PROPERTIES OF *CLOSTRIDIUM PERFRINGENS* (WELCHII) TYPE-A α -TOXIN (PHOSPHOLIPASE C) PURIFIED BY ELECTROFOCUSING

C. J. SMYTH* AND J. P. ARBUTHNOTT

*Department of Microbiology, Alexander Stone Building, University of Glasgow,
Garscube Estate, Bearsden, Glasgow*

PLATE III

CLOSTRIDIUM PERFRINGENS type-A α -toxin has occupied a central position in the study of bacterial toxins, being the first to have its mode of action defined biochemically. The observations of Nagler (1939), Seiffert (1939), van Heyningen (1941*a*), Macfarlane and Knight (1941) and Macfarlane, Oakley and Anderson (1941) led to its identification as a phospholipase C—E.C. 3.1.4.3. phosphatidylcholine cholinephosphohydrolase (Florkin and Stotz, 1964).

Since this discovery, various attempts have been made to purify α -toxin by salt and solvent fractionation, column chromatography and electrophoretic techniques (van Heyningen, 1941*b*; van Heyningen and Bidwell, 1948; Roth and Pillemer, 1953; Meduski and Volkova, 1957; Habermann, 1958, 1959; Stephen, 1961; Bangham and Dawson, 1962; Ispolatovskaya and Levdikova, 1962; Ispolatovskaya, Levdikova and Larina, 1962; Ikezawa, Yamamoto and Murata, 1964; Shemanova, Vlasova and Tsvetkov, 1965; Macchia and Pastan, 1967; Bernheimer, Grushoff and Avigad, 1968; Ito, 1968; Shemanova *et al.*, 1968; Diner, 1970; Sugahara and Ohsaka, 1970; Teodorescu, Bittner and Ceacăreanu, 1970; Casu *et al.*, 1971). Some of these procedures are exceedingly complex, requiring large volumes of starting material, and yields have been generally low. Although purified products have been shown to be homogeneous by immunoelectrophoretic criteria, rarely have purified preparations of α -toxin been examined by disk-gel electrophoretic procedures or by ultracentrifugation. Whereas the preparation of Habermann (1959) and those of Shemanova *et al.* (1968), Sugahara and Ohsaka (1970) and Casu *et al.* (1971) appeared homogeneous by cellulose-acetate and polyacrylamide disk-gel electrophoresis respectively, that of Diner (1970) was stated to be 60–70% pure on polyacrylamide disk-gel electrophoresis, containing an unspecified number of minor contaminants. Ultracentrifugation revealed that the preparations of Ispolatovskaya and Levdikova (1962) and Teodorescu *et al.* (1970) were not homogeneous, whilst that of Shemanova *et al.* (1965) contained contaminants of low molecular weight. Only the preparation of Shemanova *et al.* (1968) was homogeneous by all three criteria. However, the freedom of purified α -toxin preparations from other toxins and enzymes of type-A strains has rarely been stated as evidence of purity.

The molecular weight (MW) of α -toxin has been variously determined as 106,000 (Meduski and Volkova, 1957, 1958); 31,000 (Bernheimer and Grushoff, 1967); 51,200 (Shemanova *et al.*, 1968); 35,000–42,000 (Glushkova, Nenashev and Golshmid, 1969); 33,500 (Fraction I) and 26,300 (Fraction II) (Teodorescu *et al.*, 1970); 42,000 [calculated from the data of Shemanova *et al.* (1965) by Goodenough and Revel, 1971]; 90,000 (Casu

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* Present address: Department of Bacteriology, Karolinska Institute and Department of Bacteriology, State Bacteriological Laboratory, Stockholm, Sweden.

et al., 1971). A c'sparsity also exists in the sedimentation coefficients attributed to α -toxin: 7.9S (Meduski and Volkova, 1957, 1958); 3.8S (Shemanova *et al.*, 1965); 3.8–3.9S (Shemanova *et al.*, 1968). Moreover the toxin's isoelectric point (*pI*) has been reported as 5.0 and 5.8 (Bangham and Dawson, 1962) and 5.5 (Dawson, 1968). One amino-acid analysis has been reported identifying 17 amino acids (Shemanova *et al.*, 1968). Ispolatovskaya (1967, 1970) has presented evidence that α -toxin may be a zinc metalloenzyme, a finding as yet unconfirmed by other workers. Multiple forms of α -toxin have been described by several authors (Ispolatovskaya and Levdikova, 1962; Ispolatovskaya, 1964; Bernheimer *et al.*, 1968; Sugahara and Ohsaka, 1970; Teodorescu *et al.*, 1970).

