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STUDIES ON EXTRACELLULAR PROTEINS FROM *STAPHYLOCOCCUS AUREUS*

VI. PRODUCTION AND PURIFICATION OF β-HAEMOLYSIN IN LARGE SCALE

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SUMMARY

Staphylococcal β-haemolysin was purified to a 40 000-fold increase in specific activity by CM-Sephadex C-25 chromatography, isoelectric focusing, and Biogel P-10 chromatography. Lyophilization was found to be the method of choice for storage of the unstable purified β-haemolysin. The high degree of purity obtained was investigated by acrylamide electrophoresis in continuous and discontinuous buffer systems and different gel concentrations. The microheterogeneity of the purified β-haemolysin with an isoelectric point (p*I*) of 9.4 was studied by electrophoresis and isoelectric focusing in acrylamide gels. The gels were sliced and the activity eluted. No further resolution in separable subcomponents was achieved. The main component (p*I* 9.4 ± 0.1) of β-haemolysin was also devoid of all twelve enzymatic and toxic activities assayed for except sphingomyelinase, which gives further evidence for the identity of these two activities. Some of the biological properties, like the lethal effect and cytotoxicity, have been investigated (T. WADSTRÖM AND R. MÖLLBY, *Biochim. Biophys. Acta*), (1971).

INTRODUCTION

Staphylococcal β-haemolysin is recognized by its "hot–cold" action and special tendency to lyse sheep erythrocytes[1–3]. It has recently been purified and characterized by WISEMAN[4], by MAHESWARAN *et al.*[5], and by GOW AND ROBINSON[6]. MAHESWARAN *et al.*[5] showed that the final product of β-haemolysin was extensively purified which was confirmed by immunodiffusion, immunoelectrophoresis, and polyacrylamide gel electrophoresis. When separating the haemolysin on CM-cellulose and DEAE-cellulose columns, two different peaks of haemolytic activity were obtained which confirmed the previous work of HAQUE AND BALDWIN[7]. The two peaks are called

Abbreviations: HU, haemolytic unit(s); p*I*, isoelectric point.

the anionic and the cationic β-haemolysin. The anionic component formed a minor part of the starting material. Its purity and characteristics were therefore not further studied.

MAHESWARAN AND LINDORFER[8] and WISEMAN AND CAIRD[9] showed that the β-haemolysin was a phospholipase (phospholipase C, EC 3.1.4.3) acting on sphingomyelin from ox brain. Sphingomyelin in sheep erythrocyte ghosts was also degraded. Phospholipase activities from *Staphylococcus aureus* have earlier been reported and characterized by NYGREN *et al.*[10] and by DOERY *et al.*[11,12].

The aim of this investigation is to purify β-haemolysin in a large scale by the use of a continuous dialyzing process, ion exchange chromatography, isoelectric focusing, and molecular sieve chromatography. The degree of purity is studied and some properties of the main component will be described in a following paper[13].

MATERIALS AND METHODS

Production of crude β-haemolysin was performed in batch culture of *S. aureus* strain R 1 (kindly supplied by Dr. C. Chesbro) in a modified casein hydrolysate–yeast extract medium containing glucose (20 g/l) instead of β-glycerophosphate and lactate as the main carbon source and called the casein hydrolysate–yeast extract–glucose medium. The temperature, aeration, mechanical agitation, and pH were controlled as previously described[14]. These cultivations were performed in laboratory fermentors of 3 and 10 l (Biotec FL 103 and FL 110, Biotec, Kistners Labtjänst, Stockholm, Sweden). Antifoam was added before sterilization (0.05 ml/l) and was automatically added during the cultivations to a maximum amount of 0.2 m/l medium per h. After 6–8 h the cultures were centrifuged in a Sorvall RC2-B, 8 000 × *g* for 10 min.

Strain R 1 was cultivated weekly on fresh agar slants, and after each passage tests were made on sheep blood agar plates for homogeneity of the population with regard to the production of β-haemolysin. The strain was stored in a lyophilized state in ampoules.

Continuous dialysis

Crude culture supernatants were dialyzed by a continuous process in an artificial kidney based on the Kiil principle[15]. The apparatus is commercially available from A. S. Nycotron, Oslo, Norway. Mostly, volumes of 4 l were handled by this process with a flow rate of the supernatant of 30 ml/min. Deionized water was pumped on the other side of the membrane (Cuprophane 150 PT, Bemberg, Wuppertal, Germany) with a flow rate of 150 ml/min. The salt concentration was estimated by an LKB conductolyzer (LKB Produkter, Stockholm-Bromma, Sweden) using NaCl solutions of different molarities as a standard[16].

Ion exchange chromatography

By the continuous dialyzing procedure the ionic strength was diminished from 0.3 initially to less than 0.05. The total volume (4 l) was then applied to a column with CM-Sephadex, C-25 (Pharmacia, Uppsala, Sweden), equilibrated with 0.05 M phosphate buffer (pH 6.5). The column (2.5 cm × 15 cm) was packed to a height of 8–10 cm.

Concentration procedures

A rotary vacuum evaporator (Rotavapor R, Büchi) was used as previously described[17]. A capacity of removal of 1.0–1.5 l water per h was achieved after some modifications of the system. The temperature in the evaporated material was never permitted to exceed 15°. Flakes of polyethyleneglycol 20 M was used for concentrating the β-haemolysin by dialysis before and after isoelectric focusing. Lyophilization after dialysis against a volatile buffer (0.03 M ammonium acetate, pH 6.5) was used for storage of crude and purified haemolytic material.

Isoelectric focusing

Carrier ampholytes (Ampholine, 40%, w/v), were used to establish the pH gradient in a 110-ml column as earlier described[17]. All samples were dialyzed against glycine 1% (w/v) before isoelectric focusing. For primary electrofocusing of crude supernatant, carrier ampholytes pH 3–10 were chosen (final concentration 1%, w/v), and in experiments where prepurified haemolysin preparations were separated, ampholytes pH 8–10 were used. The carrier ampholytes and the 110-ml columns equipped with double cooling jackets were obtained from LKB-Produkter.

If not otherwise stated electrofocusing was performed in a gradient of glycerol (20–70%, v/v). Experiments were also performed in columns containing 6 M urea as previously described[18].

Biogel P-10 chromatography

Biogel P-10 was suspended in deionized water for 3 days and washed with 0.1 M ammonium acetate buffer (pH 6.5) as previously described[37]. Columns (2.5 cm × 35 cm) were packed to a height of 25 cm and used for separation of β-haemolysin from carrier ampholytes.

