Properties of the Gamma Haemolysin of *Staphylococcus aureus* 'Smith 5R'

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SUMMARY

Purified gamma haemolysin of *Staphylococcus aureus* was characterized in relation to the alpha, beta and delta haemolysins. The sedimentation coefficient of the gamma lysin was 2.65, somewhat higher than the $s_{20,w}$ 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 45000 daltons. The pI of gamma lysin was 6.0, while that of the alpha, beta and delta lysins ranged from 8.5 to 9.6.

The amino acid analysis of gamma lysin was characterized by low levels of methionine and histidine. Methionine was, however, the *N*-terminus, which 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 by quantitative precipitin tests; in Ouchterlony agar gel diffusion tests, single lines of precipitation were observed which showed no evidence of cross-reactions amongst the four haemolysins.

Gamma, beta and delta lysins had no effect in mice when injected at increasing doses ranging from 0 to 100 μ g. The alpha lysin killed mice, the LD₅₀ dose being 0.68 ± 0.12 μ g, or 27 to 34 μ g/kg mouse tissue. Gamma lysin was, however, lethal for guinea pigs when 50 μ g quantities were injected intracardially. Gamma lysin also lysed human leucocytes and destroyed C-6 (human lymphoblast) cells.

Some nitrogen and phosphorus was released from human erythrocyte membranes treated with gamma lysin, when compared to untreated cells, and the rate of this release was linear over a 3 h period. Gamma lysin had no detectable effect on phospholipids extracted from erythrocytes or on lipid-free membrane protein. However, the haemolytic reaction was inhibited by erythrocyte membranes when these were added to lysin-red cell suspensions. Furthermore, human red cell phospholipids competitively inhibited haemolysis. Haemolysis was also inhibited by EDTA and activity could be restored by dialysis. The haemolytic reaction required sodium ions.

INTRODUCTION

The gamma haemolysin 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).

We have compared the chemical and biological properties of purified gamma lysin with those of purified alpha, beta and delta lysins.

METHODS

Production and purification of gamma lysin. These procedures with the 'Smith 5R' strain of *Staphylococcus aureus* are described in the preceeding paper (Fackrell & Wiseman, 1976). Alpha lysin was purified by the method of Wiseman, Caird & Fackrell (1974), and beta lysin by a modification of the method of Wiseman & Caird (1967) in which a column of Sephadex

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G-75 was equilibrated with the buffer of Hallander (1963). Haemolysin, measured in haemolytic units (h.u.), in the column effluent was pooled, concentrated and lyophilized. Delta lysin was purified as before (Caird & Wiseman, 1970).

Haemolysin titrations. These were performed as described by Wiseman & Caird (1972). Sheep blood was used in the titration of beta lysin, human blood with delta and gamma lysins, and rabbit blood with alpha lysin. All lysin titrations were supplemented with 1 mM-Mg²⁺ as described by Wiseman & Caird (1967). The activity of beta haemolysin is enhanced in the presence of Mg²⁺ ions, but they have no effect on alpha, gamma and delta lysins. Blood from other animal species was obtained from animals kept in this Department or from the National Biological Laboratories, Winnipeg, Canada. Human erythrocytes (Rh⁺, group O) were supplied by the Canadian Red Cross in Winnipeg.

Kinetics of haemolysis. Kinetic experiments were performed in an automated SP 800B, double beam recording spectrophotometer (Pye-Unicam, Cambridge), the cuvette holder of which was equipped with a constant temperature water jacket set at 37 °C. Washed red cells were suspended in buffered saline (0.01 M, pH 7.0) in a cuvette of 1 cm light path so that a 1:2 dilution of the cells would have an E_{650} of 1.8. An equal volume of gamma lysin at the appropriate concentration was added to the cells, after which the extinction was monitored continuously or at specified time intervals as required. The slope of the line which depicted a decrease in extinction versus time, expressed as extinction change/min, reflected the concentration of haemolysin present. The effect of membrane phospholipid fractions, lipid-free membrane protein or red cell cytoplasm on the haemolysis of red cells by the gamma lysin was measured in the same way. The phospholipids or membrane proteins were added to the red cell suspension just before the addition of lysin and extinction changes were monitored. Controls were present and automatically compensated for by the double beam spectrophotometer.

