Applied Microbiology 22: 233-241.

COULTER, J.R. (1966). Production, purification, and composition of staphylococcal alpha toxin. Journal of Bacteriology 92: 1655-1662.

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

FACKRELL, H.B., & WISEMAN, G.M. (1974a). Production and purification of the gamma hemolysin of Staphylococcus aureus Smith 5R. Journal of General Microbiology. In Press.

FACKRELL, H.B., & WISEMAN, G.M. (1974b). Immunogenicity of the delta hemolysin of Staphylococcus aureus. Journal of Medical Microbiology. In Press.

FRAENKEL-CONRAT, H., HARRIS, J.I., & LEVY, A.L. (1955). Recent developments in techniques for terminal and sequence studies in peptides and proteins. Methods in Biochemical Analysis 2: 359-425.

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

GOW, J.A., & ROBINSON, J. (1969). Properties of purified staphylococcal beta hemolysin. Journal of Bacteriology 97: 1026-1032.

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

HALLANDER, H.O. (1963). Fractionation of staphylococcal toxins by gel filtration. Acta Pathologica et Micro-biologica Scandinavica 59: 543-552.

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

IDA, S., SAIRENJI, T., & HINUMA, Y. (1972). Antibodies to a new antigen induced by Epstein-Barr virus in patients with nasopharyngeal carcinoma, leukemia, malignant lymphoma and in control groups. Gann 63: 45-55.

KANTOR, H.S., TEMPLES, B., & SHAW, W.V. (1972). Staphylo-coccal delta hemolysin: purification and characterization. Archives of Biochemistry and Biophysics 151: 142-156.

KREGER, A.S., KIM, K.S., ZABORETSKY, F., & BERNHEIMER, A.W. (1971). Purification and properties of staphylococcal delta hemolysin. Infection and Immunity 3: 449-465.

LELOIR, L.F., & CARDINI, C.E. (1955). Methods in Enzymology. Vol. I. New York. Academic Press.

MARKHAM, R. (1942). A steam distillation apparatus suitable for Micro-Kjeldahl analysis. Biochemical Journal 36: 790-791.

McNIVEN, A.C., OWEN, P., & ARBUTHNOTT, J.P. (1972). Multiple forms of staphylococcal alpha toxin. Journal of Medical Microbiology 5: 113-122.

MOLLBY, R., & WADSTROM, T. (1971). Separation of gamma hemolysin from Staphylococcus aureus Smith 5R. Infection & Immunity 3: 633-635.

PATAKI, G. (1968). Techniques of thin-layer chromatography in amino acid and peptide chemistry. Ann Arbor. Ann Arbor Science Publishers.

SIX, H.R., & HARSHMAN, S. (1973). Purification and pro-perties of two forms of staphylococcal alpha toxin. Biochemistry 12: 2672-2677.

SMITH, M.L., & PRICE, S.A. (1938). Staphylococcus gamma hemolysin. Journal of Pathology and Bacteriology 47: 379-393.

STAHL, E. (1965). Thin Layer Chromatography. New York. Academic Press.

WADSTROM, T. (1968). Studies on extracellular proteins from Staphylococcus aureus. IV. Separation of toxin by isoelectric focusing. Biochimica et Biophysica Acta 133: 228-242.

WADSTROM, T., & MOLLBY, R. (1972). Some biological properties of purified staphylococcal hemolysins. Toxicon 10: 511-519.

WISEMAN, G.M., & CAIRD, J.D. (1967). The nature of staphylococcal beta hemolysin. I. Mode of action. Canadian Journal of Microbiology 13: 369-376.

WISEMAN, G.M., & CAIRD, J.D. (1972). Further observations on the mode of action of the alpha toxin of Staphylococcus aureus "Wood-46". Canadian Journal of Microbiology 18: 987-992.

WISEMAN, G.M., CAIRD, J.D., & FACKRELL, H.B. (1974). Trypsin-mediated activation of the alpha hemolysin of Staphylococcus aureus "Wood-46". Journal of Medical Microbiology. In Press.

YOSHIDA, A. (1963). Staphylococcal delta hemolysin. I. Purification and Properties. Biochimica et Biophysica Acta 71: 544-553.

TABLE 1

STAPHYLOCOCCAL HEMOLYSINS

AMINO ACID COMPOSITION*

AMINO ACID	ALPHA	BETA	GAMMA	DELTA
Ammonia	High	441.6	141.4	218.5
Aspartic Acid	121.2	135.1	142.0	164.1
Threonine	56.9	53.8	68.0	119.9
Serine	65.1	98.7	77.4	96.3
Glutamic Acid	93.0	116.9	102.4	66.4
Proline	81.2	–	50.2	14.0
Glycine	170.1	121.2	125.0	112.4
Alanine	75.8	78.2	63.2	75.2
Valine	50.1	57.8	51.0	76.0
Cysteine	–	12.2	–	–
Methionine	12.1	–	3.5	–
Isoleucine	40.4	52.5	44.4	102.7
Leucine	46.0	58.8	57.8	61.2
Tyrosine	33.5	6.3	30.6	9.3
Phenylalanine	28.3	41.4	35.8	53.5
Lysine	70.9	98.0	78.4	24.3
Histidine	20.0	24.8	27.2	3.5
Arginine	34.5	39.6	38.6	21.4

*Residues/1,000 amino acid residues.

TABLE 2

HEMOLYTIC ACTIVITY* OF STAPHYLOCOCCAL LYSINS

SPECIES	ALPHA	BETA	GAMMA	DELTA
Rabbit	76,422	81,300	113,210	1,644
Dog	2,906	<750	29,764	882
Rat	2,497	4,960	44,041	1,700
Cat	2,214	4,760	6,445	1,500
Sheep	1,026	758,830	89,906	2,929
Mouse	1,023	10,000	25,754	1,037
Chicken	83	2,950	306	803
Guinea pig	56	63,740	6,620	803
Human	53	10,360	44,041	7,527
Monkey	26	<750	3,208	749
Pigeon	5	9,930	160	165

*HU/mg protein

TABLE 3

SUMMARY OF PHYSICO-CHEMICAL DATA FOR THE STAPHYLOCOCCAL HEMOLYSINS

PARAMETER	ALPHA	BETA	GAMMA	DELTA
Sedimentation constant, S_{20w}	1.4,3.0	1.8	2.6	2.8,9.8*
Extinction coefficient, 1% 280 nm	13.56	4.24	28.35	29.08
Molecular weight gel filtration	45,000	26,000	45,000	200,000**
Isoelectric point	8.5	9.5	6.0	9.6
N-terminal amino acid	histidine	***	methionine	proline

*Data from Caird, J.D., & Wiseman, G.M. (1970).
**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.   Electrofocusing 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.
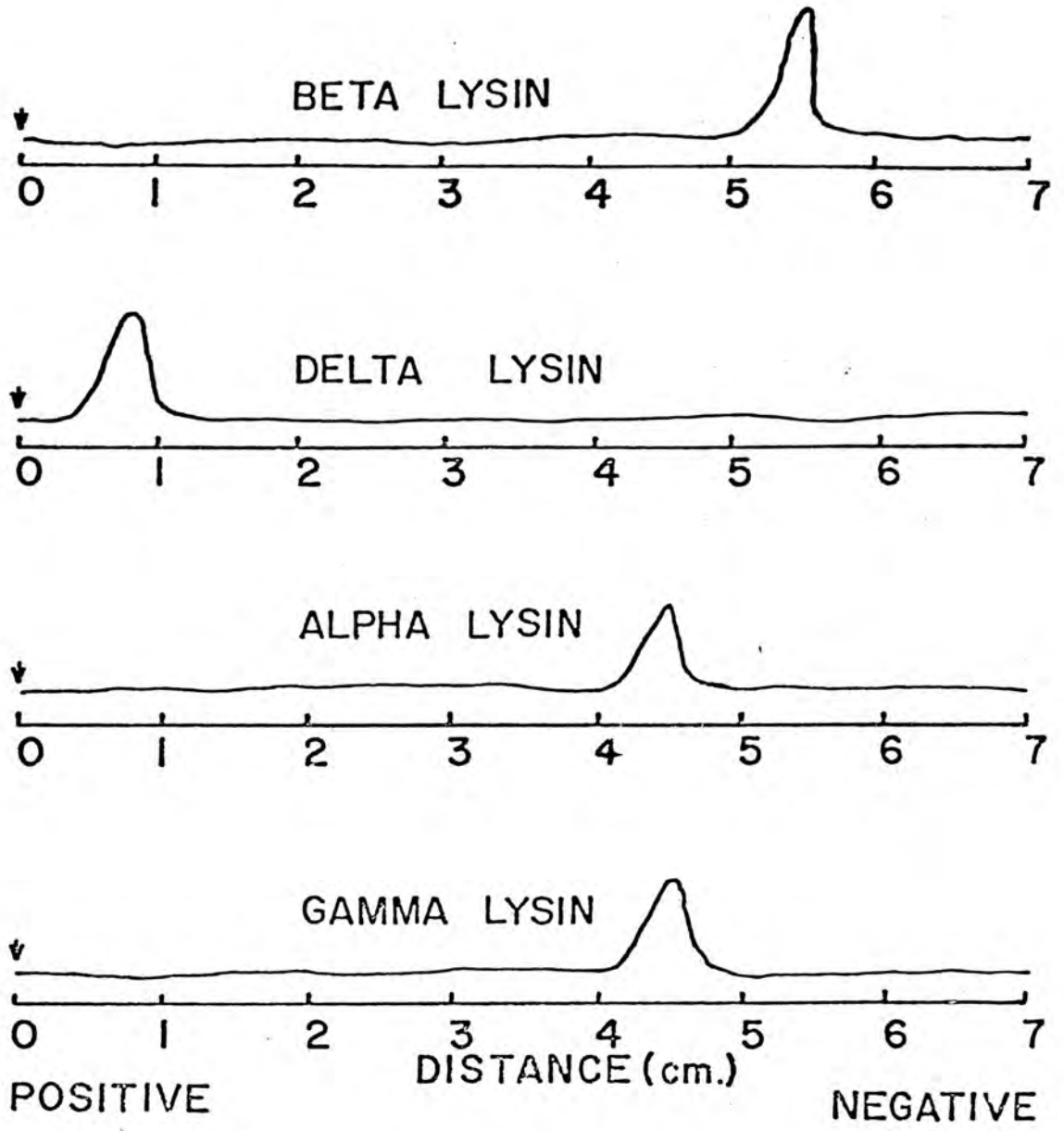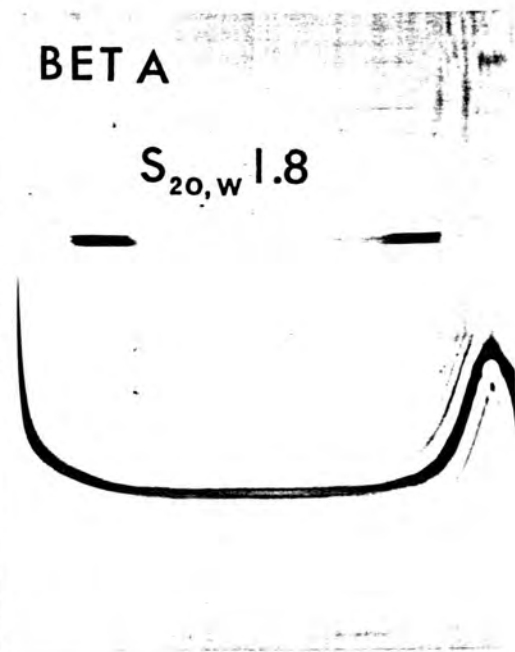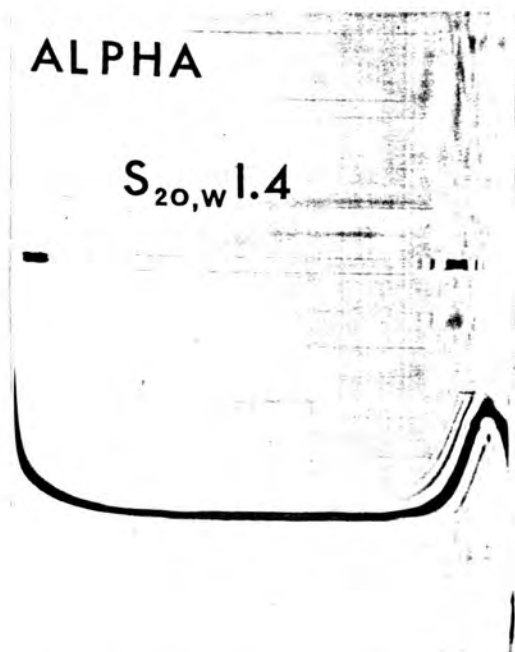
Fig. 1

Fig. 2
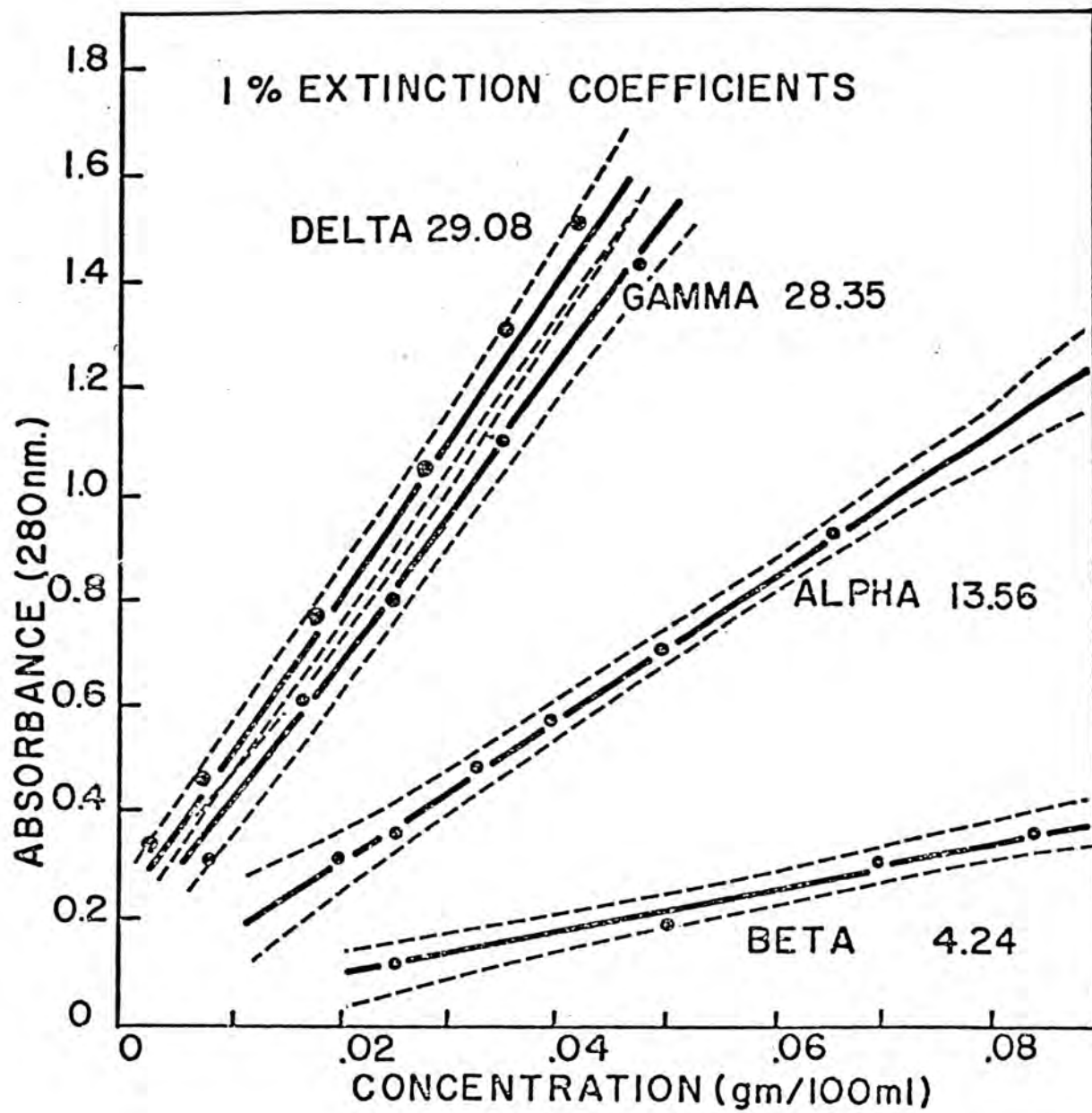
Fig. 3
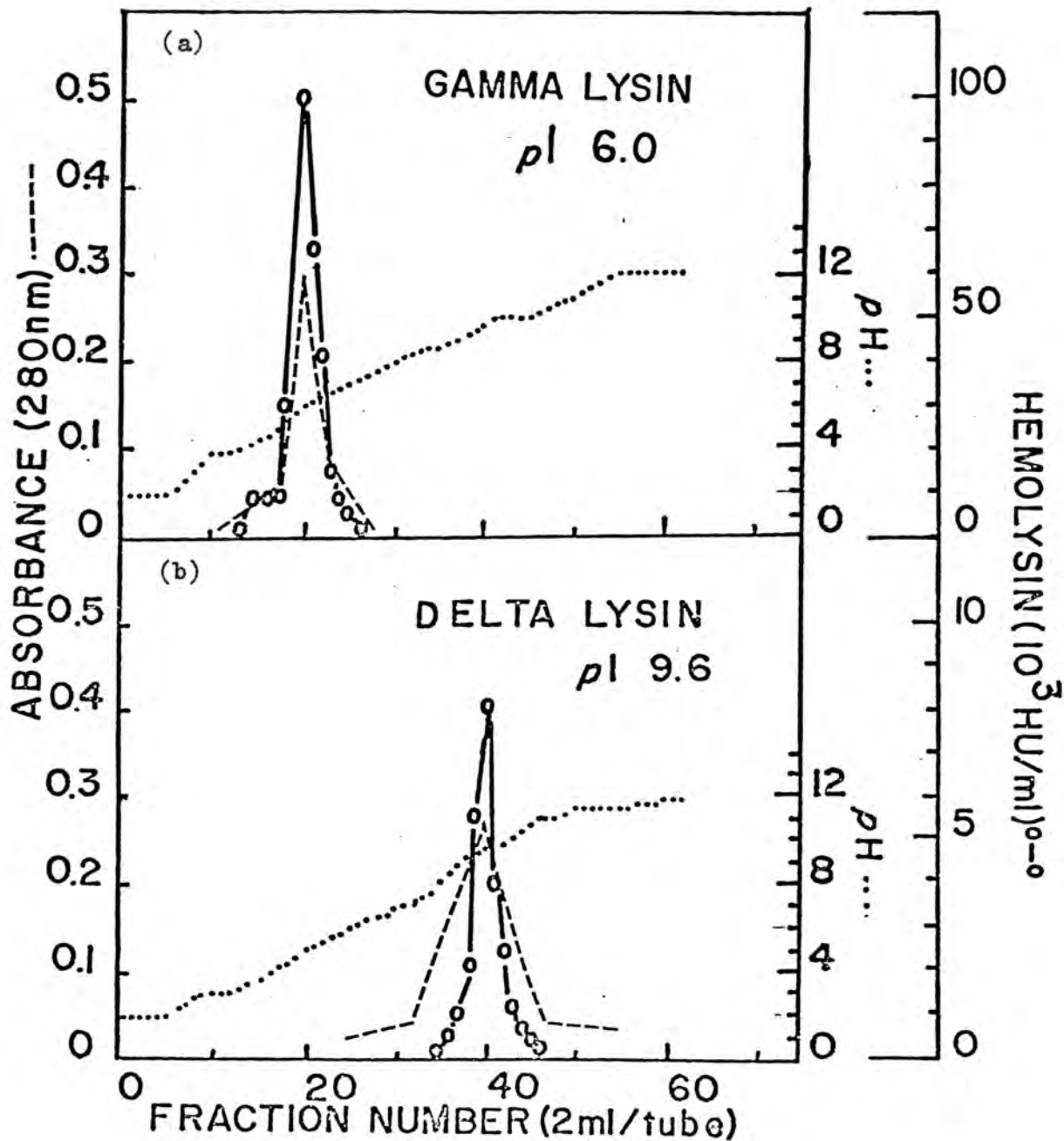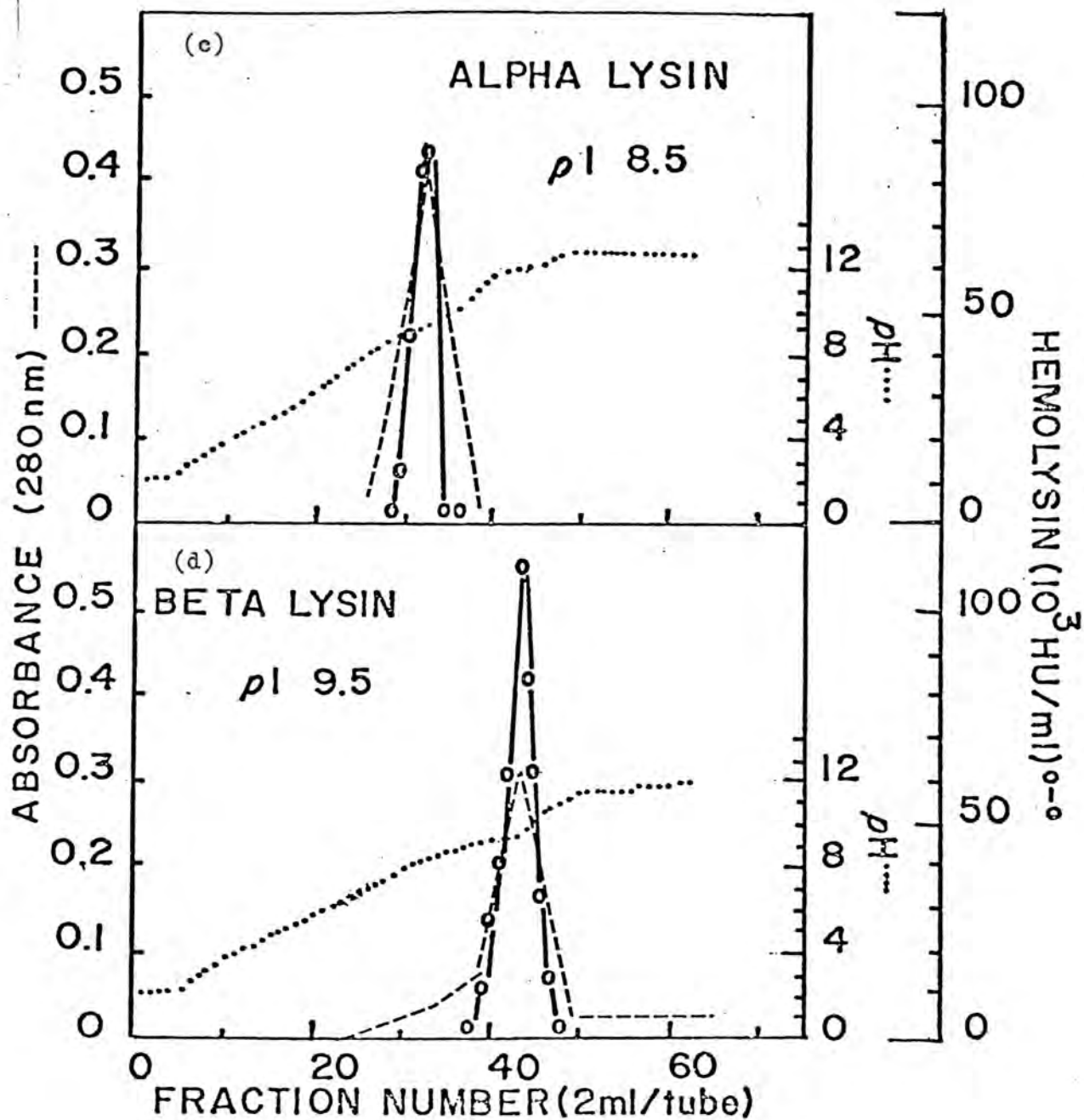
Fig. 4