The biological properties of α -toxin have been recently reviewed by Ispolatovskaya (1971). In addition to its phospholipase activity, it is lethal to laboratory animals and exhibits hot and hot-cold haemolysis of various mammalian erythrocyte species (van Heyningen, 1941*b*; Macfarlane and Knight, 1941; Roth and Pillemer, 1953; and others). Most data support the view that these activities are manifestations of a single molecular species (Zamecnik, Brewster and Lipmann, 1947; Roth and Pillemer, 1953; Habermann, 1959; Ispolatovskaya, Levdikova and Larina, 1961; Ispolatovskaya and Levdikova, 1962; Shemanova *et al.*, 1965; Ispolatovskaya and Klimacheva, 1966; Bernheimer *et al.*, 1968; Sugahara and Ohsaka, 1970), but some workers have questioned this (Roth and Pillemer, 1953; Dolby and Macfarlane, 1956; Lynch and Moskowitz, 1968).

It has long been assumed that α -toxin hydrolyses both phosphatidylcholine (PC) and sphingomyelin (Macfarlane, 1942, 1948; Matsumoto, 1961; Saito and Mukoyama, 1968). However, Macchia and Pastan (1967, 1968), Macchia, Bates and Pastan (1967), Pastan, Macchia and Katzen (1968) and Macchia, Tamburrini and Pastan (1970) have purified a specific sphingomyelinase (MW 30,000) that was not activated by Ca^{2+} and was distinct from α -toxin. Moreover, Matsumoto (1961) proposed that α -toxin preparations might contain different enzymes hydrolysing PC, sphingomyelin and phosphatidyl ethanolamine (PE).

Using the technique of isoelectric focusing, Bernheimer *et al.* (1968) and Sugahara and Ohsaka (1970) demonstrated the existence of two forms of α -toxin differing in charge with *pI*s of 5.2 and 5.5 and 5.3 and 5.6 respectively. In the present electrofocusing study we have attempted to investigate (a) the relationship of these two forms to each other, (b) the production of milligram quantities of purified toxin, (c) the distribution of other extracellular products of *C. perfringens* in the *pH* gradient, (d) the molecular weight of purified α -toxin, and (e) the biological properties of these forms of α -toxin activity. Such studies required standardisation of the methods of production and concentration of α -toxin.

MATERIALS AND METHODS

Bacteria. Two strains of *Clostridium perfringens* type A were used throughout these studies. Strain S107 was obtained from the National Collection of Type Cultures, Colindale (NCTC 8237), and Strain BP6K (ATCC 10543) from the National Collection of Industrial Bacteria, Aberdeen (NCIB 8875).

Media. Two media used for toxin production were dispensed in 500-ml screw-capped bottles. The medium of Adams, Hendee and Pappenheimer (1947) as modified by Bangham and Dawson (1962) was further modified as follows, and is referred to as M-I. DL-tryptophan and L-cystine hydrochloride were used in the basal salts solution. Tryptone (Oxoid Limited) was substituted for Tryptone (Oxo Ltd). Thioglycollic acid at a final concentration of 1% (v/v) was neutralised by addition of 1N-NaOH before sterilisation by Millipore filtration and subsequent addition to the sterilised medium. Dextrin was autoclaved at 121°C for 15 min. as a 10% (w/v) aqueous suspension. Basal medium was adjusted to *pH* 7.5 before being autoclaved at 121°C for 15 min.

The medium of Murata, Yamada and Kameyama (1956) as modified by Murata,

Yamamoto, Soda and Ito (1965) was further modified as follows, and is referred to as M-II. Fructose was sterilised by Millipore filtration as a 10% (w/v) aqueous solution and added to the autoclaved basal medium. The vitamin complex, stored as a tenfold concentrate at -20°C , was used as required for the basal medium. Basal medium was autoclaved at 121°C for 15 min. Thioglycolic acid was prepared as described for M-I.

Addition of antifoam to M-I and M-II. The addition of Silcolapse 5000 (I.C.I. Ltd) as a 10% (v/v) solution, sterilised by autoclaving at 121°C for 15 min., to a final concentration of 0.04% to both media prevented foaming due to prolific gas production.