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

Effect of EDTA haemolysis of red cells by gamma lysin. Equal volumes of EDTA dissolved in phosphate buffered saline at pH 7.0 were added to dilutions of haemolysin such that the combined volume was 1 ml. One ml of erythrocytes was added to each tube. Incubation and determination of the haemolytic 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 with the EDTA, before addition of the erythrocytes. In one experiment, sodium or potassium ions were added to the lysin dilution, and after the addition of red cells the rate of lysis was followed in the spectrophotometer as described for kinetic experiments.

Antibodies. Antibodies to the haemolysins were prepared in rabbits as described by Fackrell & Wiseman (1974). Immunodiffusion, immunoelectrophoresis and quantitative precipitin tests were performed and antibodies purified by the methods of Campbell *et al.* (1970).

Tissue culture. Cells of the C-6 line (Ida, Sairenji & Hinuma, 1972) were obtained from Dr A. Wallbank of this Department. They were grown in stationary cultures at 37 °C in McCoy's 5A medium (Grand Island Biological Corp., New York, N.Y., U.S.A.) supplemented with 10% (v/v) foetal calf serum and harvested at a concentration of 10⁷ cells/ml. The cells were washed with the McCoy medium which contained 2.5% (v/v) calf serum and resuspended to the original volume in this medium.

Phospholipids. Thin-layer chromatography was performed as described by Fackrell (1974) for detection of phospholipids, which were identified by ascending chromatography

on glass plates coated with silica gel G (Merck). 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 acids. The technique of Fraenkel-Conrat, Harris & Levy (1955) was used with the following modification. Dinitrophenyl amino acids were chromatographed on glass plates coated with silica gel G (Merck) prepared as described by Stahl (1965). Water-soluble and ether-soluble extracts were subjected to ascending chromatography in the following solvent systems recommended by Pataki (1968) (parts by vol. in brackets): *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).

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

Total carbohydrate was estimated by the anthrone method (Kabat & Mayer, 1961). Methods cited by these authors were also applied to the determination of pentose, deoxy-pentose, reducing sugar, hexosamine and sialic acid. Nitrogen was estimated by the micro-Kjeldahl technique given by Markham (1942), while protein was assayed by Bailey's (1967) modification of the Lowry protein reaction. Amino acid analysis was done by Dr F. Labella and Dr F. Stevens of this university. Corrections were made for the partial destruction of acid-labile amino acids.

Other techniques. Polyacrylamide disc gel electrophoresis, electrofocusing and sedimentation velocity analysis were as described by Wiseman *et al.* (1974). Molecular weights from Sephadex chromatography were determined by the method of Andrews (1964).

Chemicals. Pronase was obtained from Calbiochem, purified phospholipids were purchased from General Biochemicals, Chagrin Falls, Ohio, U.S.A., and phospholipase C (*Clostridium perfringens* alpha toxin) was obtained from Worthington Corp., Freehold, New Jersey, U.S.A.

RESULTS

Physico-chemical characteristics of gamma lysin

Disc gel electrophoresis of purified gamma lysin together with purified samples of alpha, beta and delta lysins showed that the gamma lysin cannot be distinguished by this method from the alpha lysin (Fig. 1); both of these lysins migrate toward the cathode. Beta lysin is displaced to the right of the alpha and gamma lysins, while delta lysin moves very little toward the cathode under the conditions of the experiment.

A single peak ($s_{20,w} = 2.6$) for gamma lysin was obtained in the ultracentrifuge. Sedimentation coefficients of alpha and beta lysins were 1.4 and 1.8 S respectively. If the alpha lysin was allowed to stand for several days at 4 °C, the $s_{20,w}$ became 3.0 as reported elsewhere (Wiseman *et al.*, 1974).