Fig. 4
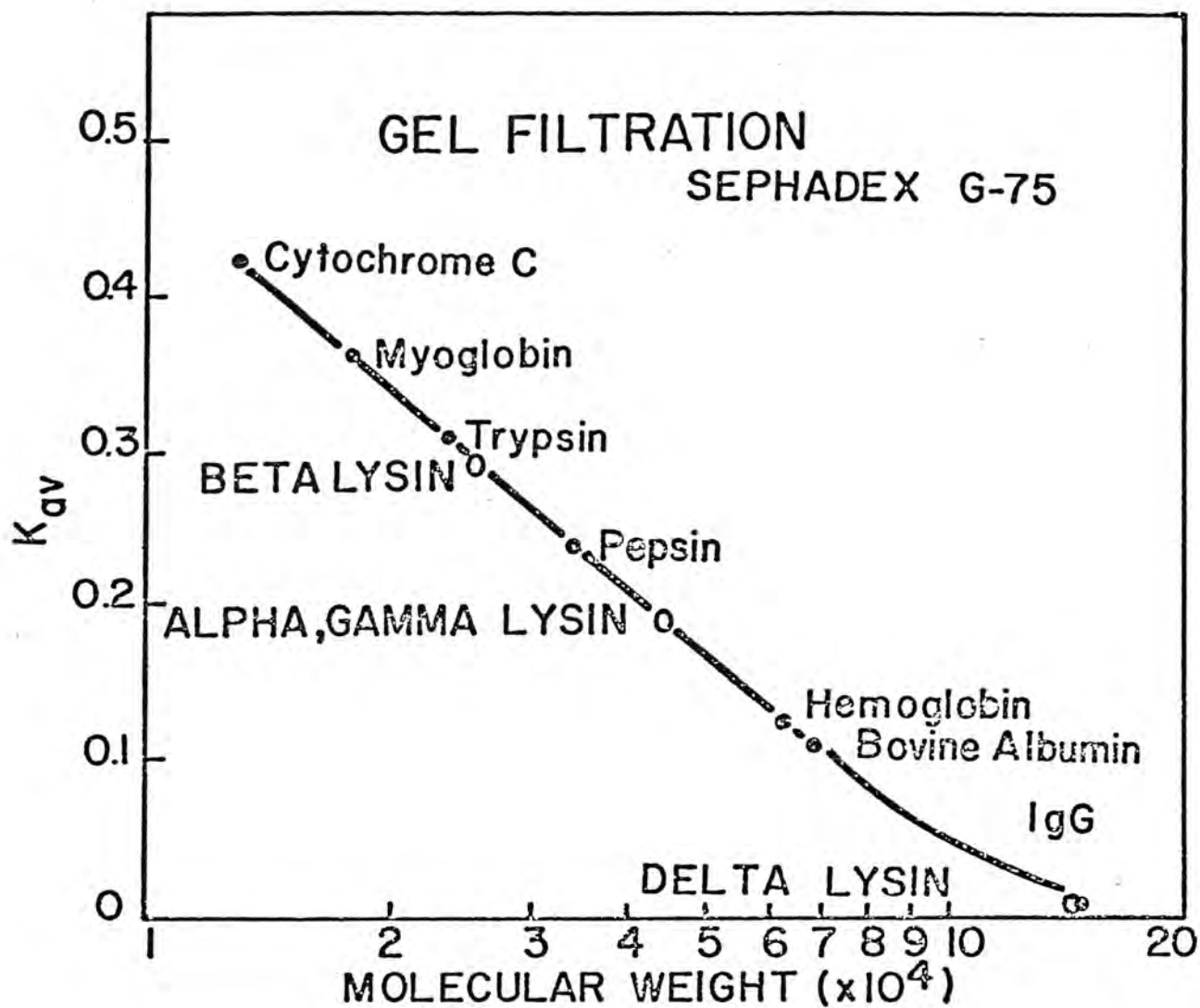
Fig. 5

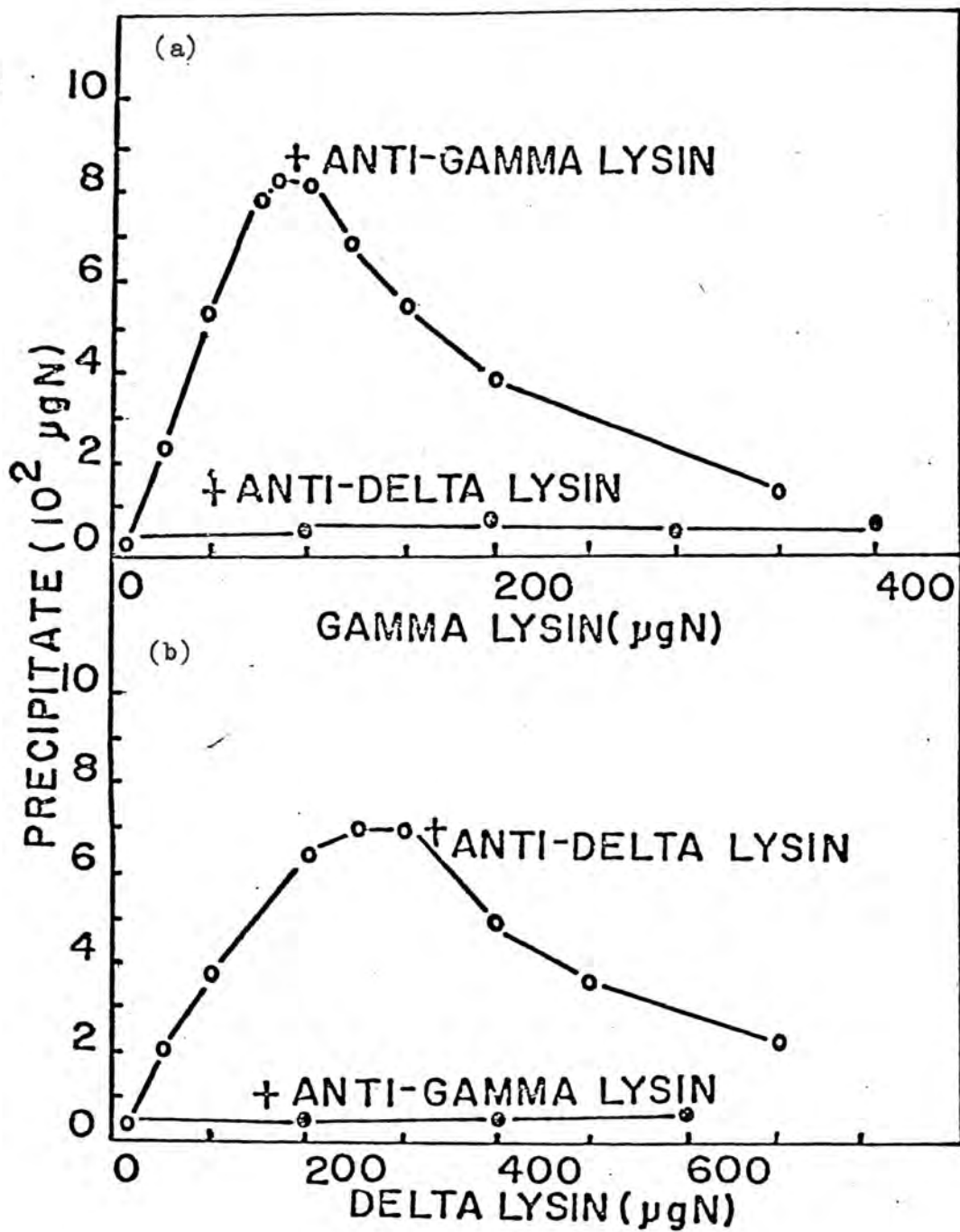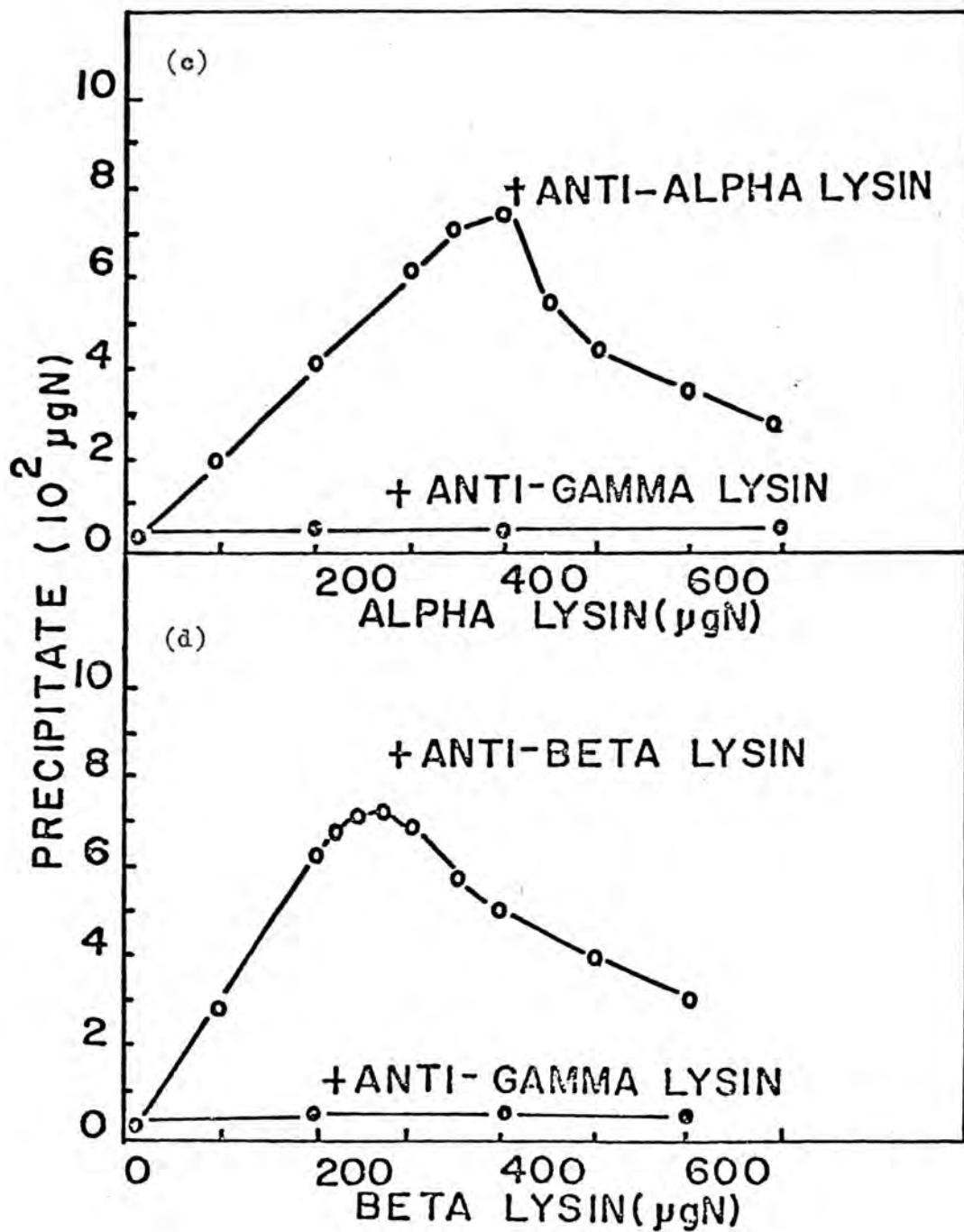HIST ALPHA GAMMA METH DELTA PRO
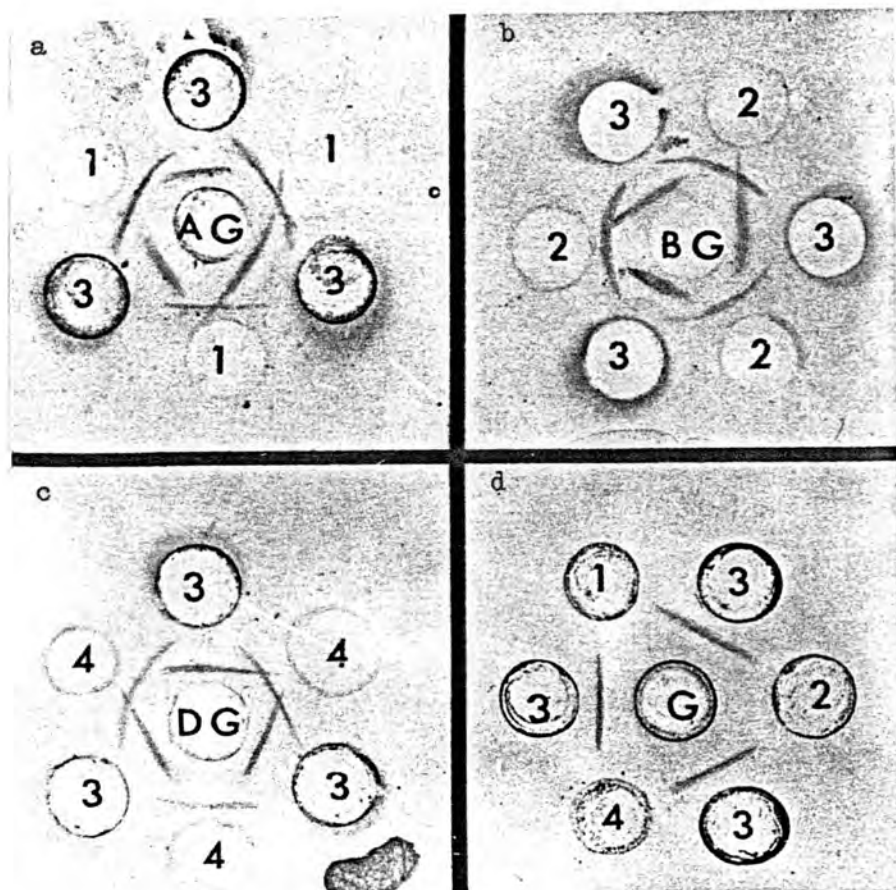
1   2   3   4   5   6
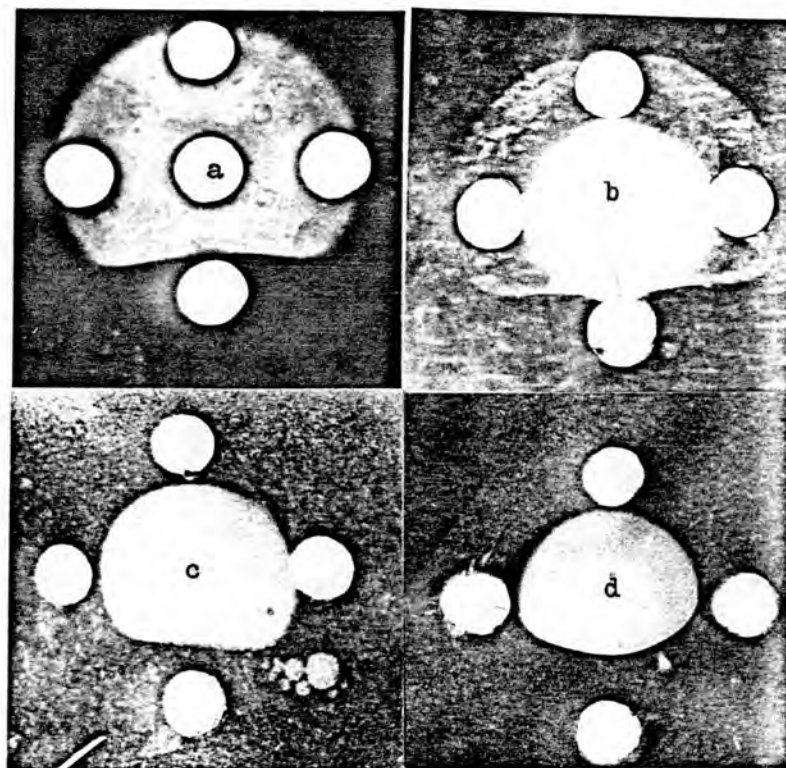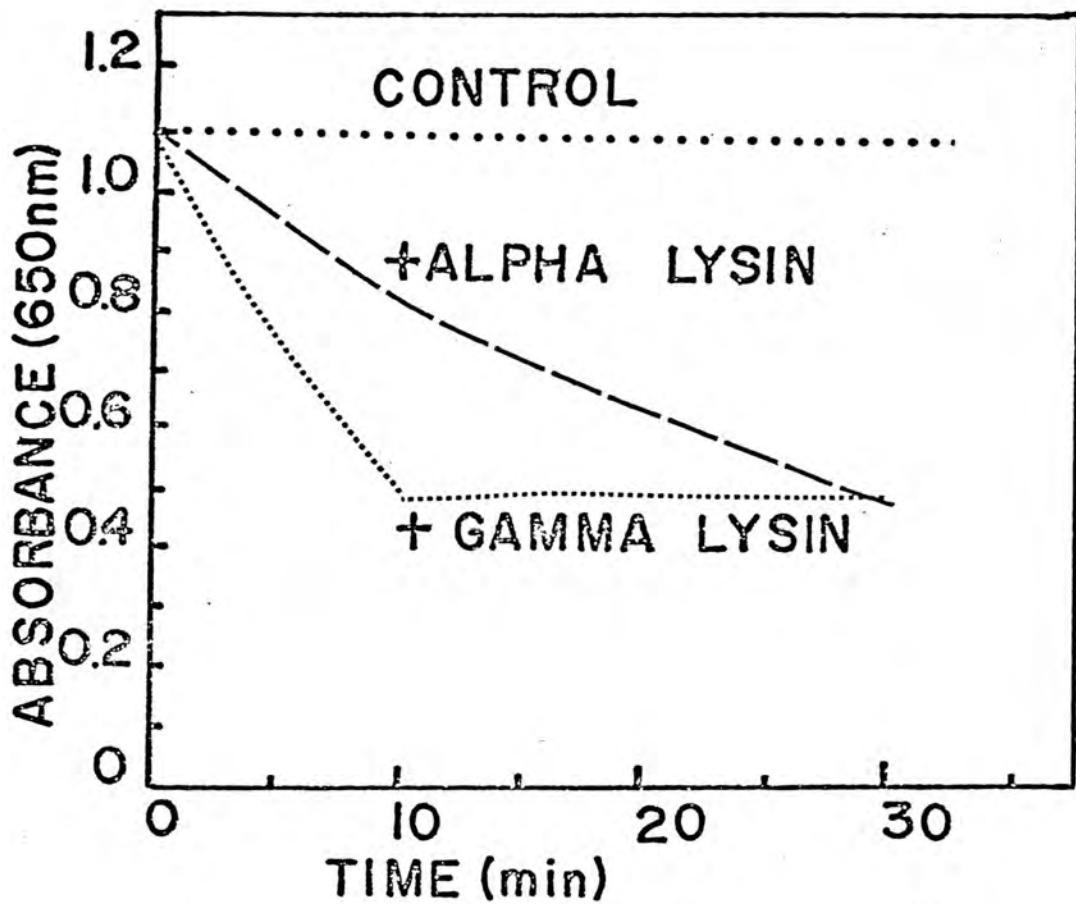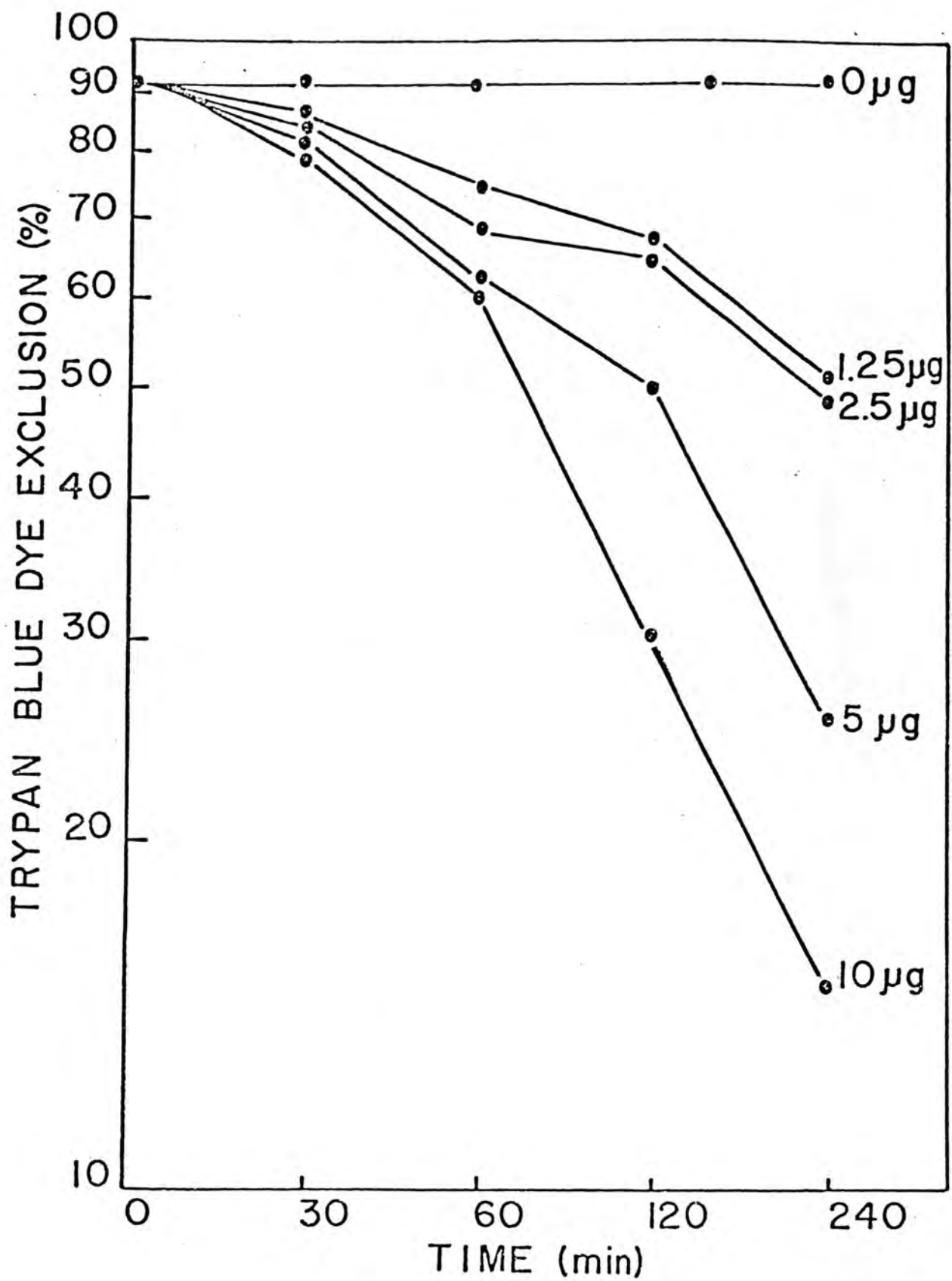
Fig. 6

