The Identification and Purification of Multiple Forms of θ -Haemolysin (θ -Toxin) of *Clostridium perfringens* Type A

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(Received 9 March 1974; revised 16 November 1974)

SUMMARY

The θ -haemolysin of *Clostridium perfringens* was purified from culture supernatant fluids of type A strains by fractional ammonium sulphate precipitation and isoelectric focusing in narrow pH 5 to 8 gradients. Four components detected on electrofocusing were designated θ_1 (pI 6.8 to 6.9), θ_2 (pI 6.5 to 6.6), θ_3 (pI 6.1 to 6.3) and θ_4 (pI 5.7 to 5.9). Specific activities ranged from 0.4×10^6 to 1.2×10^6 haemolytic units/mg protein and 2950 to 3600 LD₅₀/mg protein. Each haemolytic component was activated by cysteine hydrochloride, and inactivated by cholesterol, by addition of sheep erythrocyte ghosts and by heating at 60 °C for 10 min; mouse erythrocytes were more resistant than sheep erythrocytes to haemolysis. A reaction of identity was obtained between components in gel diffusion. Sodium dodecyl sulphate polyacrylamide disc gel electrophoresis gave molecular weights in the range 59000 to 62000 for each component. A similar value was obtained for θ_1 on density gradient ultracentrifugation. Although the multiple forms were free of 11 factors present in culture supernatants, crossed immunoelectrophoresis and disc gel electrophoresis revealed minor contaminants. These studies reveal that θ -haemolysin has physical properties in common with other oxygen-labile haemolysins.

INTRODUCTION

The production of an oxygen-labile (O-labile) haemolysin, θ -haemolysin, by *Clostridium perfringens* (*welchii*) was first described by Wuth (1923) and Neill (1926). Properties shared by O-labile haemolysins have been summarized by Bernheimer (1970, 1974). The little information available on θ -haemolysin has been reviewed by Ispolatovskaya (1971) and Hauschild (1971). Few attempts have been made to purify it (van Heyningen, 1941; Roth & Pillemer, 1955; Habermann, 1959; Stephen, 1961; Hauschild, 1965). By contrast, other O-labile haemolysins have been purified and characterized (Alouf & Raynaud, 1967; Bernheimer & Grushoff, 1967; Shumway & Klebanoff, 1971; Jenkins & Watson, 1971; Pendleton, Bernheimer & Grushoff, 1973).

Present information on θ -haemolysin is largely derived from the studies of Roth & Pillemer (1955) and Habermann (1959). The preparation of the former contained two components by electrophoresis. That of Habermann (1959) was immunologically homogeneous, whereas Stephen (1961) reported that his preparation was a complex of antigens on immunoelectrophoresis. However, the preparations of Roth & Pillemer (1955) and Habermann (1959) contained significant amounts of α -toxin (phospholipase C). A sedimentation coefficient of 6.5 s was assigned by Roth & Pillemer (1955). No other physical

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characteristics were reported until the preliminary electrofocusing studies of Smyth & Arbuthnott (1969).

Although lacking a role in the pathogenesis of *C. perfringens* (Evans, 1943, 1945*a, b*), θ -haemolysin has been reported to lyse leucocytes (Todd, 1941) and blood platelets (Bernheimer & Schwartz, 1965), to release histamine from mast cells (Habermann, 1960) and to cause morphological changes in erythrocyte ghosts (Habermann & Pohlmann, 1959). Recently it has been identified as a contaminant of crude commercially-available phospholipase C (α -toxin) from *C. perfringens* which has been used extensively as a biochemical probe of membrane structure and function (Möllby, Nord & Wadström, 1973). Thus it seemed essential to purify θ -haemolysin for assessment of its possible role in the many effects attributed to such crude phospholipase C preparations (see Möllby *et al.* 1973; Möllby, Wadström, Smyth & Thelestam, 1974). Moreover, the many cytolytic effects of streptolysin O (SLO), the O-labile haemolysin from *Streptococcus pyogenes*, lend further credence to this view (Alouf & Raynaud, 1968; Halbert, 1971; Bernheimer, 1974).

In this investigation, electrofocusing was the method chosen for purification because it has revealed information on molecular heterogeneity and isoelectric points of bacterial toxins (Arbuthnott, McNiven & Smyth, 1975). Moreover, the electrofocusing studies of Bernheimer, Grushoff & Avigad (1968) had provided interesting comparative information on SLO and cereolysin, the O-labile haemolysin produced by *Bacillus cereus*. The findings reported here have been summarized previously (Smyth & Arbuthnott, 1969; Smyth, Arbuthnott & Freer, 1972; Smyth, 1974).

METHODS

Materials. Carrier ampholytes, Ampholine®, were purchased from LKB-Produkter, Stockholm-Bromma, Sweden, and acrylamide and *N,N'*-methylene bisacrylamide (bis) from BDH; agarose for radial diffusion enzyme determinations was from L'Industrie Biologique Française, S.A., Gennevilliers, France, and for immunodiffusion, immunoelectrophoresis and crossed immunoelectrophoresis from Miles-Laboratories Ltd, Stoke Poges, Buckinghamshire; cysteine hydrochloride, glycine, cholesterol and sucrose were from Merck AG, Darmstadt, W. Germany; soybean lecithin, ovalbumin, chymotrypsinogen and bovine serum albumin were from Sigma; sperm whale myoglobin, was from Schwarz-Mann Bioresearch Inc., Orangeburg, U.S.A.; human serum albumin was from Behringwerke, Marburg-Lahn, Germany; hyaluronic acid was from Miles-Seravac Ltd, Maidenhead, Berkshire; fraction VII glycoprotein for neuraminidase assays was from the Scottish National Blood Transfusion Association, Edinburgh; Coomassie brilliant blue R-250 was from ICI, Manchester; and Phadebas® for amylase assays was from Pharmacia AB, Uppsala, Sweden. Sheep erythrocytes were obtained by venipuncture or from Oxoid. Polyvalent antiserum to the toxins and enzymes of *C. perfringens* type A was purchased from L'Institut Pasteur, Paris, France. Diphtheria toxin, highly purified, was the kind gift of Dr J. Söderholm, Statens Bakteriologiska Laboratorium, Stockholm. Goat antiserum to egg albumin was purchased from Kallestad Laboratories, Minneapolis, U.S.A. Diphtheria antitoxin was obtained from the State Serum Institute, Copenhagen, Denmark, and rabbit anti-human serum albumin from Dakopatts, Copenhagen. All other chemicals were of analytical grade.