Measurement of haemolytic activity

Determination of haemolytic activity was performed using serial 10-fold dilution of lysin in phosphate buffered saline (0.145 M NaCl + 0.02 M sodium phosphate buffer, pH 7.0) which contained 2 mM $MgSO_4$ and 0.1% (w/v) bovine serum albumin. The same 0.1-ml pipette was used for each sample of lysin in all dilution steps. 0.45 ml of the sheep red blood cell suspension, 1% (v/v) in the same buffer with albumin, was added to 0.45 ml of each dilution. Incubation was performed at 37° for 1 h and at 4° for 2 h whereafter the dilution of lysin which gave a 50% haemolysis was read. The inverted value of this dilution indicated the number of haemolytic units of the undiluted lysin/ml (HU/ml). The specific haemolytic activity was expressed as haemolytic units/mg protein.

Measurement of sphingomyelinase C activity

Quantitative determination of the sphingomyelinase activity was made as follows: 2.5 mg of sphingomyelin was sonicated in 0.5 ml of 0.1 M Tris–HCl buffer (pH 7.0) containing 0.01 M $MgCl_2$. The β-haemolysin (0.1 ml) was diluted in this buffer and added to make a final volume of 1 ml. The incubation mixture was shaken for 30 min in a water bath 37°, and the phospholipid was extracted according to BLIGH AND DYER[19]. The liberated phosphorous in the methanol water phase was determined according to BARTLETT[20]. Lecithinase C activity was estimated by the

same procedure, except that lecithin was used instead of sphingomyelin as substrate.

Other enzyme and toxin assays

α- and δ-haemolysin were assayed as β-haemolysin but on rabbit and human red blood cells respectively. Other extracellular enzymes often produced by *S. aureus* such as protease, nuclease, hyaluronate lyase, staphylokinase (fibrinolysin), lipase, coagulase and phosphatase and leucocidin (a toxin acting on polymorphonuclear leukocytes) were assayed as previously described[17]. Bacteriolytic activity was determined by a turbidimetric method measuring lysis of whole cells of *Micrococcus lysodeikticus*[21].

Protein estimation

Protein estimation was carried out according to LOWRY *et al.*[22] after thorough dialysis against 0.05 M phosphate buffer (pH 7.0) for 24 h for calculation of the specific activities during the purification procedures. Ultraviolet absorption at 280 nm was used for estimation of the protein content in fractions after Sephadex chromatography and isoelectric focusing. Microcuvettes were used for protein estimations ($A_{280\ nm-310\ nm}$) on purified β-haemolysin (total amount 0.04–0.2 mg).

Discelectrophoresis on polyacrylamide

Discelectrophoresis on polyacrylamide was carried out according to ORNSTEIN[23] and DAVIS[24] with some modifications. A discontinuous buffer system, and no stacking or sample gel according to HJERTÉN[25] and HJERTÉN *et al.*[26], was used. The gels (7.5%, 10%, and 15%; 0.6 cm × 6 cm) were photopolymerized by riboflavin and subjected to electrophoresis in a Buchler Model DEC 12 gel cell equipped with a cooling mantle (Buchler Instruments Inc., Hicksville, N.Y., U.S.A.). Different buffer systems were used: β-alanineacetate[27], lutidine–glycine[28], and one continuous buffer system (0.05 M Tris–acetate (pH 7.4); S. JERSTEDT, personal communication). The gels were used for electrophoresis (5 mA/tube; 200 V, 1–2 h run) without previous removal of the polymerizing catalysts. Usually, every sample was run in parallel experiments; one gel was stained with amido-black or coomassie blue and destained in acetic acid (7%, v/v); every second gel was rapidly frozen (−60°), later cut with a razor blade in 1–1.5 mm slices, eluted in 0.1 M phosphate buffer (pH 7.0) and assayed for haemolytic activity. In most runs one of the gels was also used for separation of a sample of β-haemolysin and a reference protein like cytochrome *c* (horse heart, p*I* 10.8)[29].

Isoelectric focusing in polyacrylamide

Isoelectric focusing in polyacrylamide was carried out in principle as previously described[30]. Gels of 5% containing ammonium persulphate were polymerized and subjected to isoelectric focusing in the Buchler Model DEC 12 gel cell. The upper tray vessel contained 0.2% (v/v) H_2SO_4 and the lower vessel 0.4% (v/v) of diethanolamine. The electrofocusing was carried out at 0–4° (3 mA/tube; approx. 100 V). Every sample (0.2 ml) was mixed with 0.1 ml Ampholine (40%, v/v; pH 3–10 or 8–10) and run in two tubes; one gel was either (1) put in a bath containing 5% (v/v) trichloroacetic acid overnight and then stained with amido-black for 2 h and destained electrophoretically[31], or (2) stained directly without previous removal of ampholytes

in a solution of bromophenol blue as recently described[32]. The second gel was frozen instantly after the run (−60°), cut, and eluted as described above. This second gel also contained 0.1 ml of cytochrome *c* (1 mg/ml) and myoglobin (1 mg/ml) as reference proteins. The coloured zones of these proteins were easily visible during the run and were also used as controls for a good focusing and for determination of R_F values for the eluted haemolytic activity. Three bands of cytochrome *c* and two bands of myoglobin were clearly visible at the end of the run. These heterogeneities are also in good accordance with previous results of isoelectric focusing in density gradients[29,33]. After the focusing of the zones of cytochrome *c* and myoglobin a certain drift of the zones was often noticed.

Immunodiffusion in gel and immunoelectrophoresis

The methods of OUCHTERLONY[34] and GRABAR AND WILLIAMS[35] were used. 0.1 ml of antigen from the various steps of purification and 0.1 ml of two polyvalent antisera, (1) "0-11" and (2) "EX 1480" (see below), were applied. The plates were photographed without previous staining after 48 h of incubation at room temperature.

Molecular weight estimation

The gel was placed between two adjustable pistons, which were fitted with Teflon collars and filter discs. The dead volume was less than 0.1%.

Sephadex G-100 was equilibrated for two days at 4° in de-aerated 0.1 M phosphate buffer (pH 7.8) containing 0.25 M NaCl. By means of repeated resuspension and sedimentation, the smallest gel particles were eliminated by decanting. The gel was sedimented in a 2 cm × 200 cm Perspex column to a height of 180 cm under constant hydrostatic pressure and flow rate. The column was washed downwards at a constant flow rate (20 ml/h) with de-aerated buffer for 2 days between each run, while the chromatography was run upwards. The column was calibrated under the same conditions with Blue Dextran 2000, human serum albumin, (mol. wt. $68 \cdot 10^3$), ovalbumin (mol. wt. $45 \cdot 10^3$), myoglobin (mol. wt. $17 \cdot 10^3$), and cytochrome *c* (mol. wt. $13 \cdot 10^3$). The columns were calibrated in 2 separate steps: (1) 2 ml of buffer contained: 10 mg blue dextran, 50 mg serum albumin, 25 mg cytochrome *c* (2) 2 ml of buffer contained: 50 mg ovalbumin and 20 mg myoglobin. To check the constancy of conditions, blue dextran, serum albumin and myoglobin were re-run on the column after the chromatography of β-haemolysin. The column effluent was measured on a constant flow adsorption meter (Uvicord I or II, LKB-Produkter) at 254 nm. The fractions between the eluted blue dextran and cytochrome *c* were read manually as well at 280 nm. The fractions containing myoglobin and cytochrome *c* were measured also at 408 nm. Experiments were performed in the same column equilibrated with 0.1 M Tris–HCl, (pH 8.4) and in 0.2 M sodium acetate (pH 6.2); both systems containing 0.25 M NaCl.