Fig. 7

Fig. 7

Fig. 8

Fig. 9

Fig. 10

Fig. 11

MODE OF ACTION OF THE GAMMA HEMOLYSIN OF

STAPHYLOCOCCUS AUREUS "SMITH 5R"

H.B. Fackrell[1] and G.M. Wiseman

Department of Medical Microbiology

University of Manitoba, Winnipeg, Canada

[1]Present address: Department of Biology, University of
Calgary, Calgary, Alberta.

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 phospho-
lipids 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 Kabat & 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) modi-
fication of the Lowry protein reaction.

Hemolytic activity of gamma lysin was assayed by
the method of Wiseman & Caird (1972). Thin layer chromato-
graphy (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 O) 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^OC. 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 spectrophoto-
meter as described for kinetic experiments.

Results

Effect of gamma lysin on human erythrocyte membranes

An attempt was made to show chemical differences in
composition of red cell membranes treated with gamma lysin.
The membranes were added to gamma lysin such that their
final concentration in terms of nitrogen was 500 μg/ml while
that of the lysin 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 tri-
chloroacetic 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-lysin mixture
increased by seven % in relation to a control. In addition,
acid-soluble phosphorus was released from the membranes by
the lysin.

These differences were further investigated as des-
cribed 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 sub-
strate. 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 $37^\circ C$. 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 $37^\circ C$ 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 mem-
branes 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 7.5×10^{-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

References

ANSELL, G.B., & HAWTHORNE, J.N. (1964). Phospholipids. pp. 50-51.

BAILEY, J.L. (1967). Techniques in Protein Chemistry. Amsterdam. Elsevier.

DODGE, J.T., MITCHELL, C., & HANAHAN, D.J. (1963). The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Archives of Biochemisty & Biophysics 100: 119-130.

FACKRELL, H.B. (1973). Studies with gamma hemolysin of Staphylococcus aureus. Ph.D. thesis. University of Manitoba.

FACKRELL, H.B., & WISEMAN, G.M. (1974a). Production and purification of the gamma hemolysin of Staphylococcus aureus. Journal of General Microbiology. In Press.

FACKRELL, H.B., & WISEMAN, G.M. (1974b). Properties of the gamma hemolysin of Staphylococcus aureus. Journal of General Microbiology. In Press.

GUYONNET, F., PLOMMET, M., BOUILLANNE, O., & LEPINE, P. (1968). Purification de l'hemolysine de Staphylococcus aureus. Comptes Rendus de l'Academie de Science (Paris) 267: 1180-1182.

KABAT, E.A., & MAYER, M.M. (1961). Experimental Immuno-chemistry. 2nd Ed. Springfield. Charles C. Thomas.

LELOIR, L.F., & CARDINI, C.E. (1955). Methods in Enzymology, Vol. I. Chapter 115. New York. Academic Press.

MARINETTI, G.V. (1962). Chromatographic separation, identification and analysis of phosphatides. Journal of Lipid Research 3: 1-20.

MARKHAM, R. (1942). A steam distillation apparatus suitable for Micro-Kjeldahl analysis. Biochemical Journal 36: 790-791.

MOLLBY, R., & WADSTROM, T. (1971). Separation of gamma hemolysin from Staphylococcus aureus Smith 5R. Infection & Immunity 3: 633-635.

ROSE, H.G., & OKLANDER, M. (1965). Improved procedure for the extraction of lipids from human erythrocytes. Journal of Lipid Research 6: 428-431.

ROSENBERG, S.A., & GUIDOTTI, G.   (1968).   The protein of
    human erythrocyte membranes.   I.   Preparation, solubili-
    zation and partial characterization.   Journal of Biological
    Chemistry 243: 1985-1992.

WISEMAN, G.M., & CAIRD, J.D.   (1967).   The nature of staphylo-
    coccal beta hemolysin.   I.   Mode of action.   Canadian
    Journal of Microbiology 13: 369-376.

WISEMAN, G.M., & CAIRD, J.D.   (1968).   Phospholipase activity
    of the delta hemolysin of Staphylococcus aureus.
    Proceedings of the Society for Experimental Biology and
    Medicine 128: 428-430.

WISEMAN, G.M., & CAIRD, J.D.   (1972).   Further observations
    on the mode of action of the alpha lysin of Staphylococcus
    aureus "Wood-46".   Journal of Medical Microbiology.   18:
    987-992.

WISEMAN, G.M., CAIRD, J.D., & FACKRELL, H.B.   (1974).
    Trypsin-mediated activation of the alpha hemolysin of
    Staphylococcus aureus "Wood-46".   Journal of Medical
    Microbiology.   In Press.

TABLE 1

INHIBITION OF GAMMA HEMOLYSIN BY EDTA

TREATMENT	EDTA (M)	TOTAL HEMOLYSIN (HU)*
Gamma lysin - EDTA solution	10^{-2}	0
	10^{-3}	0
	10^{-4}	6,500
	10^{-5}	7,300
	10^{-6}	11,200
	10^{-7}	11,800
Dialyzed gamma lysin - EDTA solution	10^{-2}	11,900
	10^{-3}	12,100
	10^{-4}	11,600
	10^{-5}	11,750
	10^{-6}	11,300
	10^{-7}	11,500
Untreated gamma lysin	0	11,800

*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
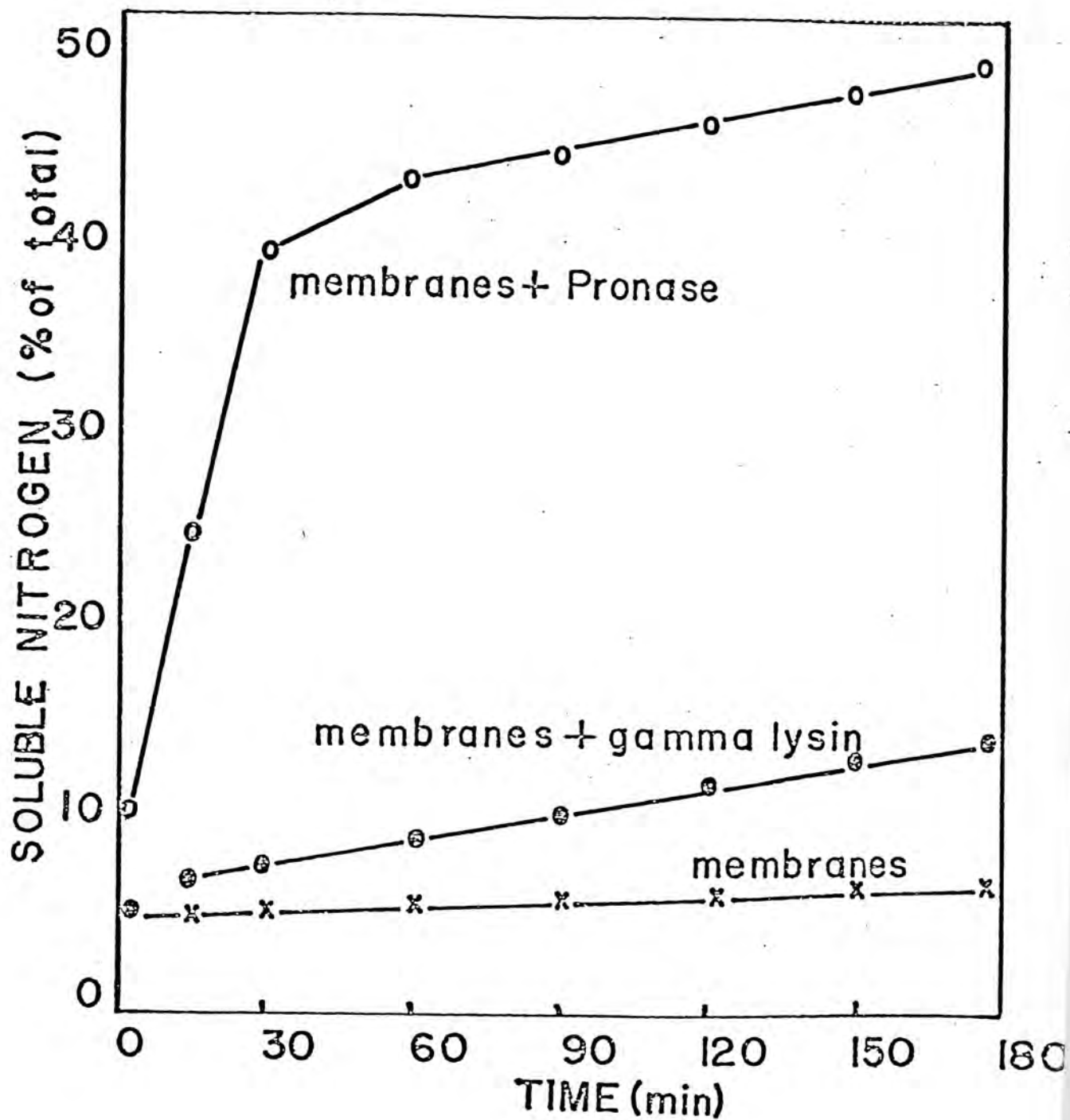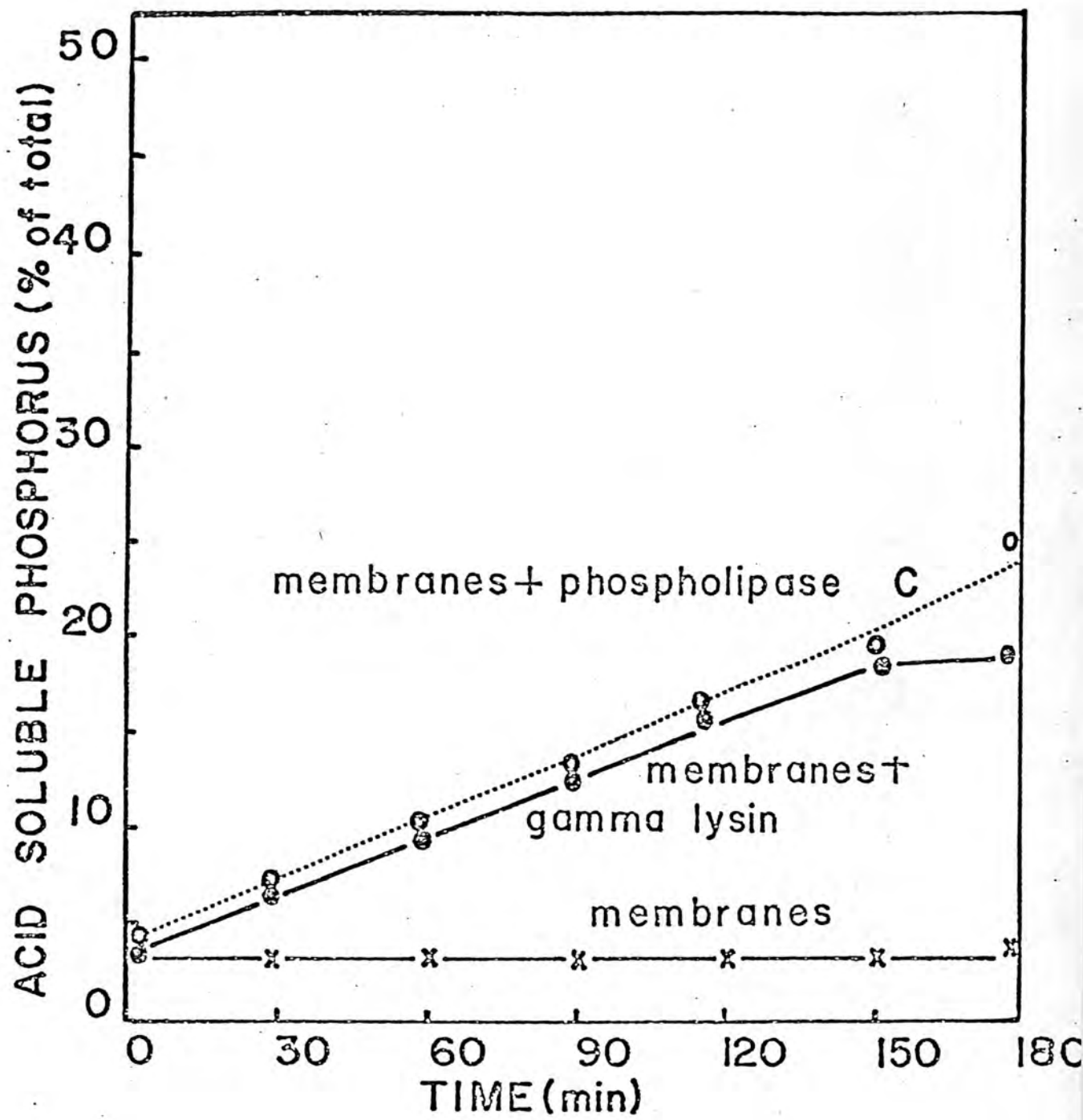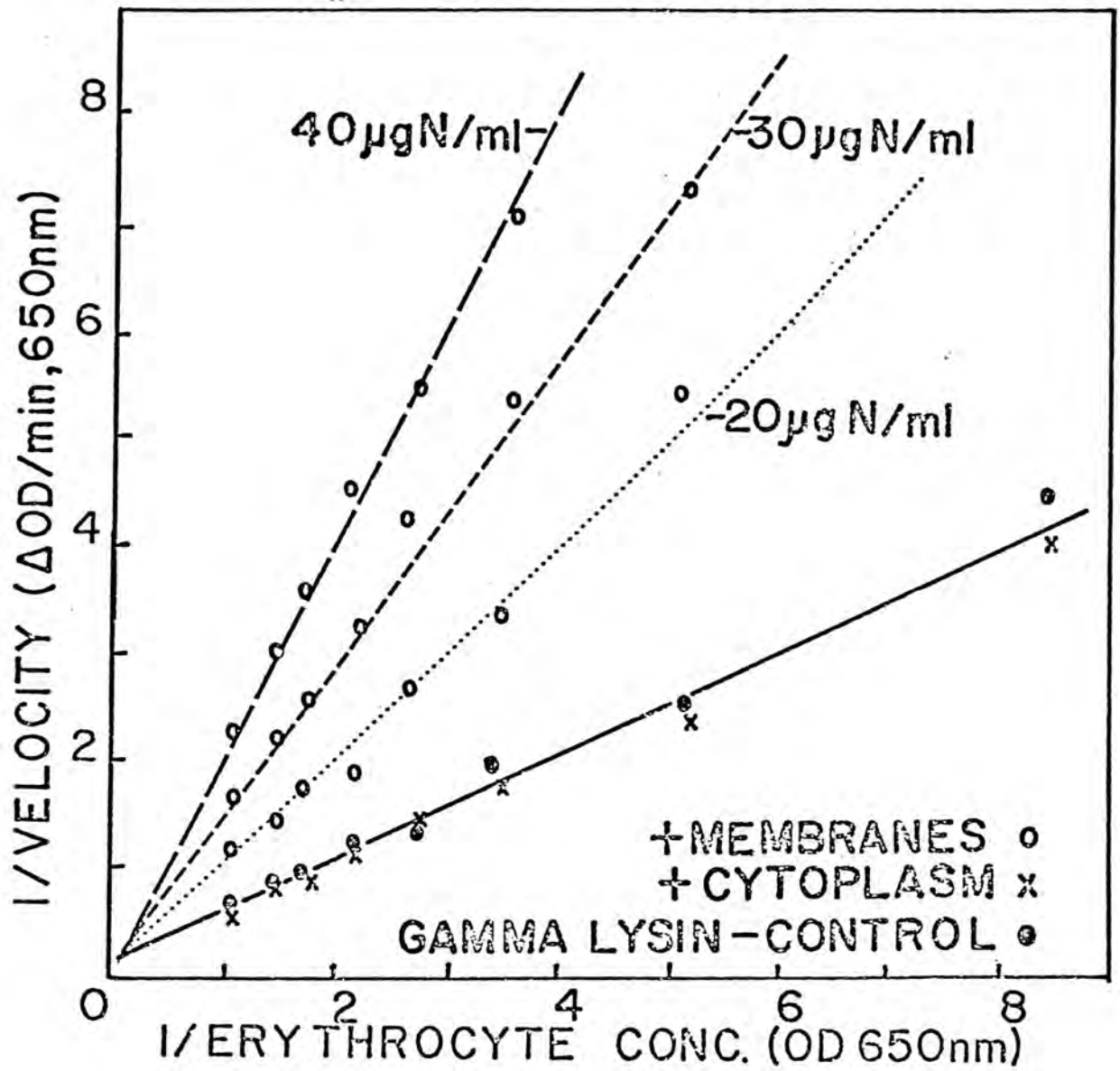of lysis of human erythrocytes in the presence
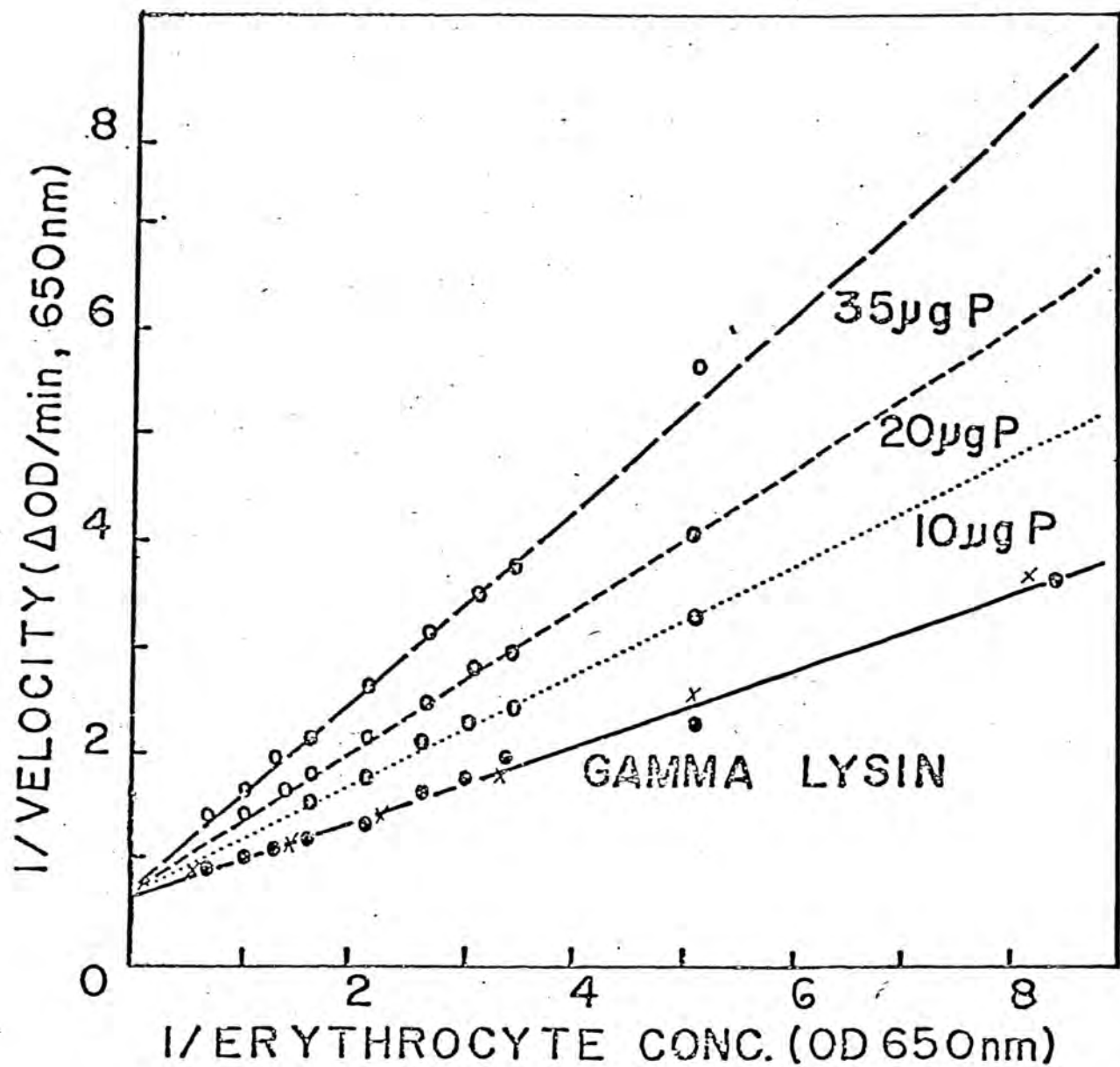of gamma lysin.