One per cent extinction coefficients were calculated for the gamma lysin from the slope of the extinction plot at 280 nm versus concentration. The $E_{280}^{1\%}$ for gamma lysin was calculated to be 28.35 compared with 29.08 for delta. The $E_{280}^{1\%}$ for alpha lysin was 13.56 while that for beta lysin was 4.24.

Isoelectric focusing of the four haemolysins is shown in Fig. 2. The pI values 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 haemolytic activity of the purified preparations in each case.

Fig. 1. Densitometer scan of purified haemolysins subjected to polyacrylamide disc gel electrophoresis. The direction of migration is from left to right.

Fig. 2. Electrofocusing of purified haemolysins. —, Haemolytic activity; ----, extinction;, pH gradient. (a) Gamma lysin (pI 6.0); (b) delta lysin (pI 9.6); (c) alpha lysin (pI 8.5); (d) beta lysin (pI 9.5).

The molecular weight of the gamma lysin based on gel filtration was 45000 daltons, as was that of the alpha lysin (Fig. 3). The molecular weights of the beta and delta lysins by gel filtration were quite different; that for beta lysin was 26000 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 200000 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 either absent or present in small amounts

Fig. 3. Molecular weight determination of purified haemolysins on a column of Sephadex G-75. K_{av} , the partition coefficient; IgG, immunoglobulin G.

Table 1. Amino acid composition of staphylococcal haemolysins

Amino acid	Composition (residues/1000 amino acid residues)			
	Alpha	Beta	Gamma	Delta
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
Ammonia	High	441.6	141.4	218.5

in the gamma lysin residues. The *N*-terminal amino acid of gamma lysin is methionine, which is present only in small quantities. Histidine is the *N*-terminus of alpha lysin, while proline is the *N*-terminus of delta lysin. No data are at present available for beta lysin.

Immunological characterization of gamma lysin

Several immunological experiments distinguish gamma lysin from the other haemolysins. The first of these is quantitative precipitation. Gamma lysin was precipitated by its purified antibody with the formation of a characteristic precipitin curve (Fig. 4*a*), while the other haemolysins were precipitated by their respective antisera and did not precipitate with anti-gamma lysin (Fig. 4*b, c, d*). Ouchterlony immunodiffusion tests of the gamma lysin (Fig. 5*a, 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-

Fig. 4. Quantitative precipitation of purified haemolysins 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.

reactions were observed. These immunodiffusion experiments were repeated in agarose which contained 1 % (v/v) washed erythrocytes. Purified haemolysins were diffused against their respective purified antibodies for 2 h at 37 °C, after which the plates were cooled to 0 °C and rapidly dried with blotting paper. Specific antibodies to the haemolysins neutralized the haemolytic activity, and no precipitin line was observed which did not correspond to the edge of the zone of haemolysis (results not shown).

Effect of gamma lysin upon animals

The toxicity of gamma lysin for mice, rabbits and guinea pigs was investigated. Ten mice per dilution were used, and over the range of 0 to 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 $0.68 \pm 0.19 \mu\text{g}$, or 27 to 34 $\mu\text{g/kg}$ mouse tissue. In contrast with the negative results obtained for mice, gamma lysin killed guinea pigs instantly if they were injected intracardially in 50 μg amounts. The same result was obtained with alpha lysin, but beta and delta lysins had no effect by this route of injection. Autopsy findings obtained for us by Dr H. Sayed of this Department were that guinea pigs which received gamma lysin intracardially showed massive haemorrhage of the kidney and serosal surfaces of the intestines and frank lysis of red cells in the major veins and arteries.

Subcutaneous injection of $> 1 \mu\text{g}$ alpha lysin caused dermonecrosis in rabbits and guinea pigs, but 100 μg quantities of beta, gamma or delta lysins had no effect.