Measurement of bacterial growth. Growth of strains S107 and BP6K was monitored as the $E_{485\text{nm}}$ of the culture medium. Where the E_{650} exceeded 0.400 the culture was diluted with formal saline until a reading within the range 0.050–0.400 was obtained. Uninoculated medium or an appropriate dilution served as a blank. Care must be taken during aseptic sampling, because flaming the neck of culture bottles can ignite hydrogen.

Preparation and harvesting of toxin. For the production of a batch of toxin, a freeze-dried ampoule of S107 or BP6K was opened and subcultured twice through Robertson's Meat Medium (Oxoid) containing 1% (w/v) dextrin or fructose for M-I and M-II respectively. A 20-ml volume of a 6- to 8-hour culture in M-I or M-II was used to seed each 500 ml of complete medium. The starter cultures were sedimented at 2750g for 10 min. and the bacterial pellets were resuspended in 5 ml of complete medium before addition to the culture bottles, which were then incubated for 4–5 hours at 37°C . The cultures were then cooled in an ice-bath for 10 min. and centrifuged at 18,000g for 15 min. at 4°C . Supernatant fluids were pooled and kept at 4°C .

Concentration of toxin preparations. Four different salt- and solvent-precipitation procedures were used to obtain toxin concentrates from culture supernatant fluids, namely, acetone precipitation in the presence of calcium phosphate (after van Heyningen, 1941b), ammonium-sulphate precipitation at 75% saturation (Bangham and Dawson, 1962), methanol precipitation (Roth and Pillemer, 1953) and sodium-chloride saturation at pH 4.3–4.4 (Shemanova *et al.*, 1965).

Fractional ammonium-sulphate precipitation. Culture supernatant-fluids were cooled at 4°C . Solid ammonium sulphate was added to give an initial 30% saturation. Saturation was increased by 10% steps to 70% saturation (Dawson *et al.*, 1969). After 2 hours at 4°C each resulting fractional precipitate was harvested by centrifugation at 38,000g for 20 min. at 4°C . Precipitates were dissolved in ice-cold distilled water and dialysed overnight at 4°C against several changes of distilled water. Preparations were clarified by centrifugation if necessary.

C. perfringens type-A toxin (Wellcome). Laboratory standard type-A test toxin batch no. AGX 1846 was kindly supplied as a freeze-dried powder by Mrs Irene Batty and Dr R. O. Thomson of Wellcome Research Laboratories, Beckenham, Kent. This material when re-constituted in 10-ml volume was stated to contain 4 units of α -toxin per 0.23 ml (mouse test). One LV unit was contained in 0.52 ml [1 LV unit is the amount of toxin that releases 100 μg of acid-soluble phosphorus from a lecithin emulsion in 15 min. at 37°C (Macfarlane and Knight, 1941)].

Electrofocusing. The method of McNiven, Owen and Arbuthnott (1972) was used with certain modifications. Toxin dialysed against 1% (w/v) glycine was applied in the less dense solution during preliminary studies, but was later applied solely in the dense solution or in both solutions in the case of preparative-scale experiments to maximise loading of crude material onto the electrofocusing columns. Up to 650 mg of protein were applied to the LKB 8101 column (110-ml capacity) and up to 3.87 g to the LKB 8102 column (440-ml capacity). Each run had a final potential of 800V and the period of focusing varied between 42 to 72 hours at 4°C . Fractions of 2 ml (8101 column) and of 4 ml (8102 column) were collected with an LKB 7000 Ultrarac fraction collector. In most experiments, columns were drained by introducing a peristaltic pump in the outlet line. The 8101 and 8102 columns were drained at flow rates of 80 ml and 180 ml per hour respectively. Carrier ampholytes (LKB Ampholine) giving pH ranges from 3–10, 4–6 and 5–8 were employed at a final concentration of 1% (w/v) in sucrose-density gradients. Electrofocusing in 6M urea was performed as described by McNiven *et al.* (1972). All pH measurements were made at 4°C against

pH 7.0 standard buffer (Electronic Instruments Ltd) prepared on the day of use and cooled to 4°C with the fractions under test. Fractions were assayed without prior dialysis to remove Ampholine and sucrose.