Fig. 1

Fig. 2
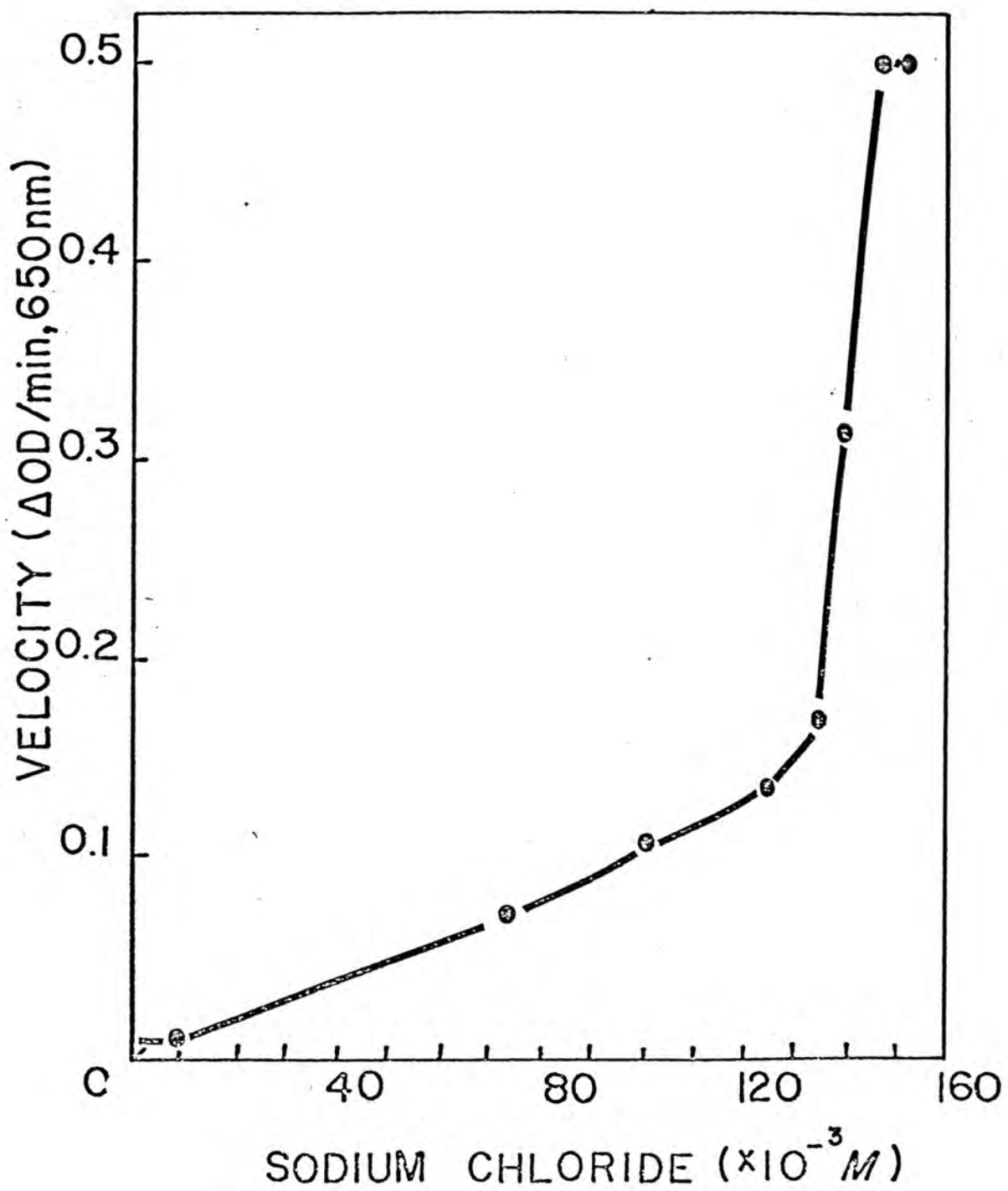
Fig. 3

Fig. 4

Fig. 5

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. WISEMAN*

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 Walbum (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 lysin 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 " lysin for sheep erythrocytes: the activity of this lysin was enhanced by Co^{++}, Mg^{++}, Mn^{++}, Ni^{++} and Fe^{++} ions,

* Present address: Department of Bacteriology and Immunology, University of Manitoba Medical School, Winnipeg 3, Canada.

but not by Zn^{++} or Ca^{++} ions, and it was inhibited by EDTA. They were, however, unable to identify the lysin 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 cellophan 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 cellophan 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 Tiselius, 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 lysin. 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 lysin was recovered by centrifuging the adsorbent at 250g for 10 min. The beta-lysin eluate was dialysed against running tap water at c. 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-MgCl$_2$ 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 lysin, commencing at 1 in 2 or 1 in 10, were made in 1 ml. phosphate-buffered saline in tubes measuring approximately 1 × 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.

A 2

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 0·0001M, and L-cysteine hydrochloride at concentrations of from 0·01 to 0·0001M; 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 0·2M ascorbic acid and others containing from 0·002 to 0·02M cysteine, and the mixtures were incubated at 4° C for *c.* 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° and 100° C. Beta-lysins 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 3·00 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 5 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. lysin 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 *p*H 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 *p*H, 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

Preparation	Haemolytic titre (units per ml.) with			Volume or weight of preparation	Total protein mg. per ml. preparation	Specific beta-lytic activity*	Per cent. recovery of beta-lysin
	rabbit cells	human cells	sheep cells				
(1) Crude haemolysin	2560	5120	40,960	850 ml.	...	...	100
(2) Dialysed crude lysin	2560	5120	40,960	850 ml.	1·000	$4·09 \times 10^4$	100
(3) NaCl eluate from adsorbent	1280	10,240	163,840	125 ml.	0·200	$8·19 \times 10^5$	59
(4) Eluate dialysed against tap water	640	5120	81,920	125 ml.	0·068	$1·20 \times 10^6$	30
(5) Lyophilised tap water dialysate (1·0 mg. per ml.)†	640	5120	81,920	67 mg.	0·070	$1·17 \times 10^6$	17

* 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

Effect of metallic cations added at a concentration of 0·001M on the rabbit-, human- and sheep-cell haemolytic titres of the haemolysins of strains R1, 252F and G143

Cation added	Haemolytic titres (units per ml.) of haemolysins from								
	strain R1			strain 252F			strain G143		
	rabbit cells	human cells	sheep cells	rabbit cells	human cells	sheep cells	rabbit cells	human cells	sheep cells
Mg⁺⁺	20	160	20,480	< 20	< 20 (40)*	160	1280	20	40
Co⁺⁺	20	160	20,480	< 20	< 20 (80)	160	640	20	40
Mn⁺⁺	20	160	10,240	< 20	< 20 (40)	320	1280	40	40
Ni⁺⁺	< 20	40	640	< 20	< 20 (40)	40	640	20	40
Ca⁺⁺	< 20	40	320	< 20	< 20	40	1280	40	40
Zn⁺⁺	< 20	< 20	< 20	< 20	< 20	< 20	< 20	< 20	< 20
None	< 20	40	640	< 20	< 20†	20	1280	20	40
Mg⁺⁺ and phosphate buffer	40	160	20,480	...	...	...	2560	20	40

* 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

A 3

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-lysins 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

| Molarity of added cation | Sheep-cell haemolytic titres (units) per ml.) in presence of added cation | | | |
| | Mg++ | | Co++ | |
	R1	252F	R1	252F
0	40	20	40	20
0·1	40	20	320	80
0·01	2560	160	1280	160
0·001	2560	160	1280	160
0·0001	320	80	640	80
0·00001	80	40	160	40
0·000001	40	20	160	40

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

Chelating agent	Molarity of chelating agent	Sheep-cell haemolytic titre (units per ml.) of lysin			
		without added Mg⁺⁺		with 0·001M Mg⁺⁺	
		R1 lysin	252F lysin	R1 lysin	252F lysin
EDTA .	0·01	< 20	< 20	< 20	< 20
	0·001	20	< 20	80	20
	0·0001	80	20	10,240	160
	0·00001	640	40	10,240	320
	0·000001	1280	40	10,240	320
	0	1280	40	10,240	320
Citric acid .	0·01	...*	...	...	...
	0·001	40	< 20	160	40
	0·0001	320	20	2560	320
	0·00001	1280	40	10,240	320
	0·000001	1280	40	20,480	320
	0	...	...	...	...

* 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 lysin 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 haemo-lysins prepared from different strains of Staph. aureus in tests with rabbit, human and sheep erythrocytes

Strain	Haemolysin preparation	Addition of magnesium ions	Haemolytic titres (units per ml.) with		
			rabbit cells	human cells	sheep cells
P92	Partially purified beta-lysin	−	< 20	< 20	20
	Partially purified beta-lysin	+	20	160	1280
G128	Partially purified beta-lysin	−	< 20	< 20*	80
	Partially purified beta-lysin	+	40	640	2560
R1	Crude lysin	−	2560	320	1280
	Crude lysin	+	2560	5120	81,920
	Partially purified beta-lysin	−	20	40	160
	Partially purified beta-lysin	+	160	320	10,240
252F	Crude lysin	−	20	40	160
	Crude lysin	+	160	640	2560
	Partially purified beta-lysin	−	< 20	20	40
	Partially purified beta-lysin	+	20	80	320

* 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 suscepti-bility 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

Erythrocyte species	Haemolytic titres (units per ml.) of beta-lysin from strain	
	R1	252F
Horse	<4	<4
Guinea-pig	<4	<4
Dog	<4 (4)*	<4
Frog	<4 (4)	<4 (4)
Mouse	<4 (8)	<4 (8)
Rat	<4 (32)	<4 (32)
Fowl	<4 (64)	<4 (64)
Pig	32	32
Rabbit	64	32
Cat	64	64
Man	256	128
Ox	512	512
Sheep	2048	512

* 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-lysins from strains R1 and 252F

| Molarity of reducing agent | Sheep-cell haemolytic titre (units per ml.) of lysin in presence of reducing agent | | | |
| | ascorbic acid | | cysteine | |
	R1 lysin	252F lysin	R1 lysin	252F lysin
0·1	20	< 20	...	...
0·01	1280	20	2560	320
0·001	2560	160	5120	320
0·0001	5120	160	5120	320
0	20,480	640	20,480	640

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-lysins from strains R1 and 252F

| Time of heating at stated temperature (min.) | Haemolytic titres with sheep cells (units per ml.) of | | | |
| | R1 lysin after heating at | | 252F lysin after heating at | |
	60° C	100° C	60° C	100° C
0	2048	2048	512	512
3	2048	512	512	512
6	1024	256	512	512
9	1024	128	512	512
12	1024	128	512	512
15	1024	128	512	256
30	256	16	512	32
60	128	< 4	256	< 4
120	64	...	256	...

Since Kodama and Kojima had stated that the beta-lysin may be subject to the same anomalous behaviour toward heat as the alphalysin, 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
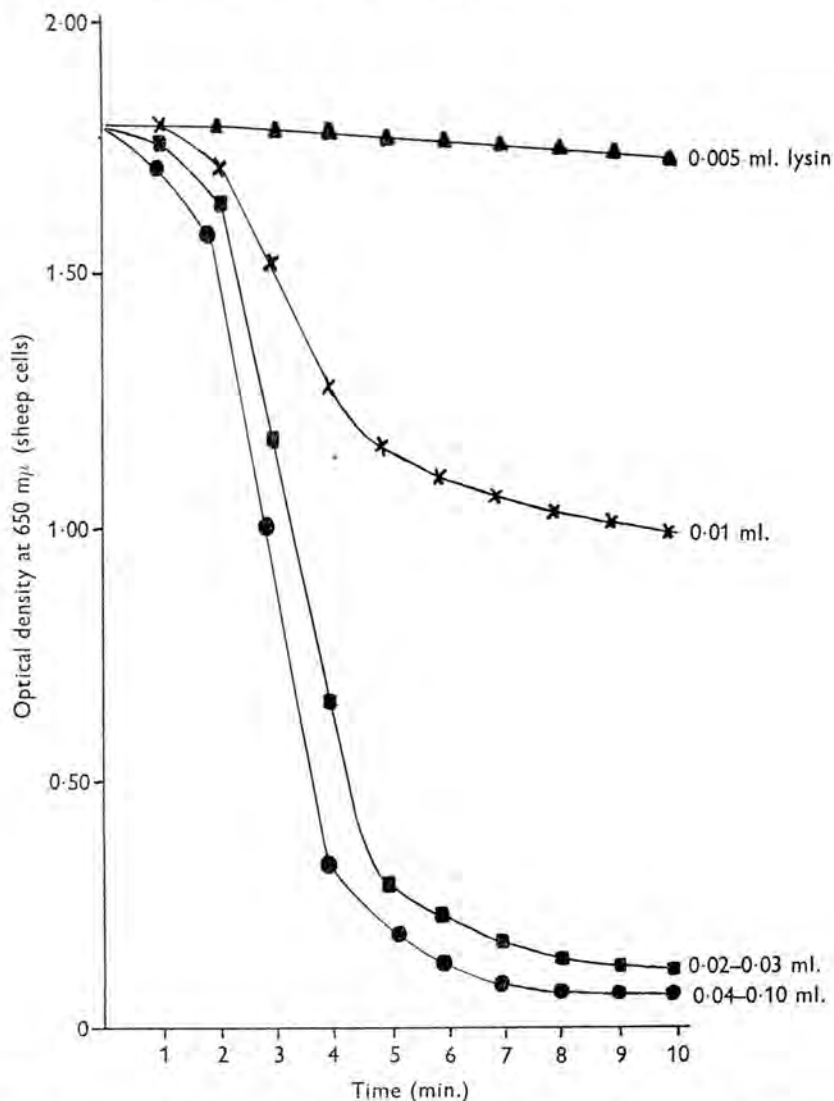little activity against mouse red cells (table VI).