Effect of purified gamma lysin on mammalian cells

Red cells obtained from eleven species of animals were used in the titration of the four haemolysins (Table 2). Some differences in the haemolytic spectra of the haemolysins were found. Rabbit erythrocytes were most sensitive to the alpha and gamma lysins, in contrast to

Fig. 5. Ouchterlony double diffusion of purified haemolysins against homologous purified antibodies. The centre wells contain antibody and the peripheral wells the lysins. Well 1 contains alpha lysin, well 2 beta lysin, well 3 gamma lysin and well 4 contains delta lysin. The centre wells contain: (a) anti-alpha and anti-gamma lysins; (b) anti-beta and anti-gamma lysins; (c) anti-gamma and anti-delta lysins; (d) only anti-gamma lysin.

Fig. 6. Lysis of human leucocytes by gamma and alpha lysins., Leucocytes only; - - - -, leucocytes + gamma lysin; — — —, leucocytes + alpha lysin.
Fig. 7. Effect of gamma lysin on the exclusion of trypan blue by C-6 cells. The concentrations of gamma lysin ($\mu\text{g/ml}$) were: $\bullet$, 0; $\circ$, 1.25; $\square$, 2.5; $\blacksquare$, 5; $\blacktriangledown$, 10.

Table 2. *Haemolytic activity of staphylococcal lysins*

	Activity (h.u./mg protein)			
	Alpha	Beta	Gamma	Delta
Rabbit	76422	81 300	113 210	1644
Dog	2906	< 750	29 764	882
Rat	2497	4960	44 041	1700
Cat	2214	4760	6445	1500
Sheep	1026	758 830	89 906	2929
Mouse	1023	10 000	25 754	1037
Chicken	83	2950	306	803
Guinea pig	56	63 740	6620	803
Human	53	10 360	44 041	7527
Monkey	26	< 750	3208	749
Pigeon	5	9930	160	165

beta and delta lysins which preferentially lysed sheep and human cells respectively. Monkey and dog cells were most resistant to beta lysin and pigeon cells were refractory to alpha, gamma and delta lysins.

Human leucocytes were incubated at 37 °C with the gamma lysin. Addition of gamma lysin (10 $\mu\text{g/ml}$) to the leucocytes caused a relatively linear decrease in extinction of the cells up to 10 min (Fig. 6). Beyond that time, no further decrease was observed. Alpha lysin included for comparison showed a nearly linear fall in extinction over a 30 min period. The effect of gamma lysin on C-6 cells was also studied. Various amounts of gamma lysin in 0.5 ml of medium were added to 4.5 ml cell suspension. At different times, 0.5 ml of this

Fig. 8

Fig. 9

Fig. 8. Liberation of nitrogen from human erythrocyte membranes in the presence of gamma lysin. Membranes alone (x); membranes treated with gamma lysin (●); membranes treated with pronase (○).

Fig. 9. Liberation of acid-soluble phosphorus from human erythrocyte membranes in the presence of gamma lysin. Membranes alone (x); membranes treated with gamma lysin (●); membranes treated with phospholipase C (*Clostridium perfringens* alpha toxin) (○).

suspension was removed and mixed with 0.2 ml of trypan blue (0.01 %, w/v). At least 700 cells were counted and their ability to exclude the dye was recorded (Fig. 7). About 93 % of the control cells excluded the dye, but after 4 h incubation with 10 μ g gamma lysin/ml (700 h.u./ml) only 15 % of the cells excluded the dye and the rate at which cells were affected was proportional to the haemolysin concentration.

Effect of gamma lysin on human erythrocyte membranes

An attempt was made to show chemical differences in composition of red cell membranes after treatment with gamma lysin. The membranes were added to gamma lysin such that their final concentration in terms of nitrogen was 500 μ g/ml while that of the lysin was 50 μ g/ml (about 6000 h.u./ml). The suspension was incubated at 37 °C for 3 h at which time the reaction was stopped by the addition of trichloroacetic acid to a final concentration of 5 % (w/v). Control (no lysin) 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 (results not shown). Furthermore, no differences in extinction at 260 or 280 nm was detected. However, the nitrogen content of the supernatant fluid of the membrane-lysin mixture increased by 7 % in relation to a control without lysin. In addition, acid-soluble phosphorus was released from the membranes by the lysin.