pH measurements in the presence of 6M Urea. Urea is known to raise the pH of solutions (Burk and Greenberg, 1930; Levy, 1958; Bull *et al.*, 1964), but its effect on the pH of carrier ampholytes used to form the pH gradient for isoelectric focusing has only recently been considered. Tests in our laboratory (Smyth, 1972) showed that the pH of the original unfocused solution containing the complete mixture of carrier ampholytes giving the pH range 5–8 at a final concentration of 1% (w/v) was raised by 0.34 of a pH unit by the addition of 6M urea whether the pH was determined at 4°C or 25°C. More recently the effect of urea on the pI of carrier ampholytes throughout the entire pH gradient has been investigated; Ui, (1971*a* and *b*) observed an increase of 0.42 pH units in the pI of carrier ampholytes independent of the pH range used in columns containing 6M urea, whereas Josephson *et al.* (1971) reported a larger increase of 0.9 pH units in the pI of carrier ampholytes throughout the pH range 3–6 in 7M urea. Thus, although differing by degree, both groups have confirmed that urea causes an upward shift in the pH of the ampholyte gradient and that correction must be made for the observed pIs of proteins when electrofocusing is performed in its presence. Because the absolute value of the correction factor to be applied to the observed pIs is far from clear, making comparison of pIs observed in the presence and absence of urea difficult, it was felt more appropriate at present to correct for the effect of urea on pH by its inclusion in the standard buffer used to calibrate the pH meter. Thus the pH-7.0 standard buffer was made 6M with respect to urea and the pH meter calibrated according to the pH value given by the manufacturer for the buffer in the absence of urea at 4°C.

Concentration of fractions for immunoelectrophoresis and disk-gel electrophoresis. Removal of Ampholine and concentration of protein was achieved as described by McNiven *et al.* (1972). Harvested precipitates were redissolved in ice-cold distilled water and dialysed overnight at 4°C.

Disk-gel electrophoresis. Disk-gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) in 11.667% (w/v) acrylamide separating gels containing 0.153% (w/v) N,N'-methylenebisacrylamide was performed as described by McNiven *et al.* (1972). The Hjertén numerals for gel density were T = 11.82% and C = 1.29% (Maurer, 1971). The molecular weight of α -toxin was determined by the method of Shapiro, Viñuela and Maizel (1967) as described by McNiven *et al.* (1972).

Double-diffusion tests. The method of McNiven *et al.* (1972) was used.

Immunoelectrophoresis. This was performed on siliconised glass sheets (20×10 cm) covered with a layer of 1% Ionagar no. 2 (Oxoid) 1.0–1.5 mm thick containing 0.01% (w/v) merthiolate in 0.05M barbitone buffer, pH 8.4. The plates were allowed to set on a Shandon levelling platform and refrigerated overnight. Troughs 0.4 cm wide by 7 cm long and wells 0.4 cm in diameter were cut out of the agar with a no. 1 cork borer, a scalpel and a ruler. The distance between the troughs and the wells was 0.4 cm. A drop of bromothymol blue added to one well acted as an electrophoretic marker. Separation was achieved with a potential of 100–150 V for 5–6 hours, the gel being cooled with an ice-bath. The agar was then removed from the troughs, antiserum was placed in the troughs and diffusion of antiserum and antigens allowed to take place over the central compartment of the electrophoretic tank or in a plastic box lined with moist blotting paper at room temperature.

Standard antiserum. *C. perfringens* type-A diagnostic serum was obtained from Wellcome Laboratories, Beckenham.

Zinc content of α -toxin preparations. Preparations for analysis were concentrated by the method of McNiven *et al.* (1972) and redissolved in distilled water. Samples were dialysed exhaustively against deionised water for 48 hours (conductivity less than 0.1 μ mhos). All glassware used had been cleaned with Decon 90 (Decon Laboratories Ltd) and rinsed thoroughly with distilled water and deionised water. Zinc was determined by atomic emission spectroscopy. The electrodes were of graphite, samples being dried into wells drilled in the ends of these electrodes. Specimens were excited in a carbon arc at 5A d.c. with an electrode gap of 4 mm and the ultraviolet spectra recorded between 370 and 240 nm

by the use of quartz optics. Analyses were performed in the Department of Geology, University of Glasgow.

Protein. This was determined by the method of Lowry *et al.* (1951), with lysozyme (Sigma, grade I) or bovine serum albumin (BSA, Sigma, type F) as a standard. The protein content of fractions from electrofocusing experiments was monitored as $E_{280}^{1\text{cm}}$.