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 lysin concentration is given as ml. of lysin
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 lysin 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.
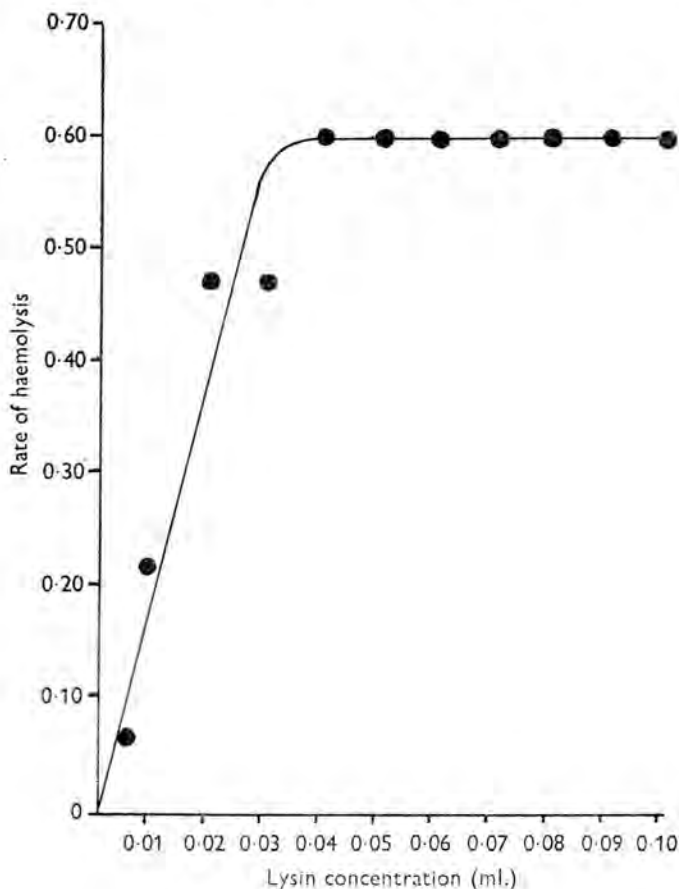

FIG. 2.—Relation between the rate of haemolysis of sheep erythrocytes at 25° C and the concentration of partially purified R1 beta-lysin (ml. per 4 ml. mixture). See text for description of the calculation of the rate of haemolysis.

Effect of beta-lysin on rabbits. Results obtained from the intra-venous 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 experi-ments 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 lysin produced very severe necrosis, but injection of crude 252F lysin 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 lysin 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° C, but lysed all three species of cells after a period of holding at 4° 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° C, and was almost completely destroyed in 2 hr, whereas the lysin from strain 252F was little affected by heating at 60° C. The activity of both preparations, however, was destroyed by heating at 100° 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° 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° 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 observations 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 substrate, 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 0·0010M and 0·0013M 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 lysin 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.

REFERENCES

BERNHEIMER, A. W. . . . . . 1947. *J. Gen. Physiol.*, **30**, 337.

BIRCH-HIRSCHFELD, LUISE . . . 1933–34. *Z. Immun.-Forsch.*, **81**, 260.

BRYCE, LUCY M., AND ROUNTREE, 1936. This *Journal*, **43**, 173.
PHYLLIS M.

CARPENTER, P. L. . . . . . 1956. Immunology and serology, *Philadelphia*, p. 301.

CRUICKSHANK, R., *et al.* . . . 1960. Mackie and McCartney's Handbook of bacteriology, 10th ed., *Edinburgh* and *London*, p. 283.

DOLMAN, C. E., AND WILSON, R. J. 1940. *Canad. Publ. Hlth J.*, **31**, 68.

ELEK, S. D. . . . . 1959. *Staphylococcus pyogenes* and its relation to disease, *Edinburgh* and *London*, p. 238.

ELEK, S. D., AND LEVY, E. . . 1950. This *Journal*, **62**, 541.

EVANS, J. B. . . . . 1957. *In* Bergey's Manual of determinative bacteriology, 7th ed., edited by R. S. Breed, E. G. D. Murray and N. R. Smith, *Baltimore*, p. 464.

EVELETH, D. F. . . . . 1937.   *J. Biol. Chem.*, **119**, 289.

FLAUM, A. . . . . 1938.   *Acta path. microbiol. scand.*, Suppl. 35.

GLENNY, A. T., AND STEVENS, 1935.   This *Journal*, **40**, 201.
MURIEL F.

HEYDRICK, F. P., AND CHESBRO, 1962.   *Bact. Proc.*, **62**, 63.
W. R.

JACKSON, A. W. . . . . . . 1963.   *Canad. J. Biochem.*, **41**, 755.

JACKSON, A. W., AND BENNS, 1961   *Canad. Soc. Microbiol. Abstr.*, p. 17.
GEORGINA

JACKSON, A. W., AND LITTLE, R. M. 1958.   *Canad. J. Microbiol.*, **4**, 435.

JACKSON, A. W., AND MAYMAN, D. 1958.   *Ibid.*, **4**, 477.

KODAMA, T., AND KOJIMA, T. . . 1939.   *Kitasato Arch. Exp. Med.*, **16**, 36.

LOMINSKI, I., AND ARBUTHNOTT, J. P. 1962.   This *Journal*, **83**, 515.

LOWRY, O. H., ROSEBROUGH, N. J., 1951.   *J. Biol. Chem.*, **193**, 265.
FARR, A. L., AND RANDALL, ROSE J.

MERCIER, P. . . . . 1938.   *C. R. Soc. Biol. (Paris)*, **127**, 297,

OUCHTERLONY, Ö. . . . . . 1958.   *Progr. Allergy*, **5**, 1.

RIAZ-UL-HAQUE, AND BALDWIN, J. N. 1963.   *Bact. Proc.*, **63**, 78.

ROBINSON, J., THATCHER, F. S., AND 1958.   *Canad. J. Microbiol.*, **4**, 345.
GAGNON, JEANNINE

ROBINSON, J., THATCHER, F. S., AND 1960.   *Ibid.*, **6**, 183.
MONTFORD, JEANNINE

ROY, T. E. . . . . 1937.   *J. Immunol.*, **33**, 437.

SMITH, M. L., AND PRICE, S. A. . 1938.   This *Journal*, **47**, 361.

THATCHER, F. S., AND MATHESON, 1954–55.   *Canad. J. Microbiol.*, **1**, 382.
B. H.

THAYSEN, E. H. . . . . . . 1948.   *Acta path. microbiol. scand.*, **25**, 529.

TISELIUS, A., HJERTÉN, S., AND 1956.   *Arch. Biochem.*, **65**, 132.
LEVIN, Ö.

WALBUM, L. E. . . . . . . 1922.   *Biochem. Z.*, **129**, 367.

WILLIAMS, R. E. O., AND HARPER, 1947.   This *Journal*, **59**, 69.
G. J.

WISEMAN, G. M. . . . . . . 1963.   Ph.D. Thesis, Univ. Edinburgh

YOSHIDA, A. . . . . 1963.   *Biochim. biophys. Acta (Amst.)*, **71**, 544.

FACTORS AFFECTING THE SENSITIZATION OF SHEEP ERYTHROCYTES TO STAPHYLOCOCCAL BETA LYSIN

G. M. WISEMAN

Department of Bacteriology and Immunology, University of Manitoba Medical School, Winnipeg, Manitoba

Received May 7, 1964

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, lysin 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 lysin 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 $MgCl_2$ 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 mμ. 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 $\pm$ 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 (% T_{30}).

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 I

Effect of NaCl gradient on sheep erythrocytes
sensitized to beta lysin

Final % NaCl	% transmittance
0.43	54.5*
0.55	52
0.66	53
0.745	55.5
0.80–2.50	0

*Complete lysis corresponds to 57 ± 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

TABLE II

Effect of pH gradient on sheep erythrocytes sensitized to beta lysin

Final concn. beta lysin	Final pH	% transmittance
64	6.0	48
"	6.3	25
"	6.5	13
"	6.6	3
"	6.9–9.8	0
0	6.0	0
0	9.8	0
64	7.0	60*

*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.

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
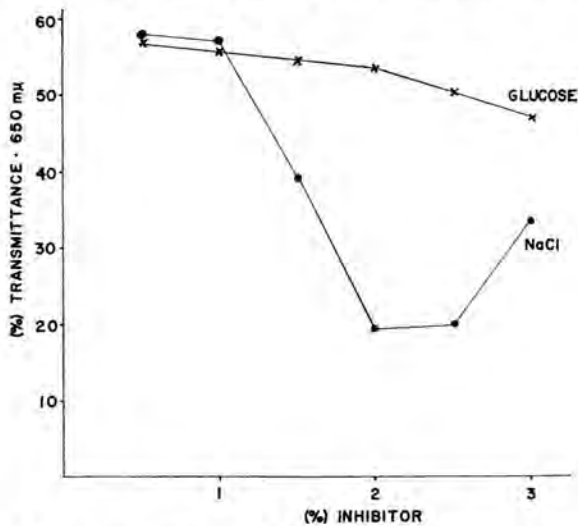

FIG. 1. Effect of inhibitors on hemolysis of sheep erythrocytes sensitized to staphylococcal beta lysin. The degree of inhibition increases with a decrease in % transmittance.

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 $\%\ T_{30}$ 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).
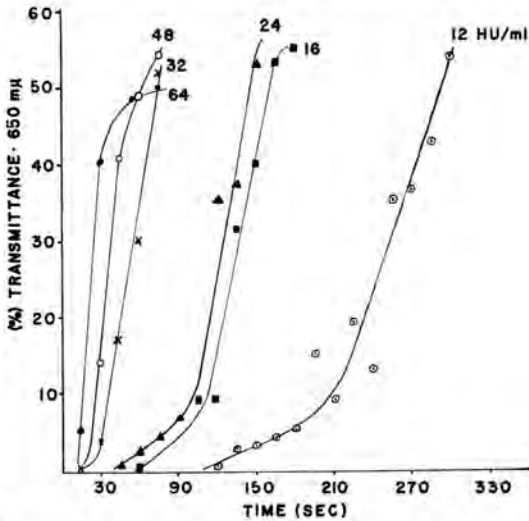

FIG. 2. Relationship of lysin concentration to time required for sensitization of sheep erythrocytes to beta lysin.
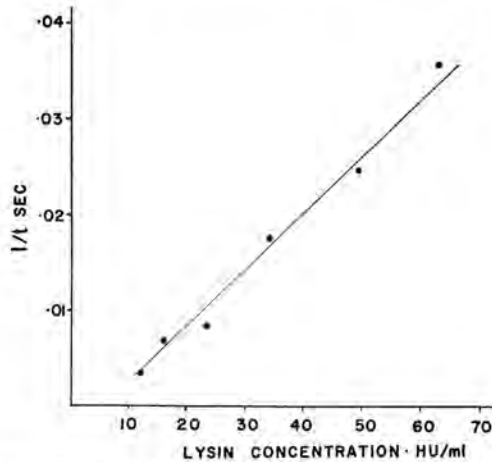

FIG. 3. Relationship of beta lysin concentration to the rate of its reaction with sheep erythrocytes. See text for calculation of reaction rates.

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-
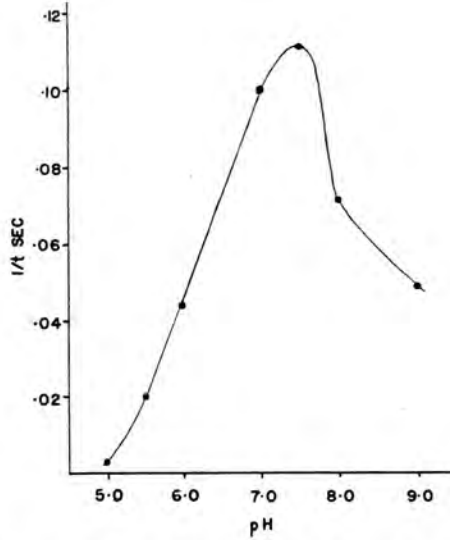

FIG. 4. The effect of variation of pH on the rate of reaction of beta lysin with sheep erythrocytes.
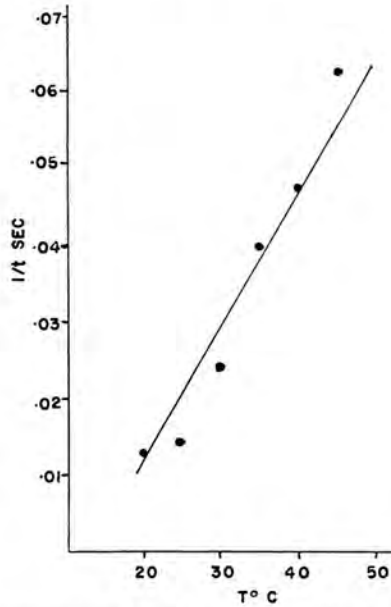

FIG. 5. The effect of temperature variations on the rate of reaction of beta lysin with sheep erythrocytes.

siderable 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, μ, of beta lysin was calculated to be 14,100 cal.
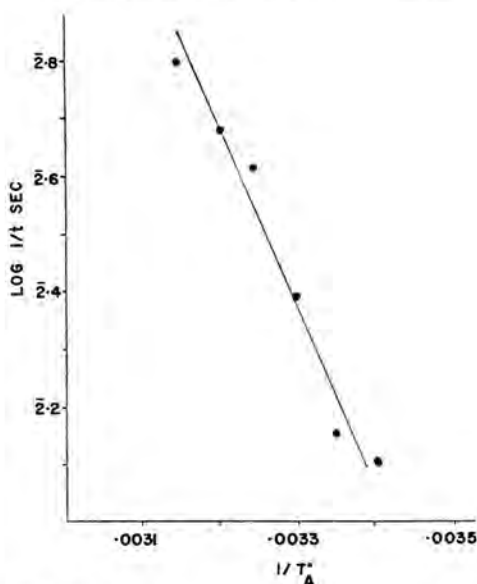
FIG. 6. Arrhenius plot of data on sensitization of sheep erythrocytes to beta lysin. The value of μ was calculated from the slope.

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 μ 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 μ 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 lysin. 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 lysin. Final proof, however, must await its more complete purification.

References

1. BERNHEIMER, A. W. Comparative kinetics of hemolysis induced by bacterial and other hemolysins. J. Gen. Physiol. **30**, 337–353 (1947).
2. BIGGER, J. W. The production of staphylococcal hemolysin with observations on its mode of action. J. Pathol. Bacteriol. **36**, 87–114 (1933).
3. DOERY, H. M., MAGNUSSON, B. J., CHEYNE, I. M., and GULASEKHARAM, J. A phospholipase in staphylococcal toxin which hydrolyzes sphingomyelin. Nature, **198**, 1091–1092 (1963).
4. IKEZAWA, H. and MURATA, R. Comparative kinetics of hemolysis of mammalian erythrocytes by *Clostridium perfringens* alpha toxin (phospholipase C). J. Biochem. **55**, 217–224 (1964).
5. JACKSON, A. W. and MAYMAN, D. Staphylococcal toxins. IV. Factors affecting hemolysis by beta lysin. Can. J. Microbiol. **4**, 477–486 (1958).
6. PONDER, E. H. Hemolysis and related phenomena. Grune and Stratton, New York. 1948. pp. 112–113.
7. PULSFORD, M. F. Factors affecting the lysis of erythrocytes treated with staphylococcal beta toxin. Australian J. Exptl. Biol. Med. Sci. **32**, 347–352 (1954).
8. SIZER, I. W. Effects of temperature on enzyme kinetics. Advan. Enzymol. **3**, 35 (1943).
9. TURNER, J. C. Absence of lecithin from the stromata of the red cells of certain animals (ruminants), and its relation to venom hemolysis. J. Exptl. Med. **105**, 189–193 (1957).
10. WISEMAN, G. M. Some characteristics of the beta hemolysin of *Staphylococcus aureus*. J. Pathol. Bacteriol. **89**, 187–207 (1965).

THE NATURE OF STAPHYLOCOCCAL BETA HEMOLYSIN
I. MODE OF ACTION

G. M. WISEMAN AND J. D. CAIRD

Department of Bacteriology and Immunology, University of Manitoba Medical School,
Winnipeg, Manitoba

The mode of action of highly purified beta hemolysin derived from the R-1 strain of *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 phosphatidylcholine 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 *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, mucopeptides, 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 *S. aureus*, methods of production and quantitative titration of the beta hemolysin have been published elsewhere (17). The E-delta strain of *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 × 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 mμ 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

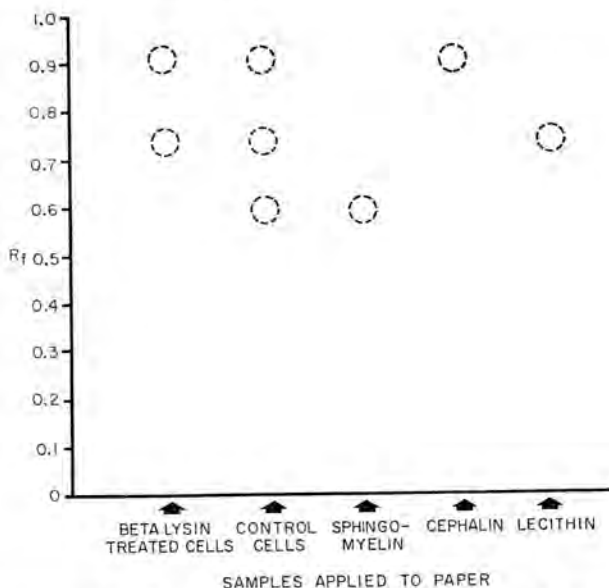Cell walls were prepared by the method of Chesbro et al. (2).

FIG. 1. Chromatogram of normal and beta hemolysin-treated sheep erythrocyte phospholipids. These were chromatographed on paper impregnated with silicic acid. The solvent was chloroform/methanol 4:1 with 2.5% water.