Nitrogen and phosphorus released from membranes were determined at various intervals of time ranging from 0 to 180 min in the presence of twice the amount of substrate (Fig. 8). The rate of nitrogen liberation was linear over the 3 h period. The rate of release was, however, much less than that obtained by treatment of the membranes with pronase. The addition of phospholipase C to the membranes liberated an amount of phosphorus similar to that given by the lysin (Fig. 9). The rate of liberation of phosphorus was greater than that of nitrogen.

Fig. 10

Fig. 11

Fig. 10. Lineweaver-Burk plot of reaction between human erythrocytes and gamma lysin. Gamma lysin-red cell control (●); gamma lysin+red cells to which red cell cytoplasm was added (×); gamma lysin+red cell suspensions at a red cell concentration ($\mu\text{g N/ml}$) of: □, 20; ■, 30; ○, 40.

Fig. 11. Lineweaver-Burk plot of reaction between human erythrocytes and gamma lysin in the presence of membrane phospholipids. Gamma lysin+human red cells alone (●); gamma lysin+red cells in presence of membrane protein (▽); gamma lysin+red cell suspensions to which have been added membrane phospholipids at concentrations ($\mu\text{g P/ml}$) of: □, 10; ■, 20; ○, 35.

The phospholipids of human erythrocyte membranes were dialysed at a concentration of 1 mg/ml against tris-saline buffer (pH 7.0) and incubated with 120 μg (about 10^4 h.u.) of gamma lysin/ml. In contrast with results obtained for red cell membranes, no detectable phosphorus was released after 3 h incubation at 37 °C. None of the phospholipids identified by t.l.c. disappeared from the phospholipid preparation after treatment with gamma lysin. The lysin also did not hydrolyse sphingomyelin and was therefore different from 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 (2 %, w/v) in phosphate buffered saline at pH 7.0 was incubated with gamma lysin (120 $\mu\text{g/ml}$). No nitrogen was detected in the supernatant liquid after 3 h incubation at 37 °C, which contrasts with observations made for membranes. No breakdown products were obtained by amino acid and *N*-terminal group detection on t.l.c. plates.

Gamma lysin might act in a manner similar to that reported for the alpha lysin by Wiseman & Caird (1972) and by Wiseman *et al.* (1974). 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. However, gamma lysin incubated with red cell membranes (known to activate alpha lysin) failed to hydrolyse this ester.

Kinetics of substrate inhibition of haemolysis

The velocity of lysis of human red cells incubated with gamma lysin was measured over a range of red cell concentrations and with erythrocyte membranes or red cell cytoplasm (1 mg/ml reaction mixture). The results, expressed in the form of a Lineweaver-Burk plot (Fig. 10), indicate that haemolysis was inhibited by as little as 20 μg of erythrocyte membrane nitrogen/ml. Cytoplasm had no effect on the rate of haemolysis. All curves have a common y -intercept.

Fig. 12. The effect of NaCl concentration upon the rate of lysis of human erythrocytes in the presence of gamma lysin. The molarity of the solutions was kept constant (0.15 M) by the addition of appropriate amounts of tris-HCl buffer or sucrose.

Table 3. Inhibition of gamma haemolysin by EDTA

Treatment	EDTA (M)	Total haemolysin (h.u.)
Gamma lysin + EDTA solution	10^{-8}	0
	10^{-7}	0
	10^{-6}	6500
	10^{-5}	7300
	10^{-4}	11200
	10^{-3}	11800
	10^{-2}	11900
Gamma lysin + EDTA solution after dialysis	10^{-8}	12100
	10^{-7}	11600
	10^{-6}	11750
	10^{-5}	11300
	10^{-4}	11500
	10^{-3}	11800
	10^{-2}	11800
Untreated gamma lysin	0	11800

The experiment was repeated with phospholipid and lipid-free membrane protein components (Fig. 11). Human erythrocyte phospholipids at several concentrations competitively inhibited haemolysis by the gamma lysin, whereas lipid-free protein had no effect.