Assay of α -toxin

The α -toxin diluent used was 0.02M 3,3(β,β) dimethylglutaric acid (Koch-Light, Puriss)-NaOH buffer, pH 7.2, containing 5mM calcium acetate and 1 mg per ml BSA (fraction V, Armour Pharmaceuticals). This is referred to as DICaB. α -Toxin was assayed by four methods.

1. *Turbidity produced in a saline extract of egg yolk.* The substrate was prepared by the method of Kushner (1957), or from egg-yolk emulsion (Oxoid) diluted 1 in 10 with 0.85% NaCl. In both cases the saline extracts were adjusted to neutrality with 0.1-N NaOH and clarified by Seitz filtration. These preparations were made on the day of use and stored at 4°C until required. To screen large numbers of fractions, spot tests containing 0.1 ml test fraction, 0.9 ml DICaB and 1.0 ml egg-yolk extract were set up and incubated at 37°C for up to 1 hour and the presence or absence of opacity recorded. Only those fractions that produced distinct turbidity or the separation of fat globules were selected for titration. Serial doubling dilution titrations of α -toxin were made in 1.0-ml volumes of DICaB. Pipettes were changed every third tube. Tubes without α -toxin served as controls. To each tube 1.0 ml of saline egg yolk extract was added and titrations were incubated at 37°C for 30 min. The highest dilution causing a distinct turbidity was taken as the end-point. On the basis of these results, fractions or test samples were suitably diluted in DICaB. Volumes of diluted toxin from 0.0 to 1.0 ml were further diluted with DICaB to a final volume of 1.0 ml and then incubated at 37°C for 2 min. before the addition of 1 ml of pre-warmed saline egg-yolk extract. Each series of tubes was incubated at 37°C for 30 min., placed immediately in an ice-bath and the $E_{520}^{1\text{cm}}$ of each dilution determined. These extinction values were plotted against the volume of diluted toxin in each tube. One unit of egg-yolk turbidity activity of α -toxin (ETU) was defined as that amount of α -toxin that produced $E_{520} = 0.500$ under the above conditions.

2. *Hot-cold haemolysis.* A standardised sheep-erythrocyte suspension (SSES) was prepared in the following manner. Sheep erythrocytes in Alsever's solution were obtained from Oxoid Ltd. Blood showing considerable haemolysis on first sedimentation was discarded. Erythrocytes were washed three times with 0.85% (w/v) NaCl and the packed erythrocytes were resuspended as a 1% (v/v) suspension in 0.85% NaCl. To 3 ml of DICaB, 1 ml of the suspension was added and the resulting suspension was haemolysed by the addition of saponin powder (BDH Chemicals Ltd). After centrifugation at 2100g for 5 min. the $E_{550}^{1\text{cm}}$ of the supernatant fluid was recorded against the supernatant of an unlysed suspension. This was the E_{550} upon 50% lysis. The erythrocyte suspension was then adjusted with 0.85% NaCl or packed erythrocytes such that the E_{550} equivalent to 50% haemolysis was 0.250.

Serial doubling dilutions of test fractions were made as described for the turbidity assay. To each tube, 1 ml of SSES was added. Sets of tubes were incubated at 37°C for 30 min., then at 0°C in an ice-bath for a further 30 min. Settled erythrocytes were resuspended every 10 min. during both phases of the titration. The titre was recorded as a visual 50% haemolysis end-point, read by comparing tubes showing haemolysis with the control tube (0% haemolysis). Alternatively, the 50% haemolysis end-point was determined by sedimenting the unlysed erythrocytes at 2100g for 5 min. at 4°C and comparing the haemoglobin released into the supernatant fluid with 40, 50 and 60% haemolysis standards prepared by dilution of a 100% haemolysed suspension. Gradient-dilution titrations were performed as previously described for the turbidity assay with 1 ml of SSES added to each tube and incubated as described for the doubling dilution titrations. Unlysed erythrocytes were sedimented at 2100g for 5 min. at 4°C and the haemoglobin released measured as the $E_{550}^{1\text{cm}}$ of supernatant fluids. E_{550} was plotted against the volume of diluted toxin in each tube and the dilution causing 50% haemolysis determined by interpolation. One haemolytic unit (HU) of α -toxin

was defined as that dilution causing haemolysis of 50% of the erythrocytes in 1 ml of SSES under the above conditions.