Spectrophotometry and pH determinations

Spectrophotometry and pH determinations were performed as earlier described[17].

Chemicals

Chemicals were of analytical grade if otherwise not stated. Brain heart infusion

was purchased from Difco Labs, Detroit, Mich., U.S.A. Ovalbumin (5 times crystallized), myoglobin (horse heart), cytochrome *c* (horse heart, salt free), sphingomyelin, and DL-α-lecithin were purchased from Sigma Chem. Comp., St. Louis, Mo. (U.S.A.), cytochrome *c* from Nutritional Biochem. Corp., Cleveland, Ohio (U.S.A.), and acrylamide from Eastman Org. Chemicals, Rochester, N.Y. (U.S.A.). Agarose was obtained from l'Ind. Biol. France S.A., Gennevilliers, Seine (France). Carrier ampholytes (Ampholine) were purchased from LKB-Produkter, Stockholm-Bromma (Sweden), Sephadex and Blue Dextran 2000 from Pharmacia, Uppsala (Sweden), and Biogel P-10 from Biorad. Labs Richmond, Calif. (U.S.A.). Polyethyleneglycol 20 M (mol. wt. 20 000) and D-glucose of technical grade were purchased from KEBO, Stockholm (Sweden). Human serum albumin was a gift from KABI, Stockholm (Sweden). Polypropyleneglycol P-2000 was obtained from Dow Chemicals, Midland. Mich. (U.S.A.).

$(NH_4)_2SO_4$ precipitated β-haemolysin (toxin batch No. S 1137), referred to as "Wellcome preparation" and rabbit antiserum "EX 1480" containing 80 units of anti-β-haemolysin were kindly supplied by Dr. P. Knight, Wellcome Res. Labs, Beckenham (England). The toxin batch proved to contain 10^6 HU/ml and had a specific activity of 100 000 HU/mg.

A polyvalent staphylococcal anti-serum "o-11" was a kind gift from Commonwealth Serum Labs, Melbourne (Australia).

RESULTS

Characterization of strain R 1

Strain R 1 is a good producer of nuclease, hyaluronate lyase, protease, lipase, and phosphatase, but produced coagulase and bacteriolytic activity only in small quantities. It did not synthesize α- and δ-haemolysin, staphylokinase, or enterotoxin B (H. O. HALLANDER, personal communication) and the crude extracellular material gave 8–10 lines on immunoelectrophoresis against polyvalent staphylococcal antisera, while crude material from other strains (*e.g.* Wood 46) gave at least 15–20 under the same conditions.

Strain R 1 was subcultured on agar slants and controlled for the production of β-haemolysin on sheep blood agar. In preliminary experiments a high percentage of colonies with a small surrounding halo of haemolysis was found after incubation at 37° for 24 h followed by 2 h at 4°. Cultivation of colonies with a large zone of haemolysis and the variant with a small zone in liquid casein hydrolysate–yeast extract medium confirmed that the first one gave a high titre of haemolysin, while the variant gave little or no haemolysin.

Cultivation experiments

Earlier β-haemolysin has often been produced on solid media covered with cellophan in an environment containing CO_2[4,7,8]. Casein hydrolysate–yeast extract–agar plates covered with cellophan 14 provided a good growth of strain R 1 (2 g dry wt./m^2 and 10^7 HU/m^2). Addition of CO_2 did not increase the yield either on solid media or in submerged culture. Shaking table experiments (5-l flasks; 1 l medium/flask) gave significantly lower yields (10^2–10^3 HU/ml; 4–6 g/l dry wt.). The maximal activity in different cultivation experiments was always found at the end

of the logarithmic growth phase after 6–8 h in flasks with indentations as recently described for several extracellular staphylococcal proteins[14]. Higher yields (10^4 HU/ml; 6–8 g/l dry wt.) were obtained in brain heart infusion broth than in casein hydrolysate–yeast extract medium in these experiments, while cultivation in casein hydrolysate–yeast extract–glucose medium in a 3-l fermentor gave supernatants containing 10^5–10^6 HU/ml (8 h cultivation time, cell dry weight: 6–10 g/l). The haemolytic titre did not decrease during the next 12 h.

Production

Cultivation for 6–8 h in a 10-l fermentor in a casein hydrolysate–yeast extract–glucose medium gave a yield of β-haemolysin of 10^7 HU/ml and a bacterial cell density of 10–12 g/l dry wt. (37°; 600 rev./min; 4 l air/min).

Purification, preliminary experiments

Crude material subjected to dialysis in tubing or by a continuous process as well as to rotary evaporation, lyophilization, or ion exchange chromatography did not lose haemolytic activity after these processes.

Isoelectric focusing of a crude not concentrated dialyzed culture supernatant from strain R 1 gave two peaks of β-haemolytic activity (Fig. 1); one major cationic peak with a pI of 9.4 $\pm$ 0.1 and one minor anionic peak with a pI of 3 $\pm$ 0.4. The latter contained less than 5% of the total activity and was found in fractions containing precipitate[18]. Centrifugation and attempts to suspend the precipitate in different buffers did not dissolve it, but the activity still remained associated with this material. Due to its inconsistency and small part of the total haemolytic activity it was not further studied. The total yield was usually more than 100% in 10 separate experiments. On separation in columns containing 6 M urea the anionic component diminished[36]. Separation in density gradients of glycerol (20–70%, v/v) gave a better resolution than obtained in gradients of sucrose (0–50%, v/v) and gradients of the former type were used for all experiments described below.

The cationic component showed a microheterogeneity already in the primary

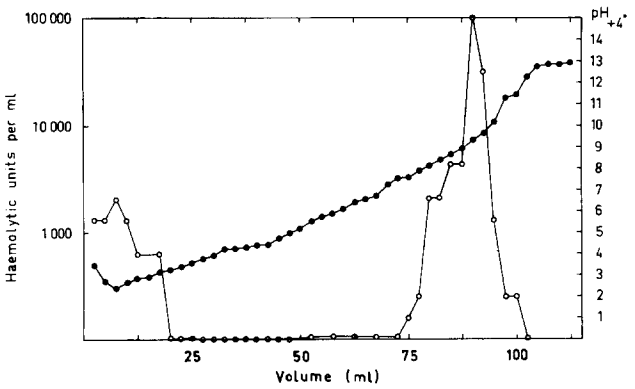

Fig. 1. Distribution of β-haemolysin after isoelectric focusing. Crude supernatant from strain R1 (1 l) was concentrated by vacuum evaporation, dialyzed against 1% (w/v) glycine, and subjected to isoelectric focusing for 48 h in a 110-ml column. 2-ml fractions were collected, and for each fractionation pH was measured at 4° (●—●), and β-haemolytic activity was assayed (○—○).