Inhibition of gamma lysin by EDTA

Haemolysis of human erythrocytes in the presence of gamma lysin was increasingly inhibited at concentrations of EDTA of 10^{-6} M or greater (Table 3). Inhibition of haemolysis by EDTA was abolished if the solution of lysin and chelating agent was dialysed against phosphate buffered saline at pH 7.0 for 24 h.

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: Mg^{2+} , Ca^{2+} , Fe^{2+} , Al^{3+} , Zn^{2+} , Mn^{2+} , Ni^{2+} , Co^{2+} , NH_4^+ and Cl^- . However, a requirement for Na^+ or K^+ was demonstrated, and at concentrations of less than 7.5×10^{-3} M-NaCl no lytic activity was present (Fig. 12). Erythrocytes were washed with tris-HCl (pH 7.5) containing various concentrations of NaCl and then incubated with gamma lysin. The total NaCl + tris-HCl concentrations were adjusted to 0.15 M to maintain isotonicity. The tris-HCl could be replaced by sucrose with no adverse effects.

Table 4. *Summary of physico-chemical data for the staphylococcal haemolysis*

	Alpha	Beta	Gamma	Delta
Sedimentation constant, $S_{20,w}$	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 by gel filtration	45000	26000	45000	200000†
Isoelectric point	8.5	9.5	6.0	9.6
N-terminal amino acid	Histidine	ND	Methionine	Proline

ND, not determined.

* Data from Caird & Wiseman (1970).

† Estimated (see text).

DISCUSSION

A comparison of the physio-chemical properties of gamma lysin in relation to the alpha, beta and delta lysins is shown in Table 4. Gamma lysin was in several respects similar to alpha lysin. For example, the molecular weights determined on Sephadex G-75 were identical and the two lysins could not be separated by disc electrophoresis (Fig. 1). The $E_{280}^{1\text{cm}}$ of beta lysin was low compared with other lysins; its pI was similar to that of the delta lysin. The sedimentation coefficient determined in our laboratory for beta lysin was close to the value obtained by Gow & Robinson (1969). The pI of gamma lysin was 6.0, which is not in agreement with the value (9.5) obtained by Mollby & Wadstrom (1971). However, the pI of alpha lysin (8.5) agreed 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 depend upon the presence or absence of some critical number of asparagine or glutamine residues, and the high ammonia content may have been due to the presence of aspartate and glutamate residues as asparagine and glutamine. Unfortunately, amino acid analyses of the beta and gamma lysins have not been published elsewhere and consequently it is not possible to make comparisons. Our amino acid analysis of the delta lysin, however, resembles those of Heatley (1971), Kantor, Temples & Shaw (1972) and Kreger *et al.* (1971); arginine, proline, tyrosine and cysteine were absent or present in low concentration while lysine, isoleucine and aspartic acids were present in all preparations in high concentration. Yoshida (1963) also presented an analysis of the delta lysin which 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 compared reasonably well with those of Coulter (1966), Bernheimer & Schwartz (1963) and Six & Harshman (1973). However, the glycine content of our preparation was somewhat higher.

Molecular weight of beta lysin calculated from its behaviour on Sephadex was 26000 daltons, somewhat higher than the value of 15500 obtained by Chesbro & Kucic (1971) for the UNH-Donita strain. However, the minimal molecular weight calculated from the amino acid analysis was 16110, and gives better agreement. The molecular weight of beta lysin by Wadstrom & Mollby (1972) is about twice the value obtained in our laboratory on Sephadex.

The gamma lysin was immunologically distinct from the alpha, beta and delta lysins. It is now abundantly clear from our 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.

Previously it has not been possible to compare the haemolytic spectra of the four haemolysins because one seldom possessed highly purified preparations or had them together at the same time. In the present study this comparison could be made 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 differences in sensitivity of rabbit and human erythrocytes to alpha lysin may vary 15- to 150-fold.