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

Relationship of sphingomyelin content to sensitivity of erythrocyte species to beta hemolysin

Erythrocyte species	μg P in sphingomyelin spot	% sphingomyelin*		Hemolytic titer (HU/ml)
		Wiseman	Dawson	
Sheep	31.0	50.5	49.0	1810
Ox	30.0	45.0	44.0	1280
Man	10.2	21.6	—	256
Rabbit	7.1	19.7	—	160
Goat	14.0	38.0	40.0	113
Horse	5.5	15.0	23.0	<20
Guinea pig	2.0	16.6	—	<20
Monkey	4.1	11.4	—	<20

*% phospholipid as sphingomyelin = $(x/y)100$ when $x = \mu$g 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

Test	Incubation time (h)	% phosphorus		% nitrogen	
		Aqueous extract	Chloroform extract	Aqueous extract	Chloroform extract
Sphingomyelin control	0	0	94	0	98
	20	5	87	0	100
Sphingomyelin and beta lysin	0	0	95	0	98
	20	93	0	52	65*

*Gives color with ninhydrin.

longer found in the choloroform 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 phosphorylcholine which is present in the aqueous extract, and is in keeping with the formation of N-acyl sphingosine as the other product of hydrolysis. 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 × 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

Specificity of action of beta hemolysin

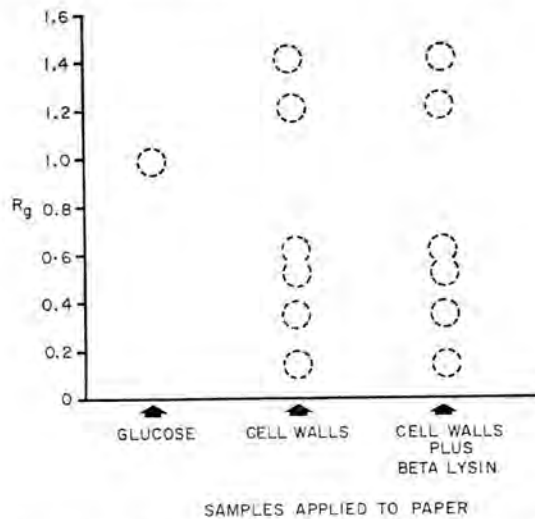| Substrate	μg P released after incubation for:		
	1 h	4 h	24 h
Phosphatidylethanolamine	0	0.6	1.0
Phosphatidylcholine	0	0.2	0.2
Ribonucleic acid	0.6	0.9	1.2
Beta glycerophosphate	0	0	0
Phenylphosphate	0.4	0.4	0.5
Sphingomyelin	3.9	10.4	25.5

SAMPLES APPLIED TO PAPER

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 I, 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 glycerophosphate, and phenylphosphate 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 glycerophosphate, 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

1. BERNHEIMER, A. W. and SCHWARTZ, L. L. 1961. Extracellular proteins of staphylococci. Proc. Soc. Exptl. Biol. Med. **106**, 776–780.

2. CHESBRO, W. R., HEYDRICK, F. P., MARTINEAU, R., and PERKINS, G. N. 1965. Purification of staphylococcal beta hemolysin and its action on staphylococcal and streptococcal cell walls. J. Bacteriol. **89**, 378–389.
3. CRUICKSHANK, R. 1965. Medical microbiology. 11th ed. Livingstone, Edinburgh and London. pp. 849–854.
4. DAWSON, R. M. C. 1964. *Cited by* L. L. M. Van Deenen and J. de Gier. *In* The red blood cell. *Edited by* C. Bishop and D. M. Surgenor. Academic Press, New York and London. p. 264.
5. DOERY, H. M., MAGNUSSON, B. J., GULASEKHARAM, J., and PEARSON, J. E. 1965. The properties of phospholipase enzymes in staphylococcal toxins. J. Gen. Microbiol. **40**, 283–296.
6. FISKE, C. H. and SUBBAROW, Y. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. **66**, 375–400.
7. JACKSON, A. W. and LITTLE, R. M. 1958. Staphylococcal toxins. III. Partial purification and some properties of delta lysin. Can. J. Microbiol. **4**, 453–461.
8. MACFARLANE, M. G. and KNIGHT, B. C. J. G. 1941. The biochemistry of bacterial toxins. I. The lecithinase activity of *Clostridium welchii* toxins. Biochem. J. **35**, 884–902.
9. MARINETTI, G. V. 1962. Chromatographic separation, identification and analysis of phosphatides. J. Lipid Res. **3**, 1–20.
10. MARKS, J. 1952. Recognition of pathogenic staphylococci: with notes on nonspecific staphylococcal hemolysin. J. Pathol. Bacteriol. **64**, 175–186.
11. HAQUE, R. and BALDWIN, J. N. 1963. Purification and properties of staphylococcal beta hemolysin. Bacteriol. Proc. **63**, 78.
12. ROBINSON, J., THATCHER, F. S., and MONTFORD, J. 1958. Studies with staphylococcal toxins. IV. The purification and metallic requirements of specific hemolysins. Can. J. Microbiol. **4**, 345–361.
13. ROSE, H. G. and OKLANDER, M. 1965. Improved procedure for the extraction of lipids from human erythrocytes. J. Lipid Res. **6**, 428–431.
14. THAYSEN, E. H. 1948. Differentiation of beta staphylolysin in two antigenically different components. Acta Pathol. Microbiol. Scand. **25**, 529–539.
15. TOLKSDORF, S., McCREADY, M. H., McCULLAGH, D. R., and SCHWENK, E. 1949. The turbidimetric assay of hyaluronidase. J. Lab. Clin. Med. **34**, 74–89.
16. TREVELYAN, W. E., PROCTER, D. P., and HARRISON, J. S. 1950. Detection of sugars on paper chromatograms. Nature, **166**, 444–445.
17. WISEMAN, G. M. 1965. Some characteristics of the beta hemolysin of *Staphylococcus aureus*. J. Pathol. Bacteriol. **89**, 187–207.

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

GORDON M. WISEMAN AND JOHN D. CAIRD (Introduced by A. M. Wallbank)

Department of Medical Microbiology, University of Manitoba Medical School, Winnipeg, Canada

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% CO_2 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

[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.

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

Erythrocyte species	Hemolytic titer (HU/ml)[a]	Total P released (%)[b]
Man	256	82
Guinea pig	64	57
Rabbit	64	54
Bovine	32	64
Goat	32	43
Sheep	32	39
Horse	16	30

[a] The titer of hemolysin, given as hemolytic units/ml, is the highest dilution which lyses 50% of the red cells.

[b] 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 serums 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 MgCl$_2$ 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 Mg^{2+} ions and EDTA. Results obtained with beta hemolysin show a 30–40% enhancement of its phospholipase activity in the presence of Mg^{2+}. The inclusion of 0.001 M EDTA resulted in a twofold inhibition of beta hemolysin activity. By contrast, neither Mg^{2+} 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

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

	P (μg) released by			
	Beta strains		Delta strains	
Additions[a]	R-1	Foggie	Newman	E-delta
None	38	44	45	30
Mg^{2+}	54	58	40	32
EDTA	20	27	39	32

[a] EDTA and Mg^{2+} 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 Mg^{2+} 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 Mg^{2+} ions.

1. Doery, H. M., Magnusson, B. J., Gulasekharam, J., and Pearson, J. E., J. Gen. Microbiol. **40**, 283 (1965).

2. Wiseman, G. M. and Caird, J. D., Can. J. Microbiol. **13**, 369 (1967).

3. Dolman, C. E. and Wilson, R. J., Can. J Public Health **31**, 68 (1940).

4. Tiselius, A., Hjerten, S., and Levin, O., Arch. Biochem. Biophys. **65**, 132 (1956).

5. Jackson, A. W. and Little, R. M., Can. J. Microbiol. **4**, 435 (1958).

6. Gladstone, G. P. and Yoshida, A., Brit. J. Exptl. Pathol. **48**, 11 (1967).

7. Gladstone, G. P. and van Heyningen, W. E., Brit. J. Exptl. Pathol. **38**, 123 (1957).

8. Rose, H. G. and Oklander, M., J. Lipid Res., **6**, 428 (1965).

9. Wiseman, G. M., J. Pathol. Bacteriol **89**, 187 (1965).

10. Fiske, C. H. and Subbarow, Y., J. Biol. Chem. **66**, 375 (1925).

11. Sizer, I. W., Advan. Enzymol. **3**, 35 (1943).

12. Marinetti, G. V., J. Lipid Res. **3**, 1 (1962).

13. Nelson, G. J., Biochim. Biophys. Acta **144**, 221 (1967).

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

G. M. WISEMAN

Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba

Received July 13, 1967

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.

Recent work suggests that the beta hemolysin of *Staphylococcus aureus* is an enzyme which hydrolyzes sphingomyelin (2, 13), liberating N-acyl sphingosine and phosphorylcholine. 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 mμ. 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* cells 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-
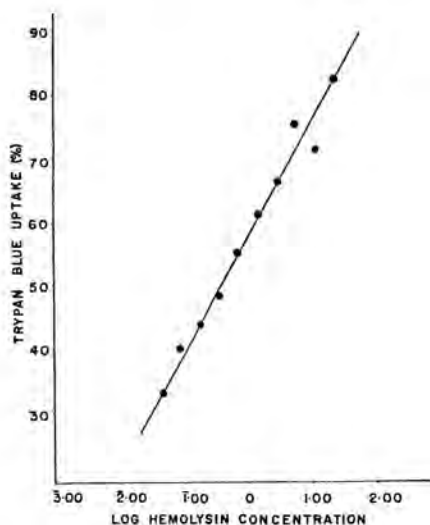

FIG. 1. Effect of beta hemolysin A concentration on trypan blue uptake by human amnion cells. Points on the graph are the means of two determinations.

TABLE I

Effect of beta hemolysin A on phagocytosis of staphylococci by leucocytes

	Sheep leucocytes			Rabbit leucocytes		
Fraction	Total counted	% with cocci	Dye reduction	Total counted	% with cocci	Dye reduction
Control	400	97	+	480	41	+
Purified beta hemolysin A	382	95	−	495	42	−
Crude		lysis	−		lysis	−

NOTE: The data represent the means of two experiments. The dye used was dichlorophenol–indophenol.

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.

1. CARPENTER. P. L. 1965. Immunology and serology. 2nd ed. Saunders, Philadelphia. p. 410.
2. DOERY, H. M., MAGNUSSON, B. J., GULASEKHARAM, J., and PEARSON, J. E. 1965. The properties of phospholipase enzymes in staphylococcal toxins. J. Gen. Microbiol. 40: 283–296.
3. EAGLE, H., HABEL, K., ROWE, W. P., and HUEBNER, R. J. 1956. Viral susceptibility of a human carcinoma cell (strain KB). Proc. Soc. Exptl. Biol. Med. 91: 361–364.
4. EDWARDS, S. W. and BALL, E. G. 1954. The action of phospholipases on succinate oxidase and cytochrome oxidase. J. Biol. Chem. 209: 619–633.
5. GLENNY, A. T. and STEVENS, M. F. 1935. *Staphylococcus* toxins and antitoxins. J. Pathol. Bacteriol. 40: 201–210.
6. HAQUE, R. and BALDWIN, J. N. 1963. Purification and properties of staphylococcal beta hemolysin. Bacteriol. Proc. 63: 78.
7. MAHESWARAN, S. K., SMITH, K. L., and LINDORFER, R. K. 1967. Staphylococcal beta hemolysin. I. Purification of beta hemolysin. J. Bacteriol. 94: 300–305.
8. McLIMANS, W. F., DAVIS, E. V., GLOVER, F. L., and RAKE, G. W. 1957. The submerged culture of mammalian cells: the spinner culture. J. Immunol. 79: 428–433.
9. ROBINSON, J., THATCHER, F. S., and MONTFORD, J. 1958. Studies with staphylococcal toxins. IV. The purification and metallic requirements of specific hemolysins. Can. J. Microbiol. 4: 345–361.
10. SYVERTON, J. T., SCHERER, W. F., and ELWOOD, P. M. 1954. Studies on the propagation *in vitro* of poliomyelitis viruses. V. The application of strain HeLa human epithelial cells for isolation and typing. J. Lab. Clin. Med. 43: 286–302.
11. THAYSEN, E. H. 1948. Differentiation of beta staphylolysin in two antigenically different components. Acta Pathol. Microbiol. Scand. 25: 529–539.
12. WILT, J. C., MILLER, D., and RUITER, J. 1964. Human amnion for tissue culture. Can. J. Microbiol. 10: 169–174.
13. WISEMAN, G. M. and CAIRD, J. D. 1967. The nature of staphylococcal beta hemolysin. I. Mode of action. Can. J. Microbiol. 13: 369–376.
14. WISEMAN, G. M. 1965. Some characteristics of the beta hemolysin of *Staphylococcus aureus*. J. Pathol. Bacteriol. 89: 187–207.
15. WOODIN, A. M. 1959. Fractionation of a leucocidin from *Staphylococcus aureus*. Biochem. J. 73: 225–237.

CHAPTER 6

The Beta- and Delta-Toxins of *Staphylococcus aureus*

Gordon M. Wiseman

I. Beta-Toxin

Glenny and Stevens (1935), investigating two preparations of α-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 α-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 phenolindo-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

EFFECT OF GASEOUS ENVIRONMENT ON β-TOXIN PRODUCTION[a]

Strain	Gas mixture	Growth (O.D. 650 mμ)	Sheep red cell titer (HU/ml)[b]
252F	Air	0.84	1,016
	CO_2-oxygen	0.76	1,280
	CO_2-air	0.91	2,560
R-1	Air	0.58	2,560
	CO_2-oxygen	0.43	20,480
	CO_2-air	0.59	32,510
P-92	Air	0.58	5,120
	CO_2-oxygen	0.51	16,260
	CO_2-air	0.54	65,010
G-128	Air	0.62	20,480
	CO_2-oxygen	0.48	16,260
	CO_2-air	0.64	130,100

[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 Mg^{2+}.

bon 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

TABLE II
EFFECT OF pH ON PRODUCTION OF β-TOXIN ON SOLID MEDIUM

Buffer	pH	Growth (O.D. 650 mμ)	Sheep red cell titer (HU/ml)[a]
Acetate	5.0	0	<20
Phosphate	6.0	0.75	81,920
Phosphate	7.0	0.87	130,000
Borate	8.0	0.36	10,240
Borate	9.0	0	<20

[a] 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
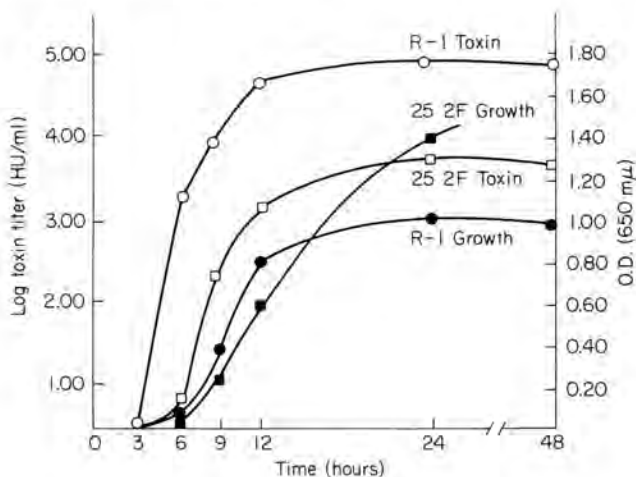

FIG. 1. Rate of β-toxin formation with respect to growth of strains R-1 and 252F. The toxins were produced on solid media and titrated in the presence of sheep erythrocytes and 0.001 M Mg^{2+}. The gaseous environment was 25% carbon dioxide in air. (Redrawn from Wiseman, 1963.)

of the same study, Wiseman also investigated amino acid requirements of growth and β-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 III

NUTRITIONAL REQUIREMENTS OF *Staphylococcus aureus* STRAIN R-1

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

[a]Optical 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.

ted. 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 Zn^{2+} 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. Wiseman 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 carboxymethylcellulose 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 β-TOXIN

Robinson et al. (1958)		Jackson (1963)		Wiseman and Caird (1967)		Maheswaran et al. (1967)	
Step	Specific activity	Step	Specific activity	Step	Specific activity	Step	Specific activity
Crude	1	Crude	1	Crude	1	Crude	1
Ethanol precipitation	15	Zinc–ethanol precipitated	3	Hydroxyapatite	20	Ammonium sulfate precipitated	23
Calcium phosphate treatment	30	Zinc–mercuric acetate precipitated	10	Acetate precipitated	35	Sephadex G-100	46
Starch electrophoresis	670	Hydroxyapatite	255	Sephadex G-100	70	Carboxymethylcellulose	245

dient 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 β-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 β-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 β-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 β-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 β-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 β-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 β-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 β-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 β-toxin. Wiseman (1965a) has tested the sensitivity of 13 species of erythrocytes to beta toxin from the R-1 and 252F strains of *S. aureus* (Table V) in the presence of 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 β-toxin derived from their Parisi strain. There was some difference in their method of titration

TABLE V

Spectrum of Erythrocyte Sensitivity to Partially Purified
β-Toxins of Strains R-1 and 252F

Erythrocyte species	Hemolytic titers (HU/ml) of β-toxins from strains	
	R-1	252F
Horse	<4	<4
Guinea pig	<4	<4
Dog	<4[a]	<4
Frog	<4[a]	<4[a]
Mouse	<4[a]	<4[a]
Rat	<4[a]	<4[a]
Fowl	<4[a]	<4[a]
Pig	32	32
Rabbit	64	32
Cat	64	64
Man	256	128
Ox	512	512
Sheep	2048	512

[a] Slight hemolysis, less than 50%, was observed at these dilutions. Titrations were performed in the presence of 0.001 M 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.

TABLE VI

INFLUENCE OF ADDITION OF 0.001 M Mg^{2+} ON HEMOLYTIC TITERS OF
β-TOXINS FROM DIFFERENT STRAINS OF *Staphylococcus aureus*

		Hemolytic Titers (HU/ml) with cells of		
Strains[a]	Mg^{2+} added	Rabbit	Man	Sheep
P-92	−	<20	<20	20
	+	20	160	1,280
G-128	−	<20	<20	80
	+	40	640	2,560
R-1	−	20	40	160
	+	160	320	10,240
252F	−	<20	20	40
	+	20	80	320

[a] Partially purified β-toxin from these strains was tested.

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 β-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 β-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 β-toxin lysed human red cells.

3. EFFECT OF METAL IONS

Jackson and Mayman (1958) showed that inactivation was the result of dialysis of β-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 β-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 β-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 VII

EFFECT OF METAL CATIONS ON HEMOLYTIC ACTIVITY OF β-TOXIN

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

[a] Robinson et al. (1958). Activity was expressed as hemin (μg)/protein (μg).

[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.

thors 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 β-toxin was an enzyme. There is no record in the literature of anions enhancing hemolytic activity of the β-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 α- and β-toxins, Doery et al. (1963) found that all hydrolyzed sphingomyelin, a common constituent of cell membranes. Ten strains producing only α-toxin had no effect on this phospholipid. Furthermore, the distribution of curtain electrophoretic fractions corresponded with those containing β-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 β-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 Rf value as an authentic sample of phosphorycholine chromatographed simultaneously. This led them to suggest the following reaction:

$$\text{Sphingomyelin} + \text{water} \xrightarrow{\ \beta\text{-toxin}\ } N\text{-acylsphingosine} + \text{phosphorylcholine}$$
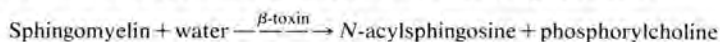
Wiseman and Caird (1966, 1967) and Maheswaran and Lindorfer (1966, 1967) were able to confirm Doery's results. The data of Wiseman and Caird (Table VIII) show that phosphorus and nitrogen were recovered quantitatively in the chloroform extract of samples initially collected in the presence and absence 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

END PRODUCT ANALYSIS OF THE REACTION OF β-TOXIN WITH SPHINGOMYELIN[a]

Test	Incubation time (hours)	Phosphorus (%)		Nitrogen (%)	
		Aqueous extract	Chloroform extract	Aqueous extract	Chloroform extract
Sphingomyelin control	0	0	94	0	98
	20	5	87	0	100
Sphingomyelin + β-toxin	0	0	95	0	98
	20	93	0	52	65

[a] After Wiseman and Caird (1967).

choline. The mode of action of the β-toxin strongly suggests that it is similar to phospholipase C. *Clostridium perfringens* α-toxin, which is a phospholipase C. preferentially hydrolyzes phosphatidylcholine. resulting in the release of phosphorylcholine and a diglyceride, but it does not attack phenylphosphate, β-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 β-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 β-toxin is activated by Co^{2+} and Mg^{2+}, Co^{2+} being more effective than Mg^{2+}. Calcium ions inhibited the reaction. Using sphingomyelin as substrate in the presence of toxin, Maheswaran and Lindorfer (1967) confirmed that 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 β-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 β-toxin attacks sphingomyelin *in vivo*. Normal rabbit serum *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 β-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 β_2. He claimed that the β_2-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 β_2-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 IX
INCIDENCE OF HEMOLYSINS IN 359 STRAINS OF STAPHYLOCOCCI[a]

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

[a] 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

lysosomes. 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.