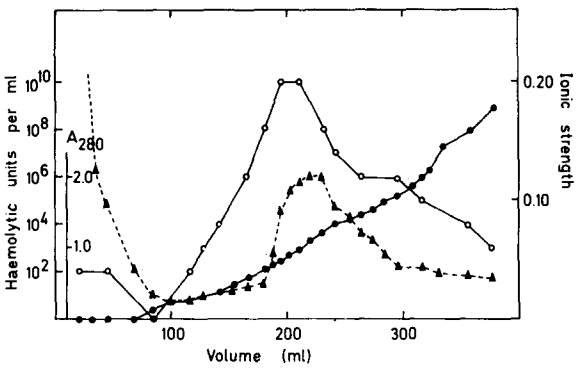

Fig. 2. Chromatography of crude culture supernatant on CM-Sephadex C-25 (Step 1, Table I). β-Haemolysin (○—○) was eluted by a linear gradient according to METHODS at an ionic strength of approx. 0.05 M. Fraction volume, 12 ml; absorbance at 280 nm (▲— — —▲) was recorded and the ionic strength was estimated from conductivity measurements (●—●), see METHODS.

electrofocusing; the major peak had a p*I* of 9.4 ± 0.1. The haemolysin in this state of purification was rather stable at −20° with a half-life of 1 month.

Samples of the Wellcome haemolysin preparation were subjected to isoelectric focusing. In principle, the same results were obtained with a major component p*I* 9.5 ± 0.1 (Fig. 2)[36]. The anionic component also proved to contain a variable percentage of the recovered activity and was undetectable in some experiments.

General purification procedure

In order to obtain purified enough β-haemolysin for physicochemical studies and further characterization of its biological properties, a large scale purification procedure was developed. Volumes of 4 to 40 l were easily handled in this process, permitting preparation of mg quantities of β-haemolysin to a high degree of purity. After one passage through the artificial kidney the ionic strength of the supernatant was usually less than 0.05 compared to the standard of NaCl, which permitted adsorption on a column containing CM-Sephadex.

CM-Sephadex chromatography (Step 1, Table I)

After washing of the column with 2 l of 0.05 M phosphate buffer (pH 6.5) β-haemolysin was eluted by a linear gradient (Fig. 2, 0.05 M phosphate buffer (pH 6.5); 0.2 M phosphate buffer (pH 6.5) + 0.2 M NaCl). More than 100% recovery and a 6000-fold increase in specific activity was obtained by both procedures (Table I). This gain of β-haemolytic activity is most likely due to a removal of inhibitory substance(s). No gain of activity was noticed after negative adsorption on DEAE-Sephadex A-25 or after any other step of purification with exception for isoelectric focusing on crude material (see above).

Isoelectric focusing (Step 2)

The fractions from chromatography on CM-Sephadex containing β-haemolytic activity were pooled, dialyzed against glycine (1%, w/v), and concentrated to 40–60 ml with polyethyleneglycol. They were then subjected to isoelectric focusing, pH interval 3–10 (Fig. 3) and 8–10 (Fig. 4). The recovery was about 100% in most

TABLE I

PURIFICATION OF β-HAEMOLYSIN

Stage	*Purification step (pooled fractions)*	*Vol. (ml)*	*Protein*		*β-Haemolysin*		*Specific activity (HU/mg)*	*% Starting material*	*Purification*
			mg/ml	*mg*	*HU/ml*	*Total HU*			
0	Crude supernatant after dialysis	4000	1.2*	4800	10^7	$4.0 \cdot 10^{10}$	$8.3 \cdot 10^6$	100	—
1	Eluate from CM-Sephadex	40	2.0	80	10^{11}	$4.0 \cdot 10^{12}$	$5.0 \cdot 10^{10}$	10 000	6 000
2	Isoelectric focusing	8.5	2.4	20.4	10^{12}	$8.5 \cdot 10^{12}$	$4.2 \cdot 10^{11}$	21 000	50 000
3	Biogel P-10 chromatography	60	0.3	18.0	10^{11}	$6.0 \cdot 10^{12}$	$3.3 \cdot 10^{11}$**	15 000	40 000

* Protein content before dialysis in artificial kidney was 1.6 mg/ml.
** 1 mg β-haemolysin was shown to release 64 μg P/min.

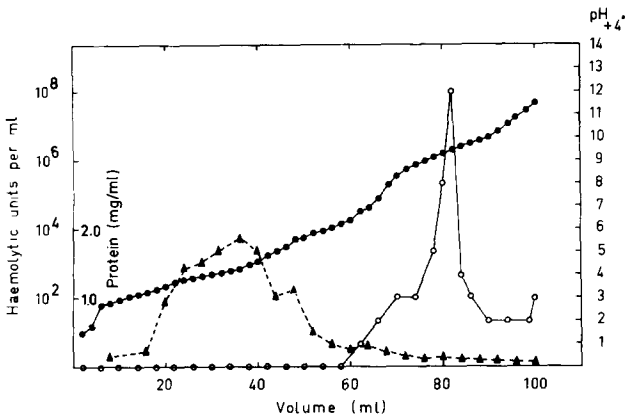

Fig. 3. Distribution of β-haemolysin after isoelectric focusing of prepurified material. 4-l supernatant from strain R1 was prepurified (Step 1, Table I), concentrated, dialyzed against glycine 1% (w/v), and subjected to isoelectric focusing for 48 h in a 110-ml column in a density gradient of glycerol containing Ampholine pH 3–10. 2-ml fractions were collected. ●—●, pH; ○—○, β-haemolytic activity; ▲— — —▲, protein, estimated by the method of LOWRY *et al.*[22].

experiments (Step 2, Table I). A broad peak of β-haemolytic activity was obtained (Fig. 4). Experiments in siliconized columns or columns containing 6 M urea did not significantly alter the separation pattern.