Strains. *Clostridium perfringens* strain BP6K (NCIB8875) was obtained from the National Collection of Industrial Bacteria, Aberdeen. Strain 13124 was obtained from the American Type Culture Collection, Rockville, U.S.A.

Media and culture conditions. Strain BP6K was grown (Smyth & Arbuthnott, 1974) in the

modified peptone medium of Murata, Yamamoto, Soda & Ito (1965) with the addition of antifoam (Silcolapse 5000; ICI). Strain ATCC13124 was grown in a pre-reduced proteose peptone medium supplemented with glucose, phosphate, cysteine hydrochloride and vitamins (Möller & Wadström, 1973; Nord, Möller, Smyth & Wadström, 1974) using a 10 l fermenter (Biotec FL 110, Biotec, Stockholm, Sweden), with the pH controlled at 7.2 by the addition of 5 M-NaOH with an automatic titrator. Inocula comprised the strains grown in the appropriate medium for several passages after resuscitation from freeze-dried ampoules or storage at -70°C in nutrient broth.

Harvesting of θ -haemolysin. Under both sets of growth conditions the strains reached stationary phase in approximately 3.5 to 4 h as monitored by $E_{650}^{1\text{cm}}$ measurements (Smyth & Arbuthnott, 1974) and glucose depletion or cessation of alkali addition (Nord *et al.* 1974). Supernatant fluids were obtained by centrifuging cultures at 10000 g for 15 min at 4°C , and θ -haemolysin concentrated by fractional ammonium sulphate saturation to obtain the precipitate between 50 and 60 % saturation (Smyth & Arbuthnott, 1974). The precipitates were harvested by centrifugation or by filtration through Hyflo supercel. In preliminary studies θ -haemolysin was also obtained by the acetone precipitation procedure of van Heyningen (1941) in the presence of calcium phosphate.

Density gradient electrofocusing. This was performed as described by McNiven, Owen & Arbuthnott (1972) and Smyth & Arbuthnott (1974), with the following modifications. In shallow gradient experiments, the anode contained 0.05 M-acetic acid and the cathode 0.09 M-imidazole. Mixtures of Ampholine of narrow pH ranges provided suitable pH gradients. Columns were usually drained by insertion of a peristaltic pump in the outlet line, but elution of the 440 ml columns (LKB8102 or LKB8100-2) used for preparative electrofocusing of crude ammonium sulphate-concentrated θ -haemolysin was best achieved by pumping 65 % (w/v) sucrose, deaerated by use of a vacuum pump, into the bottom of the column through the drain outlet. In the latter case fractions were collected from the separation-chamber loading nipple. pH measurements were made at 4°C . Individual fractions or pooled material were precipitated by dialysis against 80 % saturated ammonium sulphate pH 7.0 (Nilsson, Wadström & Vesterberg, 1970; McNiven *et al.* 1972), dissolved in 0.05 M-tris-HCl buffer pH 7.2 and dialysed against several changes of buffer or distilled water before enzymic or electrophoretic analysis.

Isoelectric focusing on polyacrylamide gel slabs. Polyacrylamide gel slabs [$T = 5\%$ (w/v), $C = 3\%$ (w/w), where T is the total percentage concentration of acrylamide + bis, and C is the percentage concentration of cross-linker relative to total concentration of the two monomers (Hjertén, 1962)] containing 2 % (w/v) Ampholine for pH 3 to 10, were prepared as described by Söderholm & Wadström (1975). Electrofocusing (LKB Multiphor 2117) was performed at 4°C at constant wattage (56 W for 1 h); final voltage in all runs was 1600 to 2000 V (Söderholm & Lidström, 1975). Filter paper wicks (Absorbent Strips, Gelman Laboratories, Ann Arbor, U.S.A.) soaked in 1 M- H_3PO_4 (anode) and 1 M-NaOH (cathode) were used as electrode solution reservoirs. Filter papers (8×8 mm, Whatman 3 MM) were placed on the gel surface 10 to 20 mm from the cathode and 20 μl samples of protein applied immediately. pH measurements were made at 4°C with a flat-membrane surface combined microelectrode (Ingold Lot 403-30, Ingold AG, Zürich, Switzerland). Gels were fixed and stained as described by Söderholm & Wadström (1975).

Immunodiffusion, immunoelectrophoresis, and crossed immunoelectrophoresis. Immunodiffusion tests were performed in agarose gels (Möller *et al.* 1973). Immunoelectrophoresis (Grabar & Williams, 1953) in agarose gels was carried out using the Beckman Microzone apparatus. Samples were run for 1 h at 100 V (approx. 12 mA). Crossed immunoelectro-

phoresis was performed as described in *A Manual of Quantitative Immuno-electrophoresis* (1973). *Clostridium perfringens* type A diagnostic antiserum was rehydrated to 1500 units/ml. Immuno-electrophoretic and immunodiffusion plates were photographed after two days at 20 °C. Crossed immuno-electrophoretic patterns were photographed after the dried and washed gels had been stained with Coomassie brilliant blue R-250 and destained (*A Manual of Quantitative Immuno-electrophoresis*, 1973).