In 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, unlike our own results. They also found that the LD₅₀ of delta lysin for mice was 125 mg, although such a large LD₅₀ is meaningless except to suggest that the lysin is relatively non-toxic for mice. Calculations based on the data of Kreger *et al.* (1971) give an LD₅₀ of 2 mg of delta lysin for mice and 7.2 mg for guinea pigs, but contamination of their preparations with alpha lysin at a concentration of one part in 2000 or less would account for these findings (see Fackrell & Wiseman, 1974).

Unfortunately, it has not been possible to elucidate the precise mode of action of gamma lysin. However, kinetics of haemolysis and inhibition are not incompatible with a catalytic reaction between gamma lysin and its substrate. Kinetic studies of a reaction in which erythrocytes are used as substrate are not ideal, but we have found such studies useful and at best they may reflect what will be 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 phospholipids were not attacked by the gamma lysin. However, the extraction procedure could have altered the substrate so that it was 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 reconcile the sodium requirement with the phospholipase-like activity of the gamma lysin.

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REFERENCES

- ANDREWS, P. (1964). Estimation of the molecular weights of proteins by Sephadex gel filtration. *Biochemical Journal* **91**, 222-223.
- ANSELL, G. B. & HAWTHORNE, J. N. (1964). *Phospholipids - Chemistry, Metabolism and Function*. New York: Elsevier.
- BAILEY, J. L. (1967). *Techniques in Protein Chemistry*, 2nd edn. Amsterdam: Elsevier.
- BERNHEIMER, A. W. (1964). Staphylococcal alpha toxin. *Annals of the New York Academy of Sciences* **128**, 112-123.
- BERNHEIMER, A. W. & SCHWARTZ, L. L. (1963). Isolation and composition of staphylococcal alpha toxin. *Journal of General Microbiology* **30**, 455-468.
- 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 edn. 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.

- DODGE, J. T., MITCHELL, C. & HANAHAN, D. J. (1963). The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Archives of Biochemistry and Biophysics* **100**, 119-130.
- ELEK, S. D. (1959). *Staphylococcus pyogenes and its Relation to Disease*. Edinburgh: Livingstone.
- FACKRELL, H. B. (1974). *Studies with Gamma Hemolysin of Staphylococcus aureus*. Ph.D. thesis, University of Manitoba.
- FACKRELL, H. B. & WISEMAN, G. M. (1974). Immunogenicity of the delta hemolysin of *Staphylococcus aureus*. *Journal of Medical Microbiology* **7**, 411-414.
- FACKRELL, H. B. & WISEMAN, G. M. (1976). Production and purification of the gamma haemolysin of *Staphylococcus aureus* 'Smith 5R'. *Journal of General Microbiology* **92**, 1-10.
- 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 propriétés. *Annales de l'Institut Pasteur* **118**, 19-33.
- HALLANDER, H. O. (1963). Fractionation of staphylococcal toxins by gel filtration. *Acta pathologica et microbiologica scandinavica* **59**, 543-552.
- HEATLEY, N. G. (1971). A new method for the preparation and some properties of staphylococcal delta-haemolysin. *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.
- KABAT, E. A. & MAYER, M. M. (1961). *Experimental Immunochemistry*, 2nd edn. Springfield: Charles C. Thomas.
- KANTOR, H. S., TEMPLES, B. & SHAW, W. V. (1972). Staphylococcal 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). In *Methods in Enzymology*, vol. 1, pp. 840-850. 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.
- MCCNIVEN, 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 and Immunity* **3**, 633-635.
- PATAKI, G. (1968). *Techniques of thin-layer chromatography in amino acid and peptide chemistry*. Ann Arbor: Ann Arbor Science Publishers.
- 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, solubilization and partial characterization. *Journal of Biological Chemistry* **243**, 1985-1992.
- SIX, H. R. & HARSHMAN, S. (1973). Purification and properties of two forms of staphylococcal alpha toxin. *Biochemistry (American Chemical Society)* **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. (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 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* **8**, 29-38.
- YOSHIDA, A. (1963). Staphylococcal delta hemolysin. I. Purification and properties. *Biochimica et biophysica acta* **71**, 655-553.