The reproducibility of the microheterogeneity was good. Further studies of

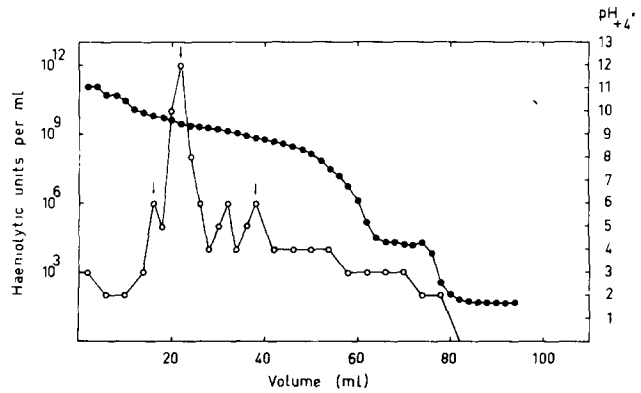

Fig. 4. Distribution of β-haemolysin after isoelectric focusing of material prepurified as in Fig. 3 (Step 1, Table I). Focusing for 48 h in a 110-ml column in a density gradient of glycerol, containing Ampholine pH 8–10, 2-ml fractions were collected. ●—●, pH: ○———○, β-haemolytic activity. Arrows indicate fractions investigated by acrylamide discelectrophoresis and isoelectric focusing in gels.

samples from single 2-ml fractions in acrylamide gel electrophoresis and gel electrofocusing have been performed (see below). The 3–4 fractions containing about 80% of the recovered activity were pooled and concentrated by polyethyleneglycol to 2–3 ml for Biogel P-10 chromatography.

Biogel P-10 chromatography (Step 3)

Recent experiments performed with radioactive ampholytes in this laboratory have shown that albumin, lysozyme, and staphylococcal α- and β-haemolysin can be freed from ampholytes by molecular sieve chromatography[37]. Separation on Biogel P-10 permitted lyophilization of the purified β-haemolysin with a total recovery (Table I). However, Sephadex G-50 or G-100 chromatography of crude and Stage 1 and 2 haemolysin always yielded a low recovery in several buffer systems used.

Sphingomyelinase activity

The sphingomyelinase activity was determined on the β-haemolytic fractions after each step of purification. Since both the crude and the purified enzyme are very unstable upon dilution, no accurate values of phospholipase activity and increase in specific activity after Steps 1–3 could be determined. The values obtained were thus not presented in Table I. However, according to this table, the specific activity of purified β-haemolysin is 10^{11} HU/mg and from the assays performed 1 mg of this Stage 3 toxin was calculated to release 64 μg P/min.

Criteria of purity

Purified β-haemolysin (Stage 3, Table I) was assayed for most of the extracellular proteins produced by strains of *S. aureus*. It was found to be devoid of α- and δ-haemolysin, nuclease, leucocidin, lipase, phosphatase, protease, hyaluronate lyase, staphylokinase, enterotoxin B, lecithinase C and bacteriolytic activity. The high degree of purity of Stage 3 β-haemolysin was also confirmed by the high specific haemolytic activity and by the fact that no extra protein peak was detected after P-10 or G-100 chromatography. This was also verified by immunodiffusion in gel, immunoelectrophoresis and discelectrophoresis (see below).

Discelectrophoresis

The progress in the purification was followed by subjection of samples from the fraction containing β-haemolysin of each purification step (Steps 1–3, Table I) to electrophoresis in acrylamide gels in several buffer systems. A crude culture supernatant, concentrated 10 times by rotary evaporation, yielded 8–10 bands after electrophoresis and staining with amido-black. A sample after CM-Sephadex gave 3 bands, while the pooled fractions after isoelectric focusing (Step 2) and concentration by polyethyleneglycol gave one faint band. About 0.05 mg of the Step 3 material in 0.1 ml was applied on gels of 7.5% or 10% of polyacrylamide and stained with coomassie blue. Elution of a second gel proved that this faint band probably contained the haemolytic activity.

Also after primary isoelectric focusing and Sephadex chromatography and lyophilization only one faint band was visible in 7.5, 10, and 15% gels when the same amount of material was applied. All these experiments were performed on the material from an initial culture volume of 4 l. Experiments in the other buffer systems showed similar results.

After separation in the Tris–acetate buffer system, parallel tubes containing cytochrome *c* as reference protein were immediately frozen and the gels were later sliced and eluted. 50% of the original activity was recovered, anodical to the band

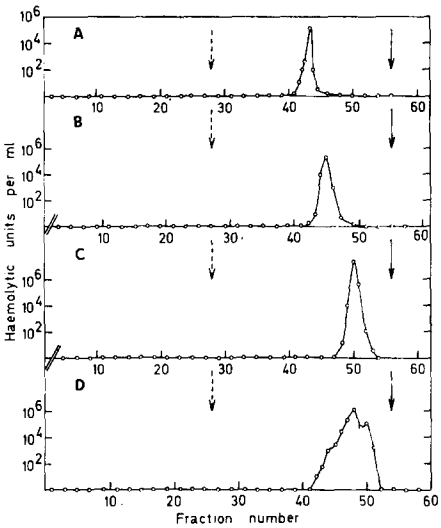

Fig. 5. Discelectrophoresis in acrylamide gels of purified β-haemolysin. 0.1 ml of 3 fractions after isoelectric focusing (marked by arrows in Fig. 4) were together with two reference proteins, cytochrome *c* and myoglobin applied on separate gels (6 cm): (A) 0.1 ml, p*I* 8.8; (B) 0.1 ml, p*I* 9.4; (C) 0.1 ml, p*I* 9.8; (D) 0.1 ml of each of these fractions. The location of the main components of cytochrome *c* (p*I* 10.8) and myoglobin (p*I* 7.3) is indicated by arrows. ○—○, β-haemolytic activity.

of cytochrome *c*, in 2–4 adjacent fractions corresponding to the faint single band in the gels coloured by amidoblack (Figs. 5A–5D). Variable recoveries were found in runs in the different buffer systems, but also in parallel runs in the same system. It was probably caused by the marked instability of this haemolysin.

These results show that a considerable purification and a high degree of purity are achieved after the primary isoelectric focusing and after large scale purification according to the general purification procedure.

Isoelectric focusing in polyacrylamide gels

Separation of prepurified β-haemolysin in a narrow pH interval (ampholytes pH 8–10) in isoelectric focusing in density gradients gave broad peaks of recovery. It was investigated in electrofocusing and electrophoresis in polyacrylamide gels if this could be explained by a microheterogeneity of β-haemolysin. Separation was performed of 0.1-ml samples of 3 fractions (p*I* 8.8, p*I* 9.4, and p*I* 9.8) as indicated by

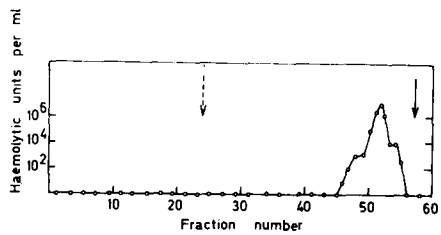

Fig. 6. Isoelectric focusing in acrylamide gel (6 cm) of purified β-haemolysin. 0.1 ml each of fraction p*I* 8.8, p*I* 9.4, and p*I* 9.8 (marked by arrows in Fig. 4) were mixed and applied together with 0.1 mg each of cytochrome *c* and myoglobin. ○—○, β-haemolytic activity.

the arrows in Fig. 4. Every second gel containing 0.1 mg of cytochrome *c* and 0.1 mg of myoglobin was frozen at −60°. The gels were sliced and the β-haemolytic activity was eluted according to the standard procedure. No resolution of the 3 fractions (p*I* 8.8, p*I* 9.4, and p*I* 9.8) was obtained either in the ordinary 6 cm or 20 cm gels (Fig. 6). Separation in electrophoresis in acrylamide gel according to Reisfeld in β-alanine acetate buffer gave similar results. Separation of a crude or partially purified β-haemolysin (Steps 1 and 2) also gave similar results both in electrofocusing and discelectrophoresis in gels.