TABLE X

EFFECT OF CARBON DIOXIDE CONCENTRATION IN AIR OR OXYGEN UPON PRODUCTION
OF δ-TOXIN BY THE E-DELTA STRAIN OF *S. aureus*[a]

	Air			Oxygen		
		Hemolytic titer (HU/ml)			Hemolytic titer (HU/ml)	
Carbon dioxide concentration (%)	Growth (O.D. 650 mμ)	α	δ	Growth (O.D. 650 mμ)	α	δ
0	0.50	100	50	—	—	—
25	0.49	200	40	0.41	160	40
35	0.51	400	40	0.32	200	40
50	0.67	2560	80	0.42	400	50
75	0.54	1590	40	0.53	640	80

[a] 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 Ouchterlony plates in the presence of antiserum. 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 re-dissolved 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. Disk electrophoresis preparations of the toxin also revealed only one strong stained band of protein.

C. CHARACTERISTICS

1. 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 lyses all spe-

TABLE XI
HEMOLYTIC SPECTRUM OF STAPHYLOCOCCAL TOXINS[a]

| | Sensitivity of red cells (HU/ml) to | | | |
| | β-Toxin | | δ-Toxin | |
Red cells	R-1	252F	Newman	E-delta
Man	512	128	512	512
Monkey	<16	—	—	256
Rabbit	64	32	128	128
Sheep	2048	512	128	64
Ox	512	512	64	64
Guinea pig	<4	<4	32	64
Horse	<4	<4	8	16

[a] 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).

cies, whereas monkey, ox, and guinea pig cells are resistant to β-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 δ-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; γ-globulin at a concentration of 1% was the most effective. Gladstone and Yoshida (1967) found that fibrinogen, α- and β-globulins, and mucoproteins markedly inhibited action of the toxin, while partial inhibition was achieved with γ-globulin and albumin (Table XII).

Wiseman and Caird (1968) also noted that δ-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 γ-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 β-toxin, metal cations have not been shown to enhance the activity of δ-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[a]

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

[a] After 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-
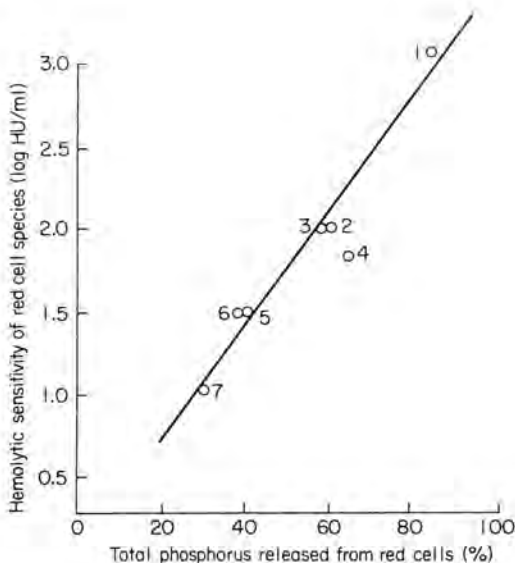

FIG. 2. The hemolytic sensitivity in hemolytic units per milliliter of all red cell species tested was plotted against the amount of acid-soluble phosphorus released from these species in the presence of δ-toxin. The linear correlation between the two phenomena is strong evidence that hemolysis is due 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.

TABLE XIII
SPECIFICITY OF THE ACTION OF δ-TOXIN ON PHOSPHOLIPIDS

Substrate	Incubation time (minutes)	Phosphorus released[a] (μg)
Sphingomyelin	60	0
Phosphatidylcholine	30	0.5
	60	0.7
Phosphatidylserine	30	0.9
	60	2.3
Phosphatidylinositol	30	3.0
	60	19.5
Toxin control	60	0

[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:

$$\text{Phosphatidylinositol} \longrightarrow \text{inositol phosphate} + \text{diglyceride}$$

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 δ-toxin is not antigenic.

Nothing is known of the *in vivo* activity of δ-toxin. It has been suggested by various authors that δ-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. aureus* strains from human sources, the association of δ-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 δ-toxin. Joiris (1952) has also noted the close association of δ-toxin production with strains isolated from lesions in man. As with other staphylococcal toxins, it is nevertheless hard to ascribe a particular role to δ-toxin in the pathogenesis of staphylococcal disease.

Recently, Hoffmann and Streitfeld (1965) found that partially purified preparations of δ-toxin inhibited growth of several species of gram-positive bacteria including *S. 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

Bergmann, F., Gutman, J., and Chaimovitz, M. (1965). *Proc. 2nd Intern. Pharmacol. Meeting, Prague, 1963* Vol. 9, p. 221. Pergamon Press, Oxford.
Bernheimer, A. W., and Schwartz, L. L. (1964). *J. Bacteriol.* 87, 1100.
Bernheimer, A. W., and Schwartz, L. L. (1965). *J. Pathol. Bacteriol.* 89, 209.
Bigger, J. W., Boland, C. R., and O'Meara, R. A. Q. (1927). *J. Pathol. Bacteriol.* 30, 271.
Birch-Hirschfeld, L. (1933). *Z. Immunitaetsforsch.* 81, 260.
Bryce, L. M., and Rountree, P. M. (1936). *J. Pathol. Bacteriol.* 43, 173.
Burns, J., and Holtman, D. F. (1960). *Ann. N.Y. Acad. Sci.* 88, 1115.
Chesbro, W. R., Heydrick, F. P., Martineau, R., and Perkins, G. N. (1965). *J. Bacteriol.* 89, 378.
Corkill, J. M. B. (1955). *Can. J. Public Health* 46, 243.
Doery, H. M., Magnusson, B. J., Cheyne, I. M., and Gulasekharam, J. (1963). *Nature* 198, 1091.
Doery, H. M., Magnusson, B. J., Gulasekharam, J., and Pearson, J. E. (1965). *J. Gen. Microbiol.* 40, 283.
Dolman, C. E., and Wilson, R. J. (1940). *Can J. Public Health* 31, 68.
Edwards, S. W., and Ball, E. G. (1954). *J. Biol. Chem.* 209, 619.

Elek, S. D. (1959). "*Staphylococcus pyogenes* and its Relation to Disease." Livingstone, Edinburgh and London.

Elek, S. D., and Levy, E. (1950). *J. Pathol. Bacteriol.* 62, 541.

Flamm, H. (1957). *Schweiz. Z. Allgem. Pathol. Bakteriol.* 20, 358.

Fulton, F. (1943). *Brit. J. Exptl. Pathol.* 24, 65.

Gladstone, G. P. (1938). *Brit. J. Exptl. Pathol.* 19, 208.

Gladstone, G. P. (1966). *Postepy Mikrobiol.* 5, 145.

Gladstone, G. P., and van Heyningen, W. E. (1957). *Brit. J. Exptl. Pathol.* 38, 123.

Gladstone, G. P., and Yoshida, A. (1967). *Brit. J. Exptl. Pathol.* 48, 11.

Glenny, A. T., and Stevens, M. F. (1935). *J. Pathol. Bacteriol.* 40, 201.

Hallander, H. O. (1963). *Acta Pathol. Microbiol. Scand.* 59, 543.

Hallander, H. O., and Bengtsson, S. (1967). *Acta Pathol. Microbiol. Scand.* 70, 107.

Haque, R., and Baldwin, J. N. (1963). *Bacteriol. Proc.* p. 78.

Haque, R., and Baldwin, J. N. (1964). *J. Bacteriol.* 88, 1304.

Heydrick, F. P., and Chesbro, W. R. (1962). *Bacteriol. Proc.* p. 63.

Hoffmann, F. M., and Streitfeld, M. M. (1965). *Can. J. Microbiol.* 11, 203.

Jackson, A. W. (1963). *Can. J. Biochem. Physiol.* 41, 755.

Jackson, A. W., and Little, R. M. (1956). *Bacteriol. Proc.* p. 88.

Jackson, A. W., and Little, R. M. (1957). *Can. J. Microbiol.* 3, 101.

Jackson, A. W., and Little, R. M. (1958a). *Can. J. Microbiol.* 4, 435.

Jackson, A. W., and Little, R. M. (1958b). *Can. J. Microbiol.* 4, 453.

Jackson, A. W., and Mayman, D. (1958). *Can. J. Microbiol.* 4, 477.

Jeljaszewicz, J., Szmigielski, S., Korbecki, M., and Zak, C. (1965). *J. Infect. Diseases* 115, 421.

Joiris, E. (1952). *Rev. Belge Pathol.* 22, 185.

Kapral, F. A. (1967). *Bacteriol. Proc.* p. 64.

Kodama, T., and Kojima, T. (1939). *Kitasato Arch. Exptl. Med.* 16, 36.

Korbecki, M., and Jeljaszewicz, J. (1965). *J. Infect. Diseases* 115, 205.

Maheswaran, S. K., and Lindorfer, R. K. (1966). *Bacteriol. Proc.* p. 44.

Maheswaran, S. K., and Lindorfer, R. K. (1967). *J. Bacteriol.* 94, 1313.

Maheswaran, S. K., Smith, K. L., and Lindorfer, R. K. (1967). *J. Bacteriol.* 94, 300.

Marandon, J., and Oeding, P. (1966). *Acta Pathol. Microbiol. Scand.* 67, 149.

Marks, J., and Vaughan, A. C. T. (1950). *J. Pathol. Bacteriol.* 62, 597.

Murphy, R. A., and Haque, R. (1967). *J. Bacteriol.* 94, 1327.

Ponder, E. H. (1948). "Hemolysis and Related Phenomena." Grune & Stratton, New York.

Pulsford, M. F. (1954). *Australian J. Exptl. Biol. Med. Sci.* 32, 347.

Pulvertaft, R. J. V., Greening, J. R., and Haynes, J. A. (1947). *J. Pathol. Bacteriol.* 59, 293.

Robinson, J., Thatcher, F. S., and Gagnon, J. (1958). *Can. J. Microbiol.* 4, 345.

Rountree, P. M. (1947). *Australian J. Exptl. Biol. Med. Sci.* 25, 359.

Roy, T. E. (1937). *J. Immunol.* 33, 437.

Slanetz, L. W., and Bartley, C. H. (1953). *J. Infect. Diseases* 92, 139.

Smith, I. M. (1965). *Ann. N.Y. Acad. Sci.* 128, 334.

Stamatin, N., Tacu, A., and Marica, D. (1949). *Ann. Inst. Pasteur* 76, 178.

Thatcher, F. S., and Matheson, B. H. (1955). *Can. J. Microbiol.* 1, 382.

Thaysen, E. H. (1948). *Acta Pathol. Microbiol. Scand.* 25, 529.

van Heyningen, W. E. (1950). "Bacterial Toxins." Blackwell, Oxford.

Williams, R. E. O., and Harper, G. J. (1947). *J. Pathol. Bacteriol.* 59, 69.

Winkler, K. C., de Waart, J., Grootsen, C., Zegers, B. J., Tellier, N. F., and Vertregt, C. D. (1965). *J. Gen. Microbiol.* 39, 321.

Wiseman, G. M. (1963). Ph.D. Thesis, University of Edinburgh.

Wiseman, G. M. (1965a). *J. Pathol. Bacteriol.* 89, 187.
Wiseman, G. M. (1965b). *Can. J. Microbiol.* 11, 463.
Wiseman, G. M. (1968). *Can. J. Microbiol.* 14, 179.
Wiseman, G. M., and Caird, J. D. (1966). *Can. Soc. Microbiol. Abstracts,* p. 16.
Wiseman, G. M., and Caird, J. D. (1967). *Can. J. Microbiol.* 13, 369.
Wiseman, G. M., and Caird, J. D. (1968). *Proc. Soc. Exptl. Biol. Med.* 128, 428.
Yoshida, A. (1963). *Biochim. Biophys. Acta* 71, 544.

Mode of action of the alpha toxin of
Staphylococcus aureus

G. M. WISEMAN AND J. D. CAIRD

Volume 16 · Number 1 · 1970

*please photostat this one
only.*

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

G. M. Wiseman and J. D. Caird

Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba

Received September 15, 1969

Wiseman, G. M., and J. D. Caird. 1970. Mode of action of the alpha toxin of *Staphylococcus aureus*. Can. J. Microbiol. **16**: 47–50.

Rabbit erythrocytes treated with the alpha toxin of *Staphylococcus aureus*, strain "Wood-46", liberate substances which contain nitrogen, absorb at 280 mμ, 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 natural membranes is questionable.

In the present study we have found that alpha toxin liberates from red cell membranes, substances which absorb at 280 mμ (millimicrons) or at 750 mμ 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 0.10 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 hydrolysis 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

	Volume (ml) in tubes						
Reagents added	1	2	3	4	5	6	7
Denatured hemoglobin	5.0	5.0	0	0	5.0	5.0	0
Erythrocyte proteolytic enzyme*	1.5	1.5	1.5	1.5	0	0	0
Alpha toxin†	1.0	0	1.0	0	1.0	0	1.0
0.01 M phosphate saline buffer pH 7.0	7.5	8.5	12.5	13.5	9.0	10.0	14.0

*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

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

*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 mμ or at 750 mμ 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 mμ. However, the presence of nucleotides or nucleosides in our test systems and their contribution to the optical density determinations at 280 mμ 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 mμ was determined at the final concentration used in the tests. No absorption at 280 mμ 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.
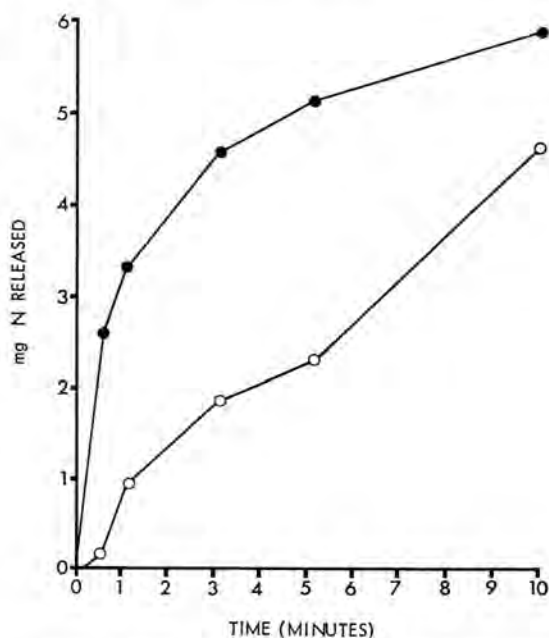

FIG. 1. Effect of alpha toxin upon intact cells and lipid-free membrane preparations of rabbit erythrocytes. ● Intact erythrocytes ○ Lipid-free protein.

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.
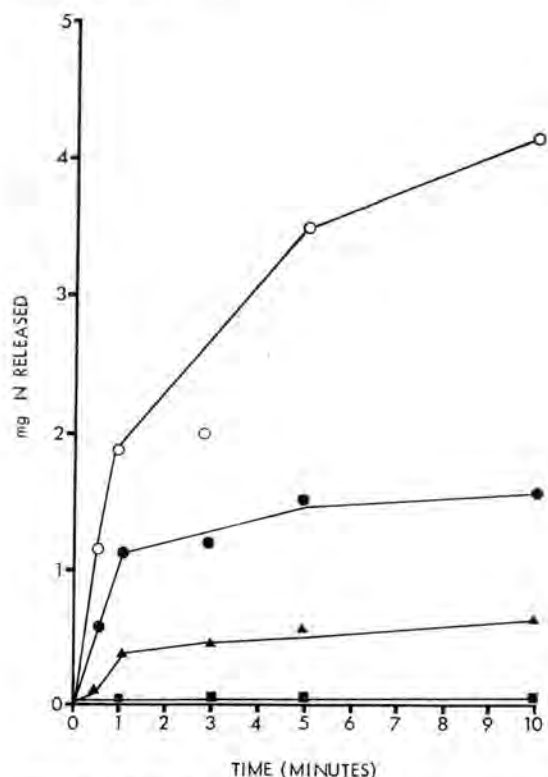

FIG. 2. Effect of alpha toxin and rabbit erythrocyte proteases upon urea-denatured hemoglobin. Erythrocyte proteases were present as a cell-free KCNS extract. ■ Alpha toxin and hemoglobin. ▲ Alpha toxin and KCNS extract. ● KCNS extract and hemoglobin. ○ Combined alpha toxin, KCNS extract, and hemoglobin.

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 mμ or at 750 mμ 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 mμ, as we have already pointed out.

Acknowledgments

1. BERNHEIMER, A. W. 1965. Staphylococcal alpha toxin. Ann. N.Y. Acad. Sci. **128**: 112–123.
2. BERNHEIMER, A. W., and L. L. SCHWARTZ. 1963. Isolation and composition of staphylococcal alpha toxin. J. Gen. Microbiol. **30**: 455–468.
3. CARPENTER, P. L. 1965. Immunology and serology. 2nd ed. Saunders, Philadelphia. p. 410.
4. COULTER, J. R. 1966. Production, purification, and composition of staphylococcal α-toxin. J. Bacteriol. **92**: 1655–1662.
5. EDELWEJN, Z., J. JELJASZEWICZ, S. SZMIGIELSKI, and C. ZAK. 1968. Brain bioelectric activity in staphylococcal α-hemolysin intoxication. Toxicol. Appl. Pharmacol. **13**: 133–145.
6. ELEK, S. D. 1959. *Staphylococcus pyogenes* and its relation to disease. Livingstone, Edinburgh and London. p. 231.
7. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265–275.
8. MORRISON, W. L., and H. NEURATH. 1953. Proteolytic enzymes of the formed elements of human blood. I. Erythrocytes. J. Biol. Chem. **200**: 39–51.
9. RAHAL, J. J., M. E. PLAUT, H. ROSEN, and L. WEINSTEIN. 1967. Purified staphylococcal alpha toxin: effect on epithelial ion transport. Science, **155**: 1118–1120.
10. ROBINSON, J., and F. S. THATCHER. 1963. Studies with staphylococcal toxins. VII. Separation of a proteolytic enzyme from alpha hemolysin. Can. J. Microbiol. **9**: 697–702.
11. ROSENBERG, S. A., and G. GUIDOTTI. 1968. The protein of human erythrocyte membranes. I. Preparation, solubilization, and partial characterization. J. Biol. Chem. **243**: 1985–1992.
12. SANGER, F. 1945. The free amino groups of insulin. Biochem. J. **39**: 507–515.
13. WEISSMANN, G., G. SESSA, and A. W. BERNHEIMER. 1966. Staphylococcal alpha toxin: effects on artificial lipid spherules. Science, **154**: 772–774.
14. WISEMAN, G. M. 1965. Some characteristics of the beta hemolysin of *Staphylococcus aureus*. J. Pathol. Bacteriol. **89**: 187–207.

Purification of the delta toxin of
Staphylococcus aureus

J. D. CAIRD AND G. M. WISEMAN

Purification of the delta toxin of *Staphylococcus aureus*

J. D. Caird and G. M. Wiseman

Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba

Received February 23, 1970

Caird, J. D., and G. M. Wiseman. 1970. Purification of the delta toxin of *Staphylococcus aureus*. Can. J. Microbiol. **16**: 703–708.