Sodium dodecyl sulphate (SDS) polyacrylamide disc gel electrophoresis. For electrophoresis (McNiven *et al.* 1972) the separating gel contained 11.7 % (w/v) acrylamide and 0.153 % (w/v) *N,N'*-methylene bisacrylamide ($T = 11.8$ %, $C = 1.3$ %). Stacking gels were strengthened by a modification of the method of De Vito & Santomé (1966) omitting EDTA. Samples were made 1 % (w/v) with SDS and 1 % (w/v) with β -mercaptoethanol and boiled in a water bath for 2 min before dilution with an equal volume of 40 % (w/v) sucrose for layering on top of gels. Electrophoresis was performed at 2 mA/gel at 4 °C in a disc gel apparatus (Buchler Instruments, Fort Lee, New Jersey, U.S.A.). Gels were fixed and stained for 1 h in 0.25 % (w/v) Coomassie brilliant blue, in 10 % (v/v) acetic acid and 50 % (v/v) ethanol. The gels were rehydrated in 7.5 % (v/v) acetic acid containing 5 % (v/v) ethanol for 1 h before horizontal electrophoretic destaining (Schwabe, 1966). A mixture of proteins of known molecular weights (bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin, cytochrome *c*) was included in each run (McNiven *et al.* 1972).

Cysteine activation of θ -haemolysin. Cysteine hydrochloride as a neutralized 0.1 M solution in phosphate buffered saline (PBS) pH 6.8 (Roth & Pillemer, 1955) was used for activation (Smyth & Arbuthnott, 1974) at a final concentration of 20 mM.

Haemolytic and lethality assays of θ -haemolysin. The assay for haemolytic activity (Smyth & Arbuthnott, 1974) employed PBS as diluent (Roth & Pillemer, 1955) and a standardized 1 % (v/v) suspension of washed sheep erythrocytes. The reciprocal of the dilution of haemolysin causing haemolysis of 50 % of the erythrocytes in the assay was taken as the number of haemolytic units (h.u.) per ml of undiluted sample. In certain experiments (see text) mouse erythrocytes (1 %, v/v; blood collected in citrated saline and erythrocytes thrice washed in saline) were used.

Lethality was determined by intravenous injection of Swiss white mice (20 to 22 g) of both sexes. The θ -haemolysin was activated with 20 mM sterile cysteine hydrochloride at 37 °C for 10 min immediately before dilution in sterile ice-cold PBS containing 0.1 % (w/v) gelatin (Bernheimer & Grushoff, 1967). The approximate minimum lethal dose was obtained by injection of 0.5 ml volumes of twofold dilutions of activated θ -haemolysin. A dilution containing the approximate minimum lethal dose in 0.5 ml was then used to inject groups of eight mice with 0.1 to 0.5 ml volumes. Deaths were recorded up to 24 h after injection. LD₅₀ values were calculated by probit analysis according to Finney (1952).

Assays for enzymes and toxins as possible contaminants of purified θ -haemolysin. Phospholipase C was always assayed by three methods: (i) by turbidity in a saline extract of egg yolk (Smyth & Arbuthnott, 1974) using 0.05 M-tris-HCl buffer pH 7.2 containing 5 mM-calcium chloride and incubating at 37 °C for 24 h; (ii) the titrimetric method of Zwaal, Roelofsen, Comfurius & van Deenen (1971), modified according to Möllby & Wadström (1973); (iii) by radial diffusion in 1.5 % (w/v) agarose (L'Industrie Française) containing 10 % (v/v) egg yolk extract or 1.5 % (w/v) lecithin (Habermann & Hardt, 1972; R. Möllby, M. Kjellgren and T. Wadström, unpublished). The agarose was buffered with 0.15 M-NaCl in 0.02 M-tris-HCl pH 7.4 containing 1 mM-CaCl₂ and 0.1 mM-ZnCl₂. Agarose gels were 1.5 mm thick; samples (50 μ l) were placed in wells (6 mm diam) and the plates incubated in a moist chamber at 37 °C for 1 to 24 h.

Assays of hyaluronidase (Dorfmann, 1955) and collagenase (Delaunay, Guillaumie & Delaunay, 1949) were as modified by Smyth & Arbuthnott (1974). Neuraminidase was assayed by the method of Holding & Collee (1971). Screening of purified θ -haemolysin for the presence of deoxyribonuclease and ribonuclease (Schill & Schumacher, 1972), caseinolytic activity (Arvidson, 1973), amylase (Ceska, 1971), gelatinase (Schumacher & Schill, 1972), and endo- β -*N*-acetylglucosaminidase (Wadström & Hisatsune, 1970) was done by radial diffusion in substrate-containing agarose gels (1.5 mm thick; 6 mm diam wells). Plates were incubated in a moist chamber at 37 °C for 1 to 24 h.

Heat inactivation of θ -haemolysin. Haemolysin (0.5 ml volumes) in PBS and activated with 20 mM-cysteine hydrochloride, was heated at 60 °C for 10 min in a water bath maintained within ± 0.5 °C of the selected temperature. Control samples were stored at room temperature for 10 min. Heated samples were immediately cooled in an ice bath. Serial twofold dilutions of heated and control samples were titrated for haemolytic activity with equal volumes of 1 % sheep erythrocyte suspension and titres read after incubation at 37 °C for 30 min.

Cholesterol inactivation of θ -haemolysin. Cholesterol (0.1 g dissolved in 5 ml acetone) was dispersed in 20 ml distilled water at 95 °C and filtered, after cooling, through glass wool to remove non-dispersed material. The final dispersion was stable at room temperature and contained 2 mg cholesterol/ml. Both dispersion and control fluid (prepared by the addition of acetone to heated distilled water) were flushed with a stream of air to remove residual acetone. To one set of doubling dilutions (0.5 ml volumes) of activated θ -haemolysin 0.1 ml volumes of cholesterol dispersion were added; 0.1 ml volumes of control fluid were added to another set. After incubation at 37 °C for 10 min, haemolytic activity towards sheep erythrocytes was titrated as above.

Inactivation of θ -haemolysin by sheep erythrocyte ghosts. Packed erythrocyte ghosts were prepared (Freer, Arbuthnott & Billcliffe, 1973) and diluted with an equal volume of PBS. To one set of doubling dilutions (0.5 ml volumes) of activated θ -haemolysin, 0.1 ml volumes of ghost suspension (15 μ g protein) were added; 0.1 ml volumes of PBS were added to another set. After incubation at 37 °C for 10 min, haemolytic activity towards sheep erythrocytes was titrated as above.

Protein determinations. The method of Lowry, Rosebrough, Farr & Randall (1951) was used with bovine serum albumin as protein standard. The protein content of fractions from electrofocusing columns was monitored at $E_{280}^{1\text{cm}}$.

Determination of molecular weight of θ -haemolysin by sucrose density gradient centrifugation. This was done using linear sucrose gradients (4.8 ml volumes; 5 to 20 %, w/v, sucrose) on to which 0.2 ml samples of haemolysin were layered. After centrifugation (Beckman Spinco Model L ultracentrifuge; SW39 rotor at 100 000 *g* for 18 h), fractions (8 drops) were collected. Ovalbumin, human serum albumin and diphtheria toxin were used as reference protein markers. Each protein was detected by immunoelectro-osmophoresis (Wadström, Nord, Lindberg & Möllby, 1974) against antisera which showed no cross-reactions with the other proteins under test. θ -Haemolysin was also detected in fractions by haemolytic titration after activation with 20 mM-cysteine hydrochloride.

RESULTS

Preliminary evidence of heterogeneity

Smyth & Arbuthnott (1969, 1974) reported that θ -haemolysin had a pI of 6.56 with an average recovery of 72 % of input activity on electrofocusing in broad pH gradients from

Fig. 1. Preparative-scale, sucrose-density gradient isoelectric focusing of θ -haemolysin from strain BP6K in a narrow pH 5 to 8 gradient. The θ -haemolysin [1340 mg (1.484×10^7 h.u.) of dialysed precipitate] was applied in the light and dense solutions as 210 and 152 ml, respectively, to a 440 ml column (LKB 8102). Total focusing time was 42 h at 4 °C to a final potential of 800 V. The anode at the bottom of the column contained 5 mM- H_3PO_4 , and the cathode 50 mM-NaOH. The Ampholine mixture comprised equal volumes of the pH 5 to 7 and pH 6 to 8 ranges. Fractions of 4 ml were collected. (●) pH gradient; (■) extinction at 280 nm; (○) haemolytic activity (h.u./ml). Total haemolytic activity recovered was 82 %.

pH 3 to 10. Here, however, elution profiles of θ -haemolysin from broad pH gradients indicated heterogeneity. Shoulders were evident on the acidic side of such profiles and on two occasions the peak profile was resolved into two components in the pH ranges 6.5 to 6.6 and 6.8 to 7.0. Peak haemolytic fractions could be refocused in broad pH gradients with good recovery of activity, but not in narrow pH gradients employed to gain increased resolution.

Multiple haemolytic forms

Toxin concentrates from *C. perfringens* strain BP6K were prepared by harvesting the 50 to 60 % saturation ammonium sulphate precipitates from culture supernatant fluids, and were electrofocused in pH 5 to 8 gradients on a preparative scale (Fig. 1). Four peaks of haemolytic activity were resolved in each of four experiments. These had pI values from 6.8 to 6.9, 6.5 to 6.6, 6.1 to 6.3 and from 5.7 to 5.9, and are designated components 1, 2, 3 and 4 respectively. Identical results were obtained with concentrates from strain ATCC13124 despite the latter's different growth conditions. Drainage of columns upwards or downwards gave identical results. The relative amounts of the major components 1 and 2 varied from experiment to experiment.

All four components were refocused individually in shallow pH gradients (Fig. 2). Although components 1, 2 and 3 refocused to give distinct major peaks of activity, shoulders or minor peaks corresponding in pI to other components were apparent. Component 4 was resolved into two peaks of activity equivalent to components 3 and 4 in pI. In further refocusing experiments on component 4 from preparative scale columns, resolution of two

components was observed on one occasion and virtually homogeneous elution profiles obtained in two runs. Thus the validity of the apparent dissociation shown in Fig. 2*d* remains unresolved. Refocusing of a mixture of components 1 and 2 in a broad pH 3 to 10 gradient yielded two peaks of haemolytic activity with appropriate pI values, confirming earlier observations.

Purification procedure

This procedure is summarized in Table 1. Higher yields (80 to 95 %) were obtained by fractional ammonium sulphate precipitation of culture supernatant fluids from pH-controlled cultures in fermenters (pH 7.2) than without pH control in batch cultures (50 to 60 %) (Smyth & Arbuthnott, 1974). Components 1 to 4 were purified 800- to 1800-fold. Although recoveries of activity on refocusing were usually satisfactory as regards total activity detectable, subsequent ammonium sulphate precipitation of peak fractions led to losses in activity and partly accounted for the apparently low recoveries presented. Moreover, the strict selection of a limited number of fractions for refocusing at step 2 and the use of peak fractions only (usually pools of two fractions) from refocusing experiments, give a false impression of the efficiency of purification. Even with this apparent wastefulness, milligram quantities of purified preparations with high specific activity were obtained at step 3. No significant gains in specific activity were obtained by refocusing.

Identity of components with θ -haemolysin

The relationship of components 1 to 4 to θ -haemolysin was assessed by comparison of the effects on their haemolytic activity of inactivation with cholesterol, heat or sheep erythrocyte ghosts, activation with cysteine hydrochloride, and their haemolytic titres on mouse and sheep erythrocytes. All four components possessed properties consistent with their identity as O-labile haemolysins (Table 2). In addition, Ouchterlony double diffusion tests in agarose gels (Fig. 3) revealed a single line of identity between components. This further substantiates the four haemolysins as multiple forms of θ -haemolysin and the following nomenclature is proposed: θ_1 (pI 6.8 to 6.9), θ_2 (pI 6.5 to 6.6), θ_3 (pI 6.1 to 6.3), and θ_4 (pI 5.7 to 5.9).

Criteria of purity

Three kinds of criteria provided evidence on the purity of each θ -haemolysin: (i) immunoelectrophoretic homogeneity, (ii) homogeneity by separation techniques depending on size and charge, and (iii) freedom from other enzymically detectable factors in culture supernatant fluids of *C. perfringens* type A.

Immunoelectrophoretic analyses of θ_1 , θ_2 , θ_3 and θ_4 were compared with haemolysin at steps 1 and 2 during purification (Fig. 4). Crude concentrated supernatant material and ammonium sulphate-precipitated haemolysin each yielded 10 to 15 precipitin arcs. After primary electrofocusing, θ_1 , θ_2 and θ_3 usually gave two precipitin arcs each, one major and one minor. After refocusing, they appeared to be homogeneous (Fig. 4, *g* to *i*). By contrast, θ_4 gave up to six arcs after primary focusing and two or three after refocusing. Antiserum prepared in rabbits against θ_1 gave a single immunoprecipitate with each form, again indicating identity between components.

Crossed immunoelectrophoresis allowed further assessment of serological purity (Fig. 5). Concentrated supernatant material and ammonium sulphate-precipitated haemolysin yielded complex antigen patterns with 25 to 30 components (Fig. 5*a*, *b*). The immunoprecipitates shown for θ_2 (step 2) and θ_3 (refocused) are representative of those found for θ_1 , θ_2 and θ_3 . The skewed or double peak profiles of the immunoprecipitates, with hooks

Fig. 2. Refocusing in narrow pH 5 to 8 gradients of pooled fractions comprising components 1, 2, 3 and 4 obtained from preparative isoelectric focusing of θ -haemolysin from strain ATCC13124. Anodes contained 0.05 M-acetic acid, and the cathodes 0.09 M-imidazole. The Ampholine mixture used in each experiment was pH 5 to 7:pH 4 to 6:pH 6 to 8 = 80:10:10. All experiments were performed at 4 °C to a final potential of 1200 V in 110 ml (LKB 8100-1) columns. Fractions of 2 ml were collected. (●) pH gradient; (■) extinction at 280 nm; (○) haemolytic activity, (h.u./ml). (a) Component 1; duration of run 48 h. (b) Component 2; duration of run 47 h. (c) Component 3; duration of run 51 h. (d) Component 4; duration of run 60 h.

Fig. 2(c) and (d). For legend see p. 226.

extending around the antigen wells, were characteristic features. The major precipitin arcs in Fig. 5(c) and (e) were shown to be due to θ -haemolysin by a toxinogram procedure using sheep erythrocyte agarose overlayers in phosphate buffered saline on unwashed duplicate plates (C.J. Smyth, unpublished). Preparations of θ_1 , θ_2 and θ_3 on primary focusing contained two to four faint minor arcs, indicating the presence of other antigens; θ_4 yielded 15 to 18 precipitin arcs by crossed immunoelectrophoresis after primary isoelectric focusing, which were reduced to two or three after refocusing.

Table 1. *Summary of purification of multiple forms of θ -haemolysin from culture supernatant fluids of strain ATCC13124 grown in a fermenter at pH 7.2*

	Volume (ml)	Protein (mg)	$10^{-6} \times$ Total θ -haemolysin activity (h.u.)	Specific activity (h.u./mg)	Overall recovery* (%)	Increase in specific activity	
Culture supernatant fluid	9400	175 780	96.3	550	100	1	
Step 1. (NH ₄) ₂ SO ₄ precipita- tion, 50–60 % saturation	185	870	91.0	104 000	94.5	189	
Step. 2. Isoelectric focusing†							
Pooled component 1	3.4	20.5	17.8	868 000	18.5	86.8	1580
Pooled component 2	3.5	18.5	18.4	993 000	19.1		1800
Pooled component 3	3.4	9.3	6.7	717 500	6.9		1300
Pooled component 4	4.2	14.2	8.3	581 600	8.5		1060
Pooled residual fractions	12.4	—	32.5	—	33.8	—	—
Step 3. Refocusing†							
Component 1	2.6	1.82	1.28	702 900	1.3 (53)		1280
Component 2	2.5	1.40	1.23	878 600	1.2 (68)		1600
Component 3	2.8	1.12	0.92	820 000	1.0 (32)		1490
Component 4	1.4	0.27	0.12	431 600	0.1 (20)		784

* Total percentage recoveries of haemolytic activity in individual electrofocusing experiments are given in parentheses.

† Activities measured in pooled fractions after concentration with $(\text{NH}_4)_2\text{SO}_4$ and dialysis as described under Methods.