Immunodiffusion in gel and immunoelectrophoresis

Samples from the different steps of purification were subjected to immuno-

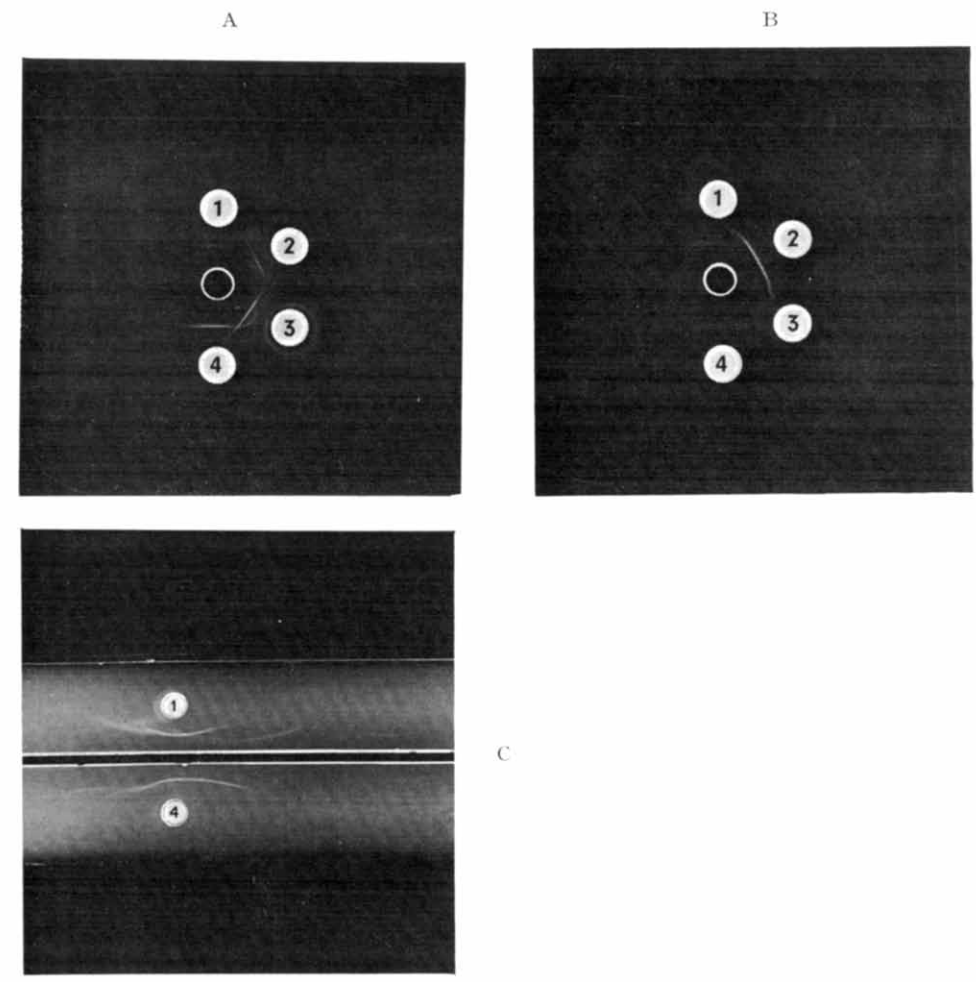

Fig. 7. Immunodiffusion and immunoelectrophoresis of β-haemolysin against polyvalent staphylococcal antisera. A and C. "0–11" serum. B. "EX 1480" serum in the middle well. Samples of β-haemolysin after different steps of purification were applied: 1, crude supernatant; 2, crude supernatant concentrated 5 times by vacuum evaporation; 3, after CM-Sephadex chromatography (Step 1, Table I); 4, after isoelectric focusing (Step 2, Table I).

diffusion and immunoelectrophoresis against polyvalent serum ("0–11", Fig. 7A, and "EX 1480", Fig. 7B). It can be seen in Fig. 7A that crude β-haemolysin showed several lines of precipitation. The β-haemolysin eluted from CM-Sephadex gave at least 3 lines, and after separation on isoelectric focusing one single line was obtained in immunodiffusion and two lines in immunoelectrophoresis (Figs. 7A–7C). The results of immunodiffusion of the same preparation against an anti-β-serum (EX 1480) are shown in Fig. 7B. This proves that the single line of antigen–antibody reaction, obtained with purified β-haemolysin as the antigen, was caused by a reaction of β-haemolysin with its specific antibody and not with a contaminant.

Sephadex G-100 chromatography. Molecular weight estimation

The molecular weight of crude and purified β-haemolysin was estimated according to ANDREWS[38] and DETERMAN[39]. From Fig. 8 a mol. wt. of 38 000 (± 4000)

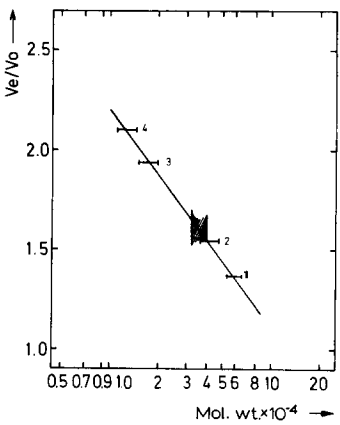

Fig. 8. Estimation of the mol. wt. of β-haemolysin by chromatography on Sephadex G-100. Reference proteins: 1, serum albumin; 2, ovalbumin; 3, myoglobin; and 4, cytochrome *c*. The points represent 4 experiments, the cross hatched area depicts the elution behaviour of β-haemolysin.

was calculated. The separation in the Tris–HCl buffer system confirmed this value of the mol. wt., while separation in 0.1 M ammonium acetate buffer showed a certain retardation and a mol. wt. of about 33 000 was calculated.

Stability

β-Haemolysin was stored after each step of purification (Table I) under a variety of conditions at 4°, −20°, and −70°. Crude and Stage 1 haemolysin was stable in a frozen state for several months. Stage 2 and 3 lysin were very unstable both at 4°, −20° (Table II) and at −70°. Probably due to the low protein content, purified β-haemolysin could not be precipitated by ammoniumsulphate and stored as a precipitate. Lyophilization of β-haemolysin at all stages of purification gave a total recovery if the protein was first equilibrated against 0.05 M ammonium acetate or bicarbonate buffer (pH 6.5). To keep the lyophilized material at −20° is hitherto the only way to store purified β-haemolysin without a loss of activity.