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 obtained 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). Immunoelectrophoresis was performed on 2.5 × 7.5 cm glass microscope slides which were layered with 3 ml of

TABLE I

Effect of pH on activity of delta toxin

Buffer	pH	Activity, HU/ml of:[†]		
		precipitation*	precipitate	supernatant fluid
Acetate	4.0	+++	2560	<20
„	4.5	+++	2560	40
„	5.0	+++	1280	320
„	5.5	+++	1280	640
Phosphate	6.0	++	640	640
„	6.5	++	640	1280
„	7.0	+	160	1280
„	7.5	+	160	1280
„	8.0	+	160	1280
Tris-HCl	8.5	±	80	2560
„	9.0	—	<20	2560

*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 *t*-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 II
Ammonium sulphate as a precipitant of delta toxin

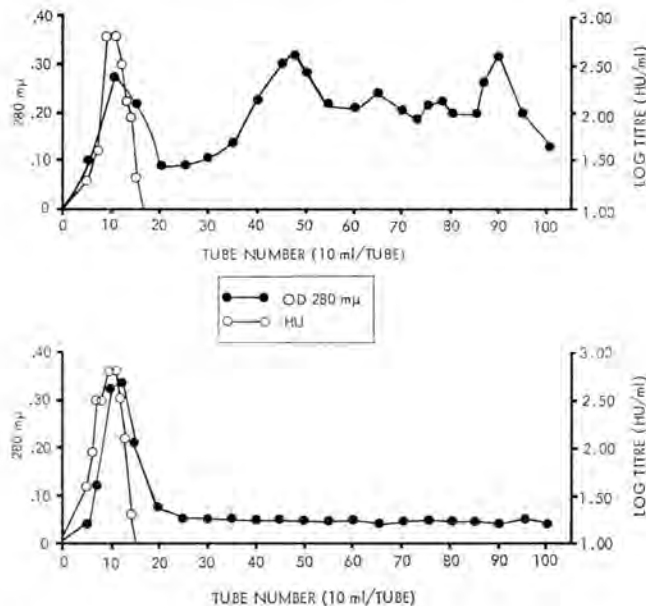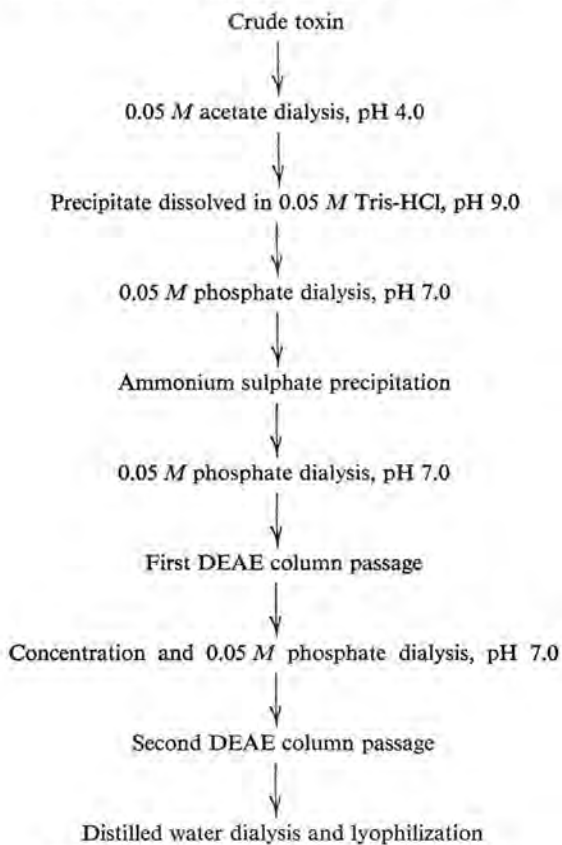| % salt	Hemolytic activity, HU/ml	Protein, µg/ml	Volume, ml	% recovery	Specific activity
0	1024	770	100	100	1.3
15	256	610	8	2	0.4
30	256	455	15	4	0.6
45	2048	425	26	52	4.9
60	256	570	15	4	0.5
75	64	440	5	0.3	0.1
90	16	360	5	1	0.04

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 mµ.

cipitated 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 20 000 *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. 1, 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

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

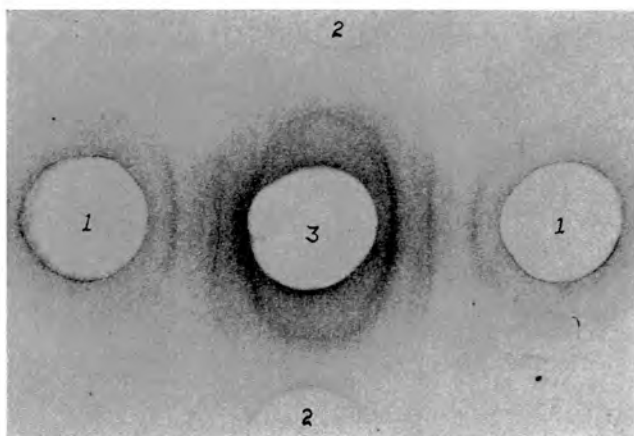*Hemolysin titrations and protein values were the means of two determinations.

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.
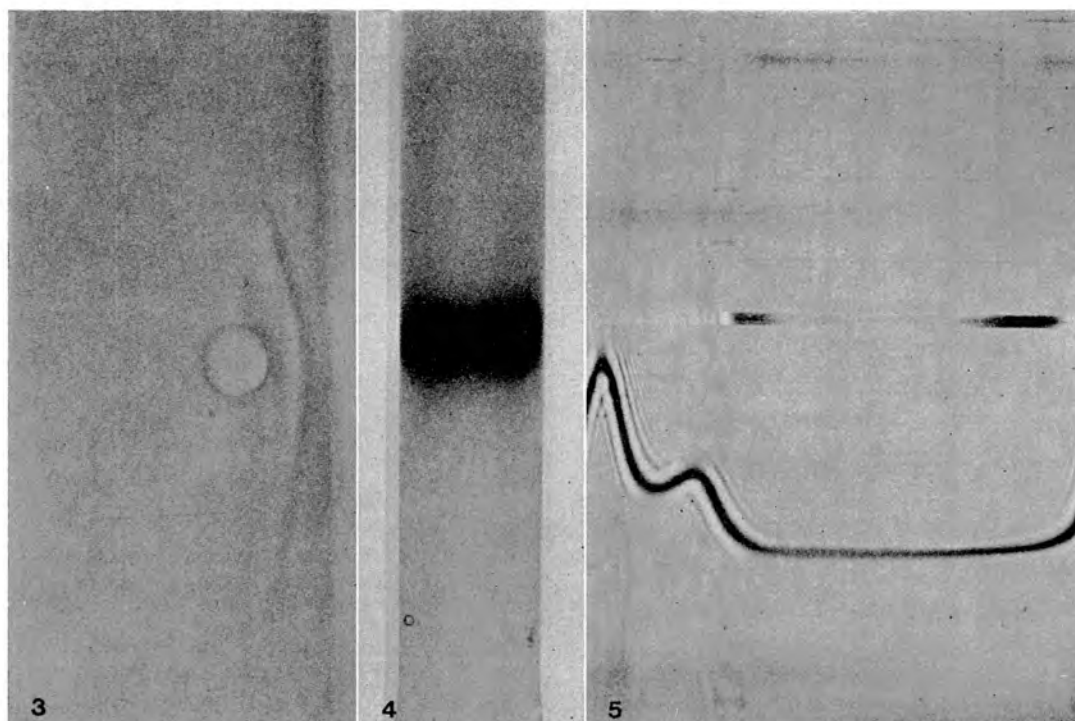

FIG. 3.   Immunoelectrophoresis of purified delta toxin. Toxin was contained in upper well and antiserum in lower trough.

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.

1. PROLINE
2. DELTA TOXIN HYDROLYSATE
3. PHENYLALANINE

FIG. 6. N-Terminal analysis of hydrolysate of purified delta toxin. Dark line at top of figure is the solvent front. Spot not encircled with pencil and which migrates with solvent front is free dinitrophenol.

pooled, concentrated by freeze-drying, and applied to a second DEAE column identical with the first. A single hemolytic protein peak was detected after the second column treatment as seen in Fig. 1. The active toxin was dialyzed against distilled water and freeze-dried.

An outline of the entire procedure is given in Scheme 1. The efficiency of the procedure for delta toxin is summarized in Table III. The overall increase in specific activity is 32-fold with a 16% recovery of toxin.

Tests of Homogeneity

Several criteria of purity were applied to the delta toxin. As observed in Fig. 2, immunodiffusion studies with crude toxin showed several strong and several weak lines of precipitation in contrast with purified material which showed one line. It is not clear whether an additional faint line appears adjacent to this single line obtained with the purified toxin. Protein concentration in the wells was 6 mg/ml. One line of precipitation was also observed when purified toxin (6 mg/ml) was subjected to immunoelectrophoresis as shown in Fig. 3. Some stained material appears above and adjacent to the single precipitation line and as with Fig. 2, we are not certain if this represents contamination.

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 immuno-diffusion 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 electrofocusing, 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 lyses 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).

1. ARBUTHNOTT, J. P., J. H. FREER, and A. W. BERNHEIMER. 1967. Physical states of staphylococcal alpha toxin. J. Bacteriol. **94**: 1170–1177.
2. BLACKBURN, S., and A. G. LOWTHER. 1951. The separation of N-2-4 dinitrophenylamino acids on paper chromatograms. Biochem. J. **48**: 126–128.
3. COULTER, J. R. 1966. Production, purification and composition of staphylococcal alpha toxin. J. Bacteriol. **92**: 1655–1662.
4. CRUICKSHANK, R. 1965. Medical microbiology. 11th ed. E. & S. Livingstone, Ltd., Edinburgh and London. pp. 849–854.
5. FRAENKEL-CONRAT, H., J. I. HARRIS, and A. L. LEVY. 1955. Recent developments in techniques for terminal and sequence studies in peptides and proteins. Methods Biochem. Anal. **2**: 359–425.
6. GLADSTONE, G. P., and A. YOSHIDA. 1967. The cytopathic action of purified staphylococcal delta hemolysin. Brit. J. Exp. Pathol. **48**: 11–19.
7. JACKSON, A. W., and R. M. LITTLE. 1958. Staphylococcal toxins. III. Partial purification and some properties of delta lysin. Can. J. Microbiol. **4**: 453–461.
8. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265–275.
9. MARKS, J., and A. C. T. VAUGHAN. 1950. Staphylococcal delta hemolysin. J. Pathol. Bacteriol. **62**: 597–615.
10. NOLL, H. 1966. Chain initiation and control of protein synthesis. Science, **151**: 1241–1245.
11. ORNSTEIN, L., and B. J. DAVIES. 1962. Disc electrophoresis. Reprinted by Distillation Products Industries, Rochester, N.Y.
12. OUCHTERLONY, O. 1958. Diffusion-in-gel method for immunological analysis. Progr. Allergy, **5**: 1.
13. WADSTROM, T. 1968. Studies on extracellular proteins from *Staphylococcus aureus*. IV. Separation of alpha toxin by isoelectric focusing. **168**: 228.
14. WILLIAMS, R. E. O., and G. J. HARPER. 1947. Staphylococcal hemolysins on sheep blood agar with evidence for a fourth hemolysin. J. Pathol. Bacteriol. **59**: 69–78.
15. WISEMAN, G. M. 1963. Studies with the beta hemolysin of *Staphylococcus aureus*. Ph.D. Thesis, University of Edinburgh.
16. WISEMAN, G. M. 1965. Some characteristics of the beta hemolysin of *Staphylococcus aureus*. J. Pathol. Bacteriol. **89**: 187–207.
17. WISEMAN, G. M., and J. D. CAIRD. 1968. Phospholipase activity of the delta hemolysin of *Staphylococcus aureus*. Proc. Soc. Exp. Biol. Med. **128**: 428–430.
18. YOSHIDA, A. 1963. Staphylococcal delta hemolysin. I. Purification and chemical properties. Biochim. Biophys. Acta, **71**: 544–553.

Canadian Journal of Microbiology

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

G. M. WISEMAN AND J. D. CAIRD

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 *Staphylococcus aureus* "Wood-46." Can. J. Microbiol. **18**: 987–992.

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* "Wood-46." Can. J. Microbiol. **18**: 987–992.

Des résultats nouveaux indiquent que la toxine alpha de *Staphylococcus aureus* (souche "Wood 46") est une protéase qui doit être activée par des protéases de la membrane érythrocytaire. Un sérum anti-protéase érythrocytaire de lapin, obtenu de souris, empêche la toxine 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 hémolytique et protéolytique. Ces propriétés sont neutralisées par l'antitoxine alpha préparée avec des lapins, mais non pas par des sérums 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 sérums de con-trôle. 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 hémolytique 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 variation 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 prepared 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).

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 centrifuged 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 spectrophotometer and plotted against the $\log_{10}$ 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

TABLE 1

Relationship of toxin adsorption to erythrocyte hemolytic sensitivity

Toxin incubated with ghosts of:	Hemolytic titer (HU/ml) of supernatant titrated against:	
	Own erythrocytes	Rabbit erythrocytes
Rabbit	256	32
Sheep	45	90
Guinea pig	16	128
Horse	8	256
Man	8	256

TABLE 2

Recovery of alpha toxin from ghost supernatant fluid

Fractions	Total hemolytic activity, HU
Initial toxin control	62 000
Initial toxin control + EDTA	62 000
Toxin-ghost supernatant	35 520
Supernatant + EDTA	114 000
Dialyzed supernatant-EDTA solution	52 500

prepared 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
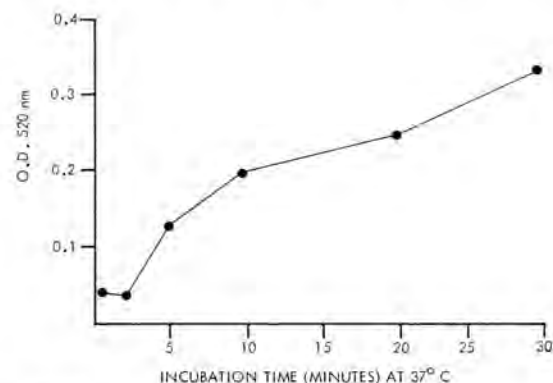

FIG. 1. Liberation of color from azocoll by a suspension of rabbit ghosts.
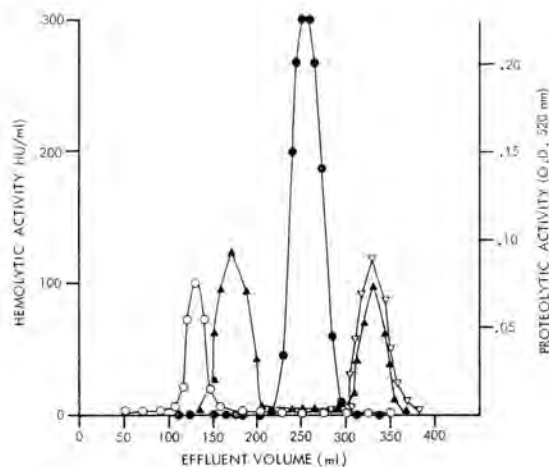

FIG. 2. Effluent patterns of EDTA toxin-ghost supernatant fluids and controls on Sephadex G-75 columns. (O), Proteolytic activity of ghost supernatant control; (●), hemolytic activity of toxin control; (▲), proteolytic activity of toxin-ghost supernatant; (△), hemolytic activity of toxin-ghost supernatant. The V_0 of blue dextran on this column was 125 ml.

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

Hemolysis and proteolytic activity of toxin-ghost supernatant fluid in the presence of alpha antitoxin

Reagents	Proteolytic activity at 30 min, O.D. 520 nm	Hemolysis, HU/ml
Toxin	0	454
Toxin + control serum	0	501
Toxin + antitoxin	0	74
Toxin + adsorbed anti-toxin*	0	454
Ghosts	0.075	<4
Ghosts + control serum	0.080	<4
Ghosts + antitoxin	0.020	<4
Ghosts + adsorbed anti-toxin*	0.080	<4
Ghosts + toxin	0.100	354
Ghosts + toxin + control serum	0.120	436
Ghosts + toxin + antitoxin	0.012	66
Column toxin†	0.100	380
Column toxin + control serum	0.100	400
Column toxin + antitoxin	0.010	<4

*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,
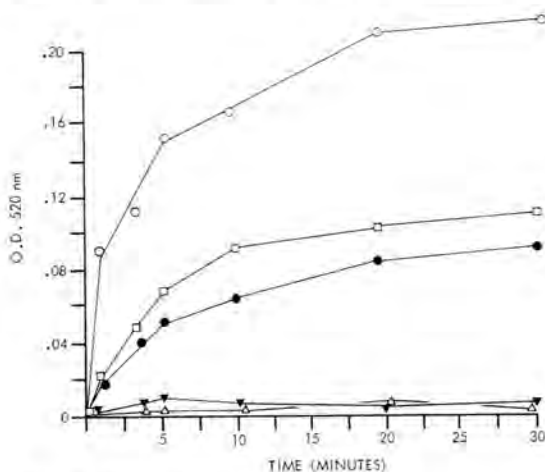

FIG. 3. Effect of antiprotease and heat on proteolytic activity of toxin-ghost preparations. (○), Ghost suspension and azocoll to which toxin was added; (●), ghost suspension incubated with azocoll; (□), ghost suspension preincubated with normal mouse serum after which azocoll and toxin were added; (△), ghost suspension preincubated with antiproteolytic mouse serum after which toxin and azocoll were added; (▼), heated ghost suspension to which toxin and azocoll were added.
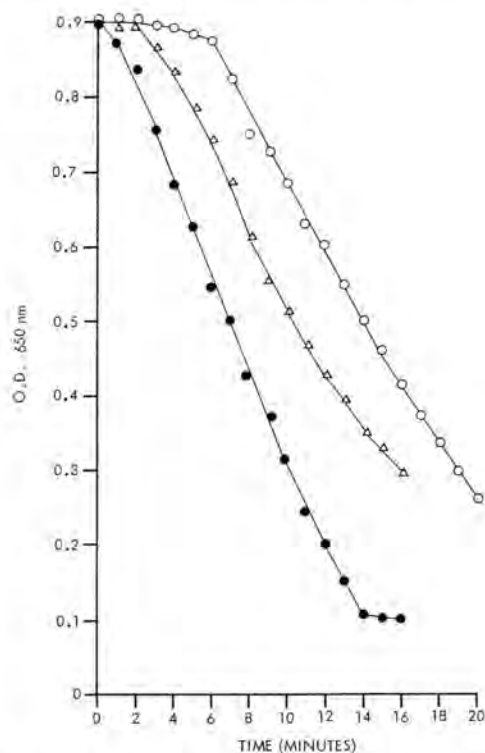

FIG. 4. Effect of mouse antiserum to rabbit ghosts upon lysis of rabbit red cells by alpha toxin. (●), Toxin and rabbit erythrocytes; (△), toxin and rabbit erythrocytes in the presence of normal mouse serum; (○), toxin and rabbit erythrocytes in the presence of antighost (antiproteolytic) serum.

incubation of ghosts alone with antitoxin reduced their proteolytic activity about four-fold. 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:

[1] $I^1 + P^2 \rightleftharpoons IP^3 \rightleftharpoons T^4 + P$
[2] $T + S^5 \rightleftharpoons TS^6 \rightarrow lysis + T$
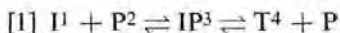
The evidence supporting this scheme is based on a number of observations, (*a*) sensitivity of red cell species to toxin is directly related to their level of proteolytic activity as cited elsewhere

[1] I = inactive toxin precursor.
[2] P = erythrocyte protease.
[3] IP = toxin-protease complex.
[4] T = active toxin.
[5] S = substrate (protein).
[6] TS = 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

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.

1. BERNHEIMER, A. W., and L. L. SCHWARTZ. 1963. Isolation and composition of staphylococcal alpha toxin. J. Gen. Microbiol. **30**: 455–468.
2. DIXON, M., and E. C. WEBB. 1964. Enzymes. 2nd ed. Longmans Green and Co., London.
3. KLAINER, A. S., M. A. MADOFF, L. Z. COOPER, and L. WEINSTEIN. 1964. Staphylococcal alpha-hemolysin: detection on the erythrocyte membrane by immunofluorescence. Science (Washington), **145**: 714–715.
4. MARKHAM, R. 1942. A steam distillation apparatus suitable for micro-Kjeldahl analysis. Biochem. J. **36**: 790–791.
5. MORRISON, W. L., and H. NEURATH. 1953. Proteolytic enzymes of the formed elements of human blood. I. Erythrocytes. J. Biol. Chem. **200**: 39–51.
6. WISEMAN, G. M. 1965. Some characteristics of the beta hemolysin of *Staphylococcus aureus*. J. Pathol. Bacteriol. **89**: 187–207.
7. WISEMAN, G. M., and J. D. CAIRD. 1970. Mode of action of the alpha toxin of *Staphylococcus aureus*. Can. J. Microbiol. **16**: 47–50.