Table 2. *Identification of refocused haemolytic components as θ -haemolysin*

Treatment	Sample*	Haemolytic titres of components			
		1	2	3	4
(i) Inactivation by cholesterol (200 μg)	Test	2	0	0	0
	Control	32 760	24 580	16 380	256
(ii) Heat inactivation (60 °C, 10 min)	Test	512	256	256	6
	Control	32 760	24 580	16 380	384
(iii) Inactivation by erythrocyte ghosts	Test	2	0	8	0
	Control	4 096	1 536	8 192	64
(iv) Activation by cysteine hydrochloride (20 mM)	Test	32 760	24 580	16 380	384
	Control	1280	640	960	20
(v) Haemolytic titres on different erythrocyte species	Mouse	6 044	3 072	2 048	32
	Sheep	32 760	24 580	16 380	384

* Test and control samples, except for (iv), were activated with cysteine hydrochloride before treatments.

In SDS electrophoresis (Fig. 6) few bands were detected in concentrated supernatant fluids. However, a distinct brown zone migrated rapidly in front of the tracking dye off the bottom of the gels. Each of the θ -haemolysin components contained a prominent major band on primary electrofocusing or refocusing which corresponded to a band in the ammonium sulphate-precipitated toxin. The θ_1 preparations contained a major satellite band of lower molecular weight. At high protein loading (50 to 100 μg), three additional minor bands were detected in refocused θ_1 and θ_2 preparations, up to five in θ_3 , and up to eight in θ_4 .

Gel electrofocusing (Fig. 7) revealed multiple protein bands in each of the refocused preparations. In each case a major component was observed corresponding in pI to the value obtained by density gradient electrofocusing, but bands corresponding to the other

(a)

(b)

Fig. 3. Immunodiffusion of θ -haemolysin from strain ATCC13124 against a polyvalent antiserum after different steps of purification. Samples ($10\ \mu\text{l}$) were applied to wells as follows. (a) Well (1): crude culture supernatant material ($1.1\ \text{mg}$), after 100-fold concentration against polyethylene glycol and dialysis against distilled water. Well (2): ammonium sulphate-precipitated θ -haemolysin ($700\ \mu\text{g}$), step 1 purification. Wells (3) to (6): pooled fractions after step 2 purification; (3) θ_1 ($56\ \mu\text{g}$), (4) θ_2 ($54\ \mu\text{g}$), (5) θ_3 ($85\ \mu\text{g}$), (6) θ_4 ($244\ \mu\text{g}$). (b) Well (1): crude culture supernatant material ($1.1\ \text{mg}$), 100-fold concentrated. Well (2): ammonium sulphate-precipitated θ -haemolysin ($840\ \mu\text{g}$), step 1 purification from strain BP6K. Wells (3) to (6): refocused components after step 3 purification; (3) θ_1 ($93\ \mu\text{g}$), (4) θ_4 ($139\ \mu\text{g}$), (5) θ_2 ($49\ \mu\text{g}$), (6) θ_3 ($85\ \mu\text{g}$). Centre wells in (a) and (b) contained 15 international units (i.u.) of *C. perfringens* type A antiserum.

Fig. 4. Immunoelectrophoresis of strain ATCC13124 θ -haemolysin. Samples ($10\ \mu\text{l}$) in each antigen well were electrophoresed at 100 V, 12 mA for 60 min; polyvalent type A antiserum, 0.15 ml (45 i.u.) in each antiserum trough. Anode to right. Well (a): crude culture supernatant material (1.1 mg), 100-fold concentrated. Well (b): ammonium sulphate-precipitated θ -haemolysin (700 μg), step 1 purification. Wells (c) to (f): pooled fractions after step 2 purification; (c) θ_1 (56 μg), (d) θ_2 (54 μg), (e) θ_3 (85 μg), (f) θ_4 (244 μg). Wells (g) to (j): refocused components after step 3 purification: (g) θ_1 (93 μg), (h) θ_2 (49 μg), (i) θ_3 (52 μg), (j) θ_4 (139 μg).

components were also present. Comparison of the SDS electrophoretic and crossed immunoelectrophoretic results with the gel electrofocusing findings, suggests that the multiple bands represent complex microheterogeneity rather than a large number of contaminants. Both the crossed immunoelectrophoretic and the gel electrofocusing data attest to the problems of purification of individual toxins of this organism.

None of the multiple forms (20 to 40 μg tested) contained the following in detectable amounts: phospholipase C, amylase, neuraminidase, deoxyribonuclease, ribonuclease, endo- β -*N*-acetylglucosaminidase, hyaluronidase, lipase, collagenase, gelatinase and caseinolytic activity.

Fig. 5. Crossed immunoelectrophoresis of strain ATCC13124 θ -haemolysin. Samples (10 μ l) in antigen wells were electrophoresed at 10 V/cm for 60 min. Agarose (8 ml) containing 20 μ l (30 i.u.) polyvalent type A antiserum was cast on the upper part of each plate (surface area of antibody-containing gel, 80 cm²). Electrophoresis was at 3 V/cm for 16 h in the 2nd dimension. Anode to left and upper edges of gels. (a) Crude culture supernatant material (1.1 mg), 100-fold concentrated. (b) Ammonium sulphate-precipitated θ -haemolysin (700 μ g), step 1 purification (60 i.u. antiserum). (c), (d) Pooled fractions after step 2 purification; (c) θ_3 (54 μ g), (d) θ_4 (244 μ g). (e), (f) Refocused components after step 3 purification; (e) θ_3 (52 μ g), (f) θ_4 (139 μ g).

Fig. 6. SDS polyacrylamide disc gel electrophoresis of strain ATCC13124 θ -haemolysin. Electrophoresis was for 2 h at 2 mA/gel at 4 °C; anode at bottom of gels. Stacking gels appear opaque.

1. Crude culture supernatant material (1.1 mg), 100-fold concentrated.
2. Ammonium sulphate-precipitated θ -haemolysin (700 μ g), step 1 purification.
- 3 to 6. Pooled fractions after step 2 purification; 3. θ_1 (48 μ g), 4. θ_2 (54 μ g), 5. θ_3 (85 μ g), 6. θ_4 (244 μ g).
- 7 to 10. Refocused components after step 3 purification; 7. θ_1 (93 μ g), 8. θ_2 (49 μ g), 9. θ_3 (52 μ g), 10. θ_4 (139 μ g).
11. Blank control gel.
12. Standard protein markers (30 μ g amounts) for molecular weight determination; top to bottom: (i) bovine serum albumin, (ii) ovalbumin, (iii) chymotrypsinogen, (iv) myoglobin and (v) cytochrome c.

Molecular weight of θ -haemolysin

By SDS disc gel electrophoresis the molecular weight of each of the multiple forms was identical (S.E.M. $\pm$ 3000; at least 4 determinations for each form) (Table 3). The major additional band in θ_1 preparations had a molecular weight in the range 38000 to 42000. The molecular weight of θ_1 by sucrose density gradient centrifugation was similar.

Lethality

For three of the θ -haemolysin forms an LD₅₀ for mice could be measured. The quantities of θ_4 available allowed an approximate mean lethal dose to be determined (Table 3). Death occurred within minutes at high doses (> 10 LD₅₀) and was usually rapid (2 to 6 h) at doses near 1 LD₅₀.

A mixture of θ_1 , θ_2 and θ_3 (in equal amounts by Lowry protein determination) was activated and administered intravenously in graded doses of between 2 and 18 μ g to a group of albino rabbits. The rabbits given 10 to 18 μ g died 2 to 24 h later; the mean lethal dose was 5 to 8 μ g/kg in rabbits; animals receiving more than 6 μ g material exhibited haemoglobinuria.

DISCUSSION

Although the method of isoelectric focusing has been used successfully in the purification of staphylococcal and clostridial toxins and enzymes, its application as the principal resolving

Fig. 7. Isoelectric focusing on polyacrylamide gel of strain ATCC13124 θ -haemolysin. Samples (20 μ l). The pH gradient was formed with an Ampholine mixture pH 3.5–10, pH 4–6, pH 5–7, pH 9–11 = 85:5:5:5 % (w/w). Gel stained with Coomassie brilliant blue.

1. Culture supernatant material (2.2 mg), after 100-fold concentration.
2. Ammonium sulphate-precipitated θ -haemolysin (2.8 mg), step 1 purification.
- 3 to 6. Pooled fractions after step 2 purification; 3. θ_1 (112 μ g), 4. θ_2 (108 μ g), 5. θ_3 (170 μ g), 6. θ_4 (488 μ g).
- 7 to 10. Refocused components after step 3 purification; 7. θ_1 (186 μ g), 8. θ_2 (98 μ g), 9. θ_3 (104 μ g), 10. θ_4 (278 μ g).

Table 3. *Physical properties and biological characteristics of purified θ -haemolysins*

Source	Component	pI	Molecular weight	Specific activities*		
				h.u./mg	LD ₅₀ /mg	LD ₅₀ (μ g/kg mouse)
This report	θ_1	6.8–6.9	{ 62 000† 60 800‡	1 043 000	3 570	13.3
	θ_2	6.5–6.6	61 600†	1 151 000	3 230	14.8
	θ_3	6.1–6.3	59 500†	609 400	2 940	16.2
	θ_4	5.7–5.9	59 000†	412 600	(714)	(70)
Mitsui <i>et al.</i> (1973)	θ_A	7.05	53 000§	889 000	130	481
	θ_B	6.65	50 000§	783 000	460	136
Hauschild <i>et al.</i> (1973)	—	—	74 000†	{ 576 000 768 000	(1 300) (1 230)	(36.7) (38.7)
Habermann (1959)	—	—	—	445 000, 544 000	—	—
Habermann (1960)	—	—	—	—	4 320	11.6
Roth & Pillemer (1955)	—	—	—	392 000	(1 300)	(36.4)

* Specific activities of θ_1 to θ_4 , in h.u./mg, are average values from three refocusing experiments. All lethality values in parentheses are mean lethal doses.

† By SDS disc gel electrophoresis.

‡ By density gradient ultracentrifugation in sucrose.

§ By gel filtration on Sephadex G-100.

step on a preparative scale has rarely been advocated (McNiven *et al.* 1972; Smyth & Arbuthnott, 1974). By comparison with previous methods, the purification of θ -haemolysin outlined here is rapid, simple, gives material of high purity in milligram yields, and minimizes the likelihood of post-synthetic modification during purification. Caution must be exercised in interpreting heterogeneity of haemolysins revealed by isoelectric focusing (McNiven *et al.* 1972). In the latter's studies on crude staphylococcal α -haemolysin six haemolytic components were detected, but only four possessed characteristics of α -haemolysin, whereas the other two appeared to be δ -haemolysin. All four haemolytic components described here behaved as isoelectric forms of θ -haemolysin and were free of phospholipase C, the hot-cold haemolysin of *C. perfringens* (Bernheimer, 1970, 1974; Halbert, 1971; Hauschild, 1971; Ispolatovskaya, 1971).

Assessment of protein homogeneity is a quantitative reflection of the methods used and their limitations, a fact ignored by some authors (Zwaal *et al.* 1971). Extensive biochemical analyses of purified toxins as well as physical and serological criteria of purity are required (Möllby *et al.* 1974). Here, clear quantitative limits of purity have been set by physical, serological and enzymic measurements for each of the θ -haemolysins. Similar information for other bacterial toxins and enzymes is difficult to find in the literature.

Mitsui, Mitsui & Hase (1973) described only two forms of θ -haemolysin from strain BP6K N5-L9, identified first by DEAE-Sephadex A-50 chromatography, which were clearly resolvable by isoelectric focusing; these correspond in pI to θ_1 and θ_2 of the four haemolysins reported here. However, R. Möllby (unpublished) has observed more than two θ -haemolysin components from strain ATCC13124 on electrofocusing of partially purified material (Möllby & Wadström, 1973) with pI values similar to those reported here. The θ -haemolysin of Möllby *et al.* (1973) and Möllby & Wadström (1973), with pI 6.8, probably corresponds to the θ_1 component.

Multiple forms of the O-labile haemolysins SLO (for review, see Smyth & Fehrenbach, 1975) and thuringiolysin (Pendleton *et al.* 1973) have been revealed by isoelectric focusing. The nature of the heterogeneity of O-labile haemolysins, in general, is unclear. Artefacts on isoelectric focusing (Smyth & Arbuthnott, 1974) and other reasons for heterogeneity (Kaplan, 1968; Markert, 1968; Epstein & Schechter, 1968; Vesterberg, 1973) have been discussed. Deamidation is a possible explanation, but has experimental support only for staphylococcal enterotoxin B (Spero, Warren & Metzger, 1974).

Alouf & Raynaud (1973) have shown that acidic forms of SLO were partially toxoided; this may explain the drop in specific haemolytic activity from θ_1 to θ_4 . Conformational relationships between multiple forms of bacterial haemolysins have been suggested by electrofocusing studies using 4 to 6 M-urea (Ui, 1971*a, b*; McNiven *et al.* 1972; Smyth & Arbuthnott, 1974); similar studies could reveal such relationships for the O-labile haemolysins. Aggregation would not appear to cause heterogeneity in θ -haemolysin as the components identified here and by Mitsui *et al.* (1973) did not differ from each other in molecular weight. Amino acid differences between Mitsui's θ_A and θ_B suggest that a small basic peptide may be hydrolysed from θ_A to form θ_B .

Reported physical and biological characteristics of θ -haemolysin(s) are compared in Table 3. Hauschild, Lecroisey & Alouf (1973) reported that θ -haemolysin, on SDS electrophoresis, migrated consistently more slowly than bovine serum albumin; the converse was true here (see Fig. 6). Hauschild *et al.* (1973) suggested that type A and type C strains could produce θ -haemolysins of differing molecular weights. Alternatively, peptide hydrolysis may occur in type A but not in type C culture fluids (Mitsui *et al.* 1973). Although the molecular weights reported here, found by two different methods, were in close agreement, chromato-

graphy of θ_1 on Biogel P-60 yielded a lower molecular weight of 46000 (Smyth, 1974), comparable with the values of Mitsui *et al.* (1973). However, as recoveries of haemolytic activity were low, this may not have been an accurate estimate. Hauschild *et al.* (1973) found a non-toxic component of 35000 to 40000 mol. wt in highly purified θ -haemolysin which may be identical to the prominent satellite band described here in θ_1 preparations on SDS electrophoresis.

The high sedimentation coefficient reported by Roth & Pillemer (1955) for θ -haemolysin is inconsistent with published molecular weight values. However, recent experiments with gradient pore electrophoresis on polyacrylamide gels have indicated molecular weights between 200000 and 250000 for the four isoelectric forms (C. J. Smyth, unpublished). The monomeric (55000) and dimeric (110000) forms of SLO (Alouf & Raynaud, 1973) and the high molecular weight of 171000 for listeriolysin (Jenkins & Watson, 1971) support the hypothesis that high molecular weight forms of O-labile haemolysins may exist. Further studies are in progress to investigate the molecular weights of each θ -haemolysin component by a number of methods and to compare the molecular weights of θ -haemolysins from different types of *C. perfringens*.

The specific activities of components θ_1 to θ_4 (Table 3) compare favourably with those for other O-labile haemolysins (Bernheimer, 1974). The specific haemolytic activities recorded previously are within the range of values found here; the activities of θ_1 to θ_4 may reflect partial toxoiding of preparations (Alouf & Raynaud, 1973) rather than distinct differences in purity. The higher lethal activity of Habermann's (1960) preparation may be related to contamination with phospholipase C as stated by this author. No definite explanation of the comparatively lower activity of the preparation of Mitsui *et al.* (1973) is possible; these authors used male ddYS mice for the determination of LD₅₀ and mouse strains might differ in susceptibility to the lethal action of θ -haemolysin. Previous studies on θ -haemolysin did not record its lethality for the rabbit, which appears to be approximately twice as sensitive as the mouse on a lethal dose/kg basis. Similar observations were noted for SLO (Halbert, 1971).

These studies illustrate that θ -haemolysin possesses similar physical properties to other O-labile haemolysins and strengthen the proposal that O-labile haemolysins are similar in size and charge as well as sharing biological properties (Smyth, 1972; Bernheimer, 1974). However, the mechanism of action of O-labile haemolysins at the cellular level is still poorly understood. The relative ease of purification and high degree of purity of θ -haemolysin make it a good model for the investigation of their cytolytic action.

The preliminary parts of this work were performed during the tenure of a S.R.C. Studentship held in the Department of Microbiology, University of Glasgow. Other parts were performed in the Department of Microbiology, The Queen's University of Belfast, Ireland and the Department of Bacteriology, Karolinska Institutet, Stockholm, Sweden. The author is grateful to Professors K. B. Fraser, T. Holme and A. C. Wardlaw and to Dr T. Wadström for facilities. Thanks are due to Drs T. Wadström, J. P. Arbuthnott and R. Möllby for helpful advice, stimulating discussions and access to unpublished data. The technical assistance of M. Kjellgren and P. Allestam, the secretarial services of I. Claesson, M. Hedqvist, K. Hugg and K. Sepänmaa, and the photographic work of L. Sjöstedt are gratefully acknowledged.

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