The great instability of the extensively purified haemolysin seems to be

TABLE II

STABILITY OF β-HAEMOLYSIN

Stage (see Table I)	*Half-life (days)*	
	$+4°$	$-20°$
Crude	7–14	120
1	3–7	120
2	1–3	10
2*	< 1	1–2
3	< 1	1–2

* After dialysis against buffer.

independent of the purification procedure. Elution of the haemolytic activity of Stage 1 or 2 haemolysins after disc electrophoresis has shown that the half-life upon storage at 4° of both was 12–24 h. The same is true for a preparation separated just twice by isoelectric focusing.

In conclusion, β-haemolysin at a certain stage of purity seems to lose its haemolytic activity rapidly, independent of the purification procedure. The inactivation of the β-haemolysin, the sphingomyelinase, the cytotoxic, and the lethal activity always seems to be simultaneous[13].

DISCUSSION

Staphylococcus aureus produces at least three different haemolysins called the α-, β-, and δ-haemolysin (for review see refs. 40, 41). These are cationic proteins with high isoelectric points[17,18,36] and of similar size as estimated by ultracentrifugation and gel filtration. All three, α-toxin[18,42], β-haemolysin[5–7,36,41,43], and δ-lysin[44–46], have previously been shown to be heterogeneous upon separation by conventional methods. The probably most extensively purified product of α-haemolysin up to now[42] has recently been found to contain δ-haemolytic activity[47], and a purified preparation of δ-haemolysin[48] has been found to be contaminated by β-haemolysin and ribonuclease[49].

THAYSEN[50] first suggested, and later HAQUE AND BALDWIN[7] and WISEMAN[4] showed the existence of two molecular species of β-haemolysin. WISEMAN[4] also proposed that different β-haemolysins might be produced by different strains of *S. aureus*. However, the evidences for this proposition are few.

CHESBRO[43] separated two components by chromatography on calcium phosphate. Previous reports[5,7,43] showed that the major component of β-haemolysin should be a cationic protein as estimated by ion exchange chromatography and electrophoresis. MAHESWARAN AND LINDORFER[8] recovered two components after chromatography on DEAE- and CM-cellulose. The major component called the cationic β-haemolysin seems not to have been adsorbed on the DEAE column which was the case for the minor one (the anionic β-haemolysin). Separation in isoelectric focusing in density gradients containing 6 M urea showed that the recovery of an anionic component diminished[36]. This haemolysin had characteristics similar to the main cationic component of β-haemolysin. This indicates that the cationic β-haemo-

lysin might form aggregates spontaneously with acidic components as previously reported for another basic protein, egg white lysozyme[51].

HAQUE AND BALDWIN[7] emphasized the importance of the choice of strain for the production of β-haemolysin. Low-producing variants within the single strain could be detected upon plating on sheep blood agar. Continued passages were found to reduce the amount of β-haemolysin produced by the organisms. Preliminary experiments showed that within strain R 1 variants could be detected producing small zones of haemolysis on the blood agar, but also yielding less β-haemolysin in submerged culture. For optimal production the strain was subcultured on sheep blood agar before every large scale production, and colonies with large zones of haemolysis were selected for the precultures.

The production of β-haemolysin per amount of cells was found to be equally good on casein hydrolysate–yeast extract–agar covered by cellophan as in submerged culture in the same medium. However, since the plate method is very laborious to handle for purification of mg amounts of β-haemolysin, cultivation under control of temperature, pH, aeration, and mechanical agitation in 8-l scale in a 10-l fermentor was chosen for practical reasons.

Previous work in this laboratory[14] showed that a casein hydrolysate–yeast extract medium gives high yields of several extracellular proteins. It was earlier emphasized that the brain heart infusion broth was superior to other media tested for the production of high yields[7], which is in accordance only with the production in shaken flasks. For all the toxins and enzymes studied including β-lysin, cultivation in a stirred fermentor with agitation and sufficient aeration increased the yields compared with experiments performed on a shaking table. CO_2 which was reported to be necessary to obtain a high yield of β-lysin[7], was not necessary for optimal production for any exoprotein studied[14,17] contradictory to previous investigations[40]. This addition is probably not essential in a cultivation with pH control. The pH of the medium proved to be of importance for production of several staphylococcal exoproteins[52,53]. HAQUE AND BALDWIN[7] reported that the addition of different fermentable sugars to complex media inhibited enzyme production. The initial and final pH in the culture flasks were estimated and found to decrease below pH 5 during cultivation while pH control in the stirred fermentors and casein hydrolysate–yeast extract–glucose medium gave an increased yield.

The cultivation experiments reported were performed to optimate the production of β-haemolysin. This is important to get enough protein to investigate chemical composition and physical properties of β-lysin (R. MÖLLBY AND T. WADSTRÖM, unpublished data). The extremely high specific haemolytic activity (10^{11} HU/mg) and low protein content of the purified Stage 3 toxin are shown in Table I. However, this value is not accurate due to the error in the many serial 10-fold dilution steps. A lower value (10^7–10^8 HU/mg) was obtained when a clean pipette was used for each dilution. The higher value is probably less accurate but was found to be as reproducible. Also for practical reasons titrations were thus always made without change of pipette. The much lower specific activities earlier reported might partly be caused by this and also the great instability of the purified toxin, especially when diluted[13].

This investigation shows that β-haemolysin can be extensively purified by the method of isoelectric focusing. Preliminary experiments showed that separation

twice, the second time in a shallow pH gradient, yield one haemolytic peak (p*I* 9.4) which after Sephadex G-50 or G-100 chromatography gives one band in acrylamide electrophoresis. Its location corresponds to the location of the haemolysin as determined by elution of the lytic activity from a second gel. However, the problem of aggregate and precipitate formation upon isoelectric focusing of a crude supernatant material has earlier been discussed upon purification of staphylococcal α-toxin[18]. The prepurification by CM-Sephadex chromatography permits separation of much higher quantities of β-haemolysin in one experiment without interference of precipitates with the draining procedure. The recovered anionic component (p*I* 3) was less than 1% after these separations.

After concentration of this material by polyethyleneglycol, chromatography on a rather short Biogel P-10 column was performed. This procedure was finished in 2–3 h and was found to be superior to dialysis (Table II) for removal of carrier ampholytes as previously reported[54]. This was apparent both from the experiments of electrophoresis on acrylamide gels and on cellulose acetate strips upon staining with amido-black, coomassie blue, or ponceau S, since carrier ampholytes interfere with all three stains. Diffuse zones were observed even after dialysis for 12 h. Attempts to precipitate both the crude and the purified β-lysin with $(NH_4)_2SO_4$ were all in vain, probably due to the small amount of protein. This has otherwise earlier been found suitable for purification of β-lysin[5,6,55] and α-toxin[42], and for storage of the latter, which is also unstable in purified state[18,42]. Repeated precipitations have also been found convenient to separate proteins from carrier ampholytes[56].