3. *Lethality titrations in mice.* Test preparations of α -toxin were diluted to contain 1 egg-yolk turbidity unit (ETU) per ml with DICA_B to provide a suitable starting dilution. Each test was performed with 10 male mice weighing 20–22 g (Tuck, Rayleigh, Essex). Pairs of mice were challenged intravenously with 0.2-, 0.25-, 0.3-, 0.4- and 0.5-ml volumes of diluted toxin. Dilutions of toxin were made just before injection and kept in an ice-bath during the period of injection. Pairs of mice were given 0.5 ml of control fluid. In testing fractions from electrofocusing columns, the control fluids were 60% (w/v) sucrose containing 1% (w/v) ampholine of the appropriate pH range in distilled water and a 1 in 4 dilution of this with DICA_B. In the case of urea columns the sucrose-ampholine mixture was made 6M with respect to urea. On other occasions, DICA_B was used as the control fluid. One lethal unit (LU) of α -toxin was defined as the amount of toxin that killed one or both mice in 24 hours.

4. *Release of water-soluble phosphorus from a phospholipid emulsion.* Crude egg-yolk phospholipid was prepared by the acetone, ethyl-alcohol, petroleum-ether extraction procedure of Hanahan, Turner and Jayko (1951). All solvents were Analar grade. This phospholipid substrate was analysed by thin-layer chromatography and contained, in addition to PC, PE, sphingomyelin and lysolecithin. An emulsion was prepared by sonic treatment of 120 mg of egg-yolk phospholipid in 20 ml of DICA_B containing only 2.5 mM Ca²⁺ in a round-bottomed flask in an ultrasonic cleaner (Allan and Hanbury, London). All glassware was cleaned with chromic acid. The assay system comprised 0.5 ml of phospholipid emulsion and 0.1 ml of test fraction or a dilution thereof in DICA_B (2.5 mM Ca²⁺). Assays in triplicate were incubated at 37°C for 30 min. without shaking. After incubation, 0.2 ml of 5% (w/v) bovine serum albumin (BSA) in distilled water and 1 ml of 10% (w/v) trichloroacetic acid (TCA) were added. Tubes were placed in an ice-bath until centrifuged at 4°C at 2100g for 5 min. This precipitation procedure greatly aided removal of diglyceride and unhydrolysed phospholipid emulsion. Supernatant fluids were decanted into micro-Kjeldahl flasks (30-ml) and the precipitates were washed once by resuspension in 1-ml volumes of ice-cold 10% TCA. After centrifugation, washes were combined with the parent supernatant fluids. To each flask, 1 ml of 10-N H₂SO₄ was added and the contents digested until the H₂SO₄ fumed. After cooling, a few drops of 100-vol MAR grade H₂O₂ were added and the flasks were reheated until bubbling stopped and the acid started fuming again. If the contents of the flasks were still discoloured this procedure was repeated. Phosphate was determined by the procedure of Allen (1940) modified as follows. The contents of each flask were made up to 12.5 ml by addition of 10.8 ml of distilled water, 1.0 ml of 1% (w/v) amidol in 20% (w/v) sodium metabisulphite and 0.5 ml of 8.3% (w/v) ammonium molybdate, added in that order. After 10 min. but not more than 30 min., the $E_{410}^{1\text{cm}}$ of each solution was read against distilled water, the results averaged and the extinction of appropriate blanks subtracted. The phosphate released in each case was determined from a standard curve plotted for amounts of inorganic phosphate between 0–120 μg . One phospholipase-C unit (PCU) of α -toxin was defined as that amount that caused the release of 1 μmole (30.2 μg) of phosphate from the phospholipid emulsion per min. One PCU is approximately equal to 4.5 LV units of α -toxin (Macfarlane and Knight, 1941).

Assay for θ -toxin

A haemolytic assay based on the method of Roth and Pillemer (1955) was employed. The SSES was prepared as described for the hot-cold haemolytic assay for α -toxin. The θ -toxin diluent was the 0.07 M phosphate-buffered saline (PBS) of Roth and Pillemer (1955). Fractions or preparations assayed for θ -toxin were first reactivated: 0.1 ml of test material, 0.9 ml of PBS and 0.25 ml of 0.1 M cysteine hydrochloride neutralised with 1N NaOH were incubated at 37°C for 15 min. and then 0.75 ml of PBS was added. Thus the starting dilution for all titrations was 1 in 20. Large numbers of fractions were screened by the method of McNiven *et al.* (1972). Serial doubling-dilution titrations were set up in 1.0-ml volumes as described for α -toxin with PBS as diluent. Titrations were incubated at 37°C for 30 min.

The highest dilution causing 50% haemolysis was recorded as the titre. Two controls were included with each set of titrations: (a) contained diluent+erythrocytes, and (b) diluent treated as for toxin activation+erythrocytes. Gradient-dilution titrations were set up as described for α -toxin with an appropriate dilution of activated θ -toxin in PBS. Again titrations were incubated at 37°C for 30 min. The volume of diluted toxin causing 50% haemolysis was determined as described for α -toxin. One haemolytic unit (HU) of θ -toxin was defined as the dilution of toxin that caused 50% haemolysis under the above conditions.

Assay for hyaluronidase

Hyaluronidase or hyaluronate lyase—E.C. 3.2.1.35—was assayed by the modification of the turbidity-reduction assay method of Dorfmann (1955), as described by Miles-Seravac in their brochure "Enzymes and Related Biochemicals". The assay was performed with half the volumes stated (final volume = 5 ml). Highly polymerised hyaluronic acid was obtained from Miles-Seravac. The hyaluronic-acid solution for use in the assay was made up so as to give an $E_{510}^{1\%} = 0.250$ when 0.5 ml of this solution was treated as a substrate control for the assay. Fractions were compared against an international standard preparation of testicular hyaluronidase standardised by the method of Humphrey (1957). Grade-I ovine hyaluronidase was obtained from Miles-Seravac. Values of hyaluronidase activity were expressed as International Units (IU).

Assay for collagenase

Collagenase—E.C. 3.4.4.19—was assayed quantitatively with azocoll powder (Wellcome Reagents) as substrate. Ten milligrams of powder were weighed out into 4-in. $\times$ $\frac{1}{4}$ -in. test-tubes and 1.9 ml of 0.05 M Tris-maleic acid buffer, pH 7.0, added. Tubes were incubated at 37°C for 5 min. and 0.1 ml of test solutions added. Incubation was continued for 1 hour. Assays were performed in duplicate. The tubes were agitated every 10 min. to resuspend settled azocoll powder. The azocoll powder was sedimented at 2100g for 2 min. and the $E_{510}^{1\%}$ of each supernatant fluid was measured. The average E_{510} of control supernatant fluids from mixtures of 10 mg azocoll powder and 2 ml buffer incubated under identical conditions was subtracted from the test values. The relationship between dye release (E_{510}) and collagenase concentration in tests with a preparation of *C. histolyticum* collagenase (Koch-Light, form II) was linear for E_{510} values between 0.000–0.250 under the above conditions. One azocoll-digesting unit (AU) of collagenase was defined as that amount of enzyme that caused an increase in the E_{510} of supernatant fluid of 0.010 per hour under the above conditions.

The identity of the enzyme causing digestion of azocoll powder as a true collagenase was confirmed by the collagen disk assay of Delaunay, Guillaumie and Delaunay (1949) as modified by Smyth (1972). Disks of collagen paper were cut with an 8-mm diameter cork borer (Fisons, Loughborough, No. 5) against a glass surface. Only disks weighing between 90 and 140 μ g were used; within any group of tests disks used fell within a 10- μ g range.

Such collagen disks were resistant to attack by chymotrypsin (Sigma, type II), proteinase (Koch-Light), subtilisin (Sigma, type-VII protease) and trypsin (Sigma) for up to 24 hours; they were readily attacked by collagenase (Koch-Light, form II) and pronase (Koch-Light) and slowly by ficin (Sigma) in 8 hours. Papain (Sigma) caused dissolution after 24 hours but did not cause visible changes within 8 hours. All enzymes were used at a final concentration of 1 mg per ml under assay conditions.

For collagenase tests, WHO complement-fixation trays were used. Each well contained 0.1 ml of test fraction and 0.9 ml of 0.05-M Tris-maleic-acid buffer, pH 6.5, containing 0.145 M NaCl and 1 mM calcium acetate. A rat-tail collagen disk was added to each well, the wells sealed with Sellotape and the trays incubated at 37°C. Plates were examined each hour for up to 8 hours. Visible dissolution of the collagen disks was taken to indicate collagenase activity. Controls comprised disks suspended in 1 ml of buffer.

Thin-layer chromatography (TLC) of phospholipids

TLC plates were prepared with a layer thickness of 0.25 mm. The slurry consisted of 36 g silica gel G (Merck), 48 ml distilled water and 24 ml methanol (Analar). Plates were activated before use by heating at 110°C for 20–30 min. Samples were applied 2 cm from the bottom edge. The TLC plates were developed with chloroform:methanol:water in the ratio 65:35:5. Development was terminated 14 cm from the origin. The plates were sprayed with 50% sulphuric acid and heated at 110°C for 10 min. to develop lipid spots.

Substrate specificity of purified α -toxin

L- α -lecithin (General Biochemicals) and sphingomyelin (L. Light & Co. Ltd) were emulsified by sonic treatment in a round-bottomed flask by means of an ultrasonic cleaner (Allan and Hanbury, London) with DICA_B containing only 2.5 mM Ca²⁺. The PC was chromatographically pure on TLC, but the sphingomyelin showed two additional spots at the solvent front. The nature of these was not resolved, but they were not contaminating PC, PE, phosphatidyl serine, phosphatidyl inositol or lysolecithin. Assay emulsions contained 1 mg PC and 1.32 mg sphingomyelin per ml. The reaction mixtures comprised 0.5 ml of emulsion, 0.4 ml DICA_B (2.5 mM Ca²⁺) and 0.1 ml α -toxin. Tests were set up in duplicate. Controls contained substrate, buffer and distilled water. One tube of each pair was assayed for phospholipid breakdown by the determination of water-soluble phosphorus (*vide supra*). To the duplicate tube of each assay, 10 ml of chloroform was added, the contents mixed thoroughly and the chloroform extracts evaporated to dryness under nitrogen. Each extract was then taken up in 0.2 ml chloroform and TLC was performed.

RESULTS

Toxinogenesis during the growth cycle

The production of α -toxin, θ -toxin, collagenase and hyaluronidase during growth was studied to determine the optimal time for harvesting the culture for α -toxin. Results of a typical experiment with M-II medium are given in fig. 1. Exponential growth began immediately after inoculation; θ -toxin and hyaluronidase production began shortly after initiation of logarithmic growth; there was a significant lag before α -toxin and collagenase were detected in the culture supernatant fluids; and production of all four extracellular products decreased as the culture entered the stationary phase. This sequence of toxin and enzyme production was observed with both strains S107 and BP6K grown in either M-I or M-II medium. Continued incubation for up to 10 hours resulted in little further growth as measured turbidimetrically. The titre of α -toxin declined, but not that of θ -toxin. The effect of prolonged incubation on hyaluronidase and collagenase production was not investigated. In the preparation of a number of batches of toxin the E₆₅₀ of cultures was found to increase to values between 14.0 and 15.0 in 4 to 5 hours, but this increased growth did not always result in a similar increase in toxin titres. Generally α -toxin yields in M-II medium were higher by a factor of 2 or 3 times than those observed with M-I medium.

Concentration of α -toxin

A summary of a study with culture-supernatant fluid from strain BP6K grown in M-II medium is shown in table I. Whereas the procedures of van Heyningen (1941b) and Bangham and Dawson (1962) yielded approximately

50% of the activity in culture-supernatant fluids, those of Shemanova *et al.* (1965) and Roth and Pillemer (1953) gave poor recoveries. The procedure of

ETU = egg-yolk turbidity units; IU = international units; AU = azocoll units; HU = haemolytic units (see *Methods*).

FIG. 1.—Toxinogenesis of *Clostridium perfringens* strain BP6K in M-II medium. ●—● = growth measured turbidimetrically at 650 nm; ○—○ = θ -toxin activity; □—□ = α -toxin activity; ■—■ = hyaluronidase (μ -toxin) activity; △—△ = collagenase (κ -toxin) activity.

van Heyningen was used to prepare toxin for most of the preliminary electrofocusing studies.

However, ammonium sulphate precipitation was chosen for later work because it was more convenient when large volumes were handled. Because

TABLE I

*Comparison of methods for the concentration of α -toxin from culture supernatant fluids of strain BP6K**

Method of	Total activity recovered (PCU)*	Percentage recovery	Total protein (mg)	Specific activity (