Purification by ion exchange chromatography and isoelectric focusing in combination with a strictly independent method of purification, such as molecular sieve chromatography, will mostly be the method of choice[54]. This is to be preferred after isoelectric focusing which mostly gives a concentration of the activity. Chromatography on Sephadex G-50, G-10 or Biogel P-10 did not increase the specific activity. This could also partly be explained by the great instability of the highly purified haemolysin on dilution. Adsorption of the small amount of purified β-lysin to the Sephadex gel as reported for several basic proteins[57] is probably more important, since chromatography on Biogel under the same conditions gave much better recoveries.

Several of the staphylococcal exoproteins have isoelectric points in the vincinity of pH 8–10. Strain R 1 has previously been used for production and purification of β-haemolysin[4,43]. While other strains of *S. aureus* seem to be good producers of several of the extracellular toxins and enzymes[14,17], strain R 1 produces only a few of these exoproteins in a large amount. This facilitates the purification procedure since several of these proteins are basic and of similar molecular weight. Staphylococcal α- and δ-haemolysin, leucocidin (T. WADSTRÖM, unpublished data), endo-β-*N*-acetylglucosaminidase[21] as well as one lipase (R. ERIKSSON AND O. VESTERBERG, to be published), one nuclease, and one protease are all basic proteins[17,36]. Enterotoxin B has also been found to be a basic protein of rather a low molecular weight[58]. The difficulty to get a staphylococcal α-haemolysin devoid of δ-haemolytic activity and a β-haemolysin devoid of α-haemolytic activity was recently reported[6,41]. HALLANDER[44] showed that most of the exoproteins mentioned also fall in the same range of molecular size as β-haemolysin. The choice of a suitable strain for optimal production of β-haemolysin, but yielding low quantities of other exoproteins such as

strain R 1, will thus facilitate a large scale purification of β-haemolysin to a high degree of purity.

Some of these proteins have been shown to possess several biological effects. For example, nuclease, leucocidin, enterotoxin B, and all three haemolysins have been reported to be cytotoxic for different types of cells[41]. However, the degree of purity, *e.g.* lack of the other extracellular activities have seldom been investigated for the preparations used in these studies. The aim of this investigation was to obtain β-haemolysin of a high degree of purity to permit studies on its cell membrane lytic action. Production and purification according to Table I will soon yield enough purified haemolysin for aminoacid analysis and ultracentrifugation studies and other physicochemical investigations.

The difficulty in obtaining purified β-haemolysin enough has prevented its biological characterization. Contradictory results, *e.g.* on the possible lethal effect of purified β-haemolysin in doses employed, have been suspected to be due to contamination by, *e.g.* α-toxin and to the great difference in the amount of toxin used. Cytotoxic and other biological studies on purified β-lysin have up to now been difficult to perform, since on the protein weight basis there is a great difference in the amount of toxin used in these experiments[6,41]. Very recently it was shown that purified β-haemolysin, devoid of α-toxin activity and possessing a high specific activity but without any criteria of purity, was lethal for rabbits (40–160 μg[6]). This is in good accordance with the results obtained by β-haemolysin devoid of other cytolytic toxins produced by *S. aureus*[13] and purified according to Table I (Steps 1–3). Thus, on the weight basis it has been found to be dermonecrotic and lethal in approximately the same amount as α-haemolysin.

Purified β-haemolysin has been reported to possess one of the highest specific activities of all toxins studied[5]. In earlier reports, yields of crude β-haemolysin of 100–1000 HU/ml culture supernatant have been reported[4,7]. In this investigation a specific activity of 10^{10} HU/mg was obtained after purification. MAHESWARAN AND LINDORFER[8] reported a value of 68 000 HU/mg, while GOW AND ROBINSON[6] obtained a product of $524 \cdot 10^6$ HU/mg, and very recently HAQUE AND BALDWIN[55] obtained a purified lysin of approx. 10^7 HU/mg. The assay procedures have in principle been the same which should permit a comparison of the results. However, the low accuracy of the haemolytic assay at high dilutions of the β-lysin probably makes a proper comparison impossible. In our opinion accurate quantitative determinations at high dilutions of β-lysin are difficult and prevent a proper comparison of the specific haemolytic activities.

Since β-haemolysin is very susceptible to denaturation in a purified state and the specific activity is very high, difficulties to get an appropriate amount for these studies, amino acid analysis and ultracentrifugation are great. Previously, CHESBRO *et al.*[43] and MAHESWARAN *et al.*[5] pointed out the instability of the purified β-haemolysin, while GOW AND ROBINSON[6] were recently able to purify a β-haemolysin stable at 4°. Many compounds have been tried in this investigation to stabilize the activity, but only a partial stabilization has been achieved, *e.g.* by glycerol and carrier ampholytes (Ampholine) (Table II). The low protein content was believed to be deleterious to the haemolysin, since a purified lysin diluted one hundred fold in different buffers destroyed the activity momentaneously.

The molecular weight of β-haemolysin was determined in sucrose-gradient

centrifugation by CHESBRO *et al.*[43]. An approximate mol. wt. of 59 000 was calculated. Recently, GOW AND ROBINSON[6] showed that α- and β-haemolysin were eluted in the same fractions from a Sephadex G-100 column, thus indicating the similarity in mol. wt. of these two proteins. Molecular weight determination in Sephadex G-100 as described by ANDREWS[38] was performed in this investigation, since in spite of the large scale purification used there was not material enough for ultracentrifugation. A mol. wt. of 38 000 was calculated (Fig. 8).

Immunoelectrophoresis and analytical polyacrylamide electrophoresis were used to study the degree of purity of β-haemolysin. It has also been confirmed by the fact that it is devoid of all enzymatic and toxic activities assayed for. Due to the low protein content, staining of the acrylamide gels after separation of approx. 0.1 mg of Stage 3 β-haemolysin gave just one very faint band. In order to prove that this was caused by β-haemolysin, the second gel was cut and the haemolytic activity was eluted. MAHESWARAN *et al.*[5] also obtained one band in both these procedures. However, the activity from the acrylamide gels was not eluted to prove that the single stained band was caused by the haemolysin.

While none of these criteria is conclusive by itself, yet as a group they provide strong evidence that the purified β-haemolysin has a high degree of purity and that the hydrolysis of sphingomyelin and haemolysis of sheep red blood cells is caused by one and the same protein molecule as previously suggested[4,8,11].

Evidence is presented in the following report[13] which indicates that the substrate specificity of enzyme is high. The toxicity of the sphingomyelinase for mice, rabbits, and cell cultures has been studied. The possibility that this in all cases is caused by a membrane destroying action of the phospholipase is discussed[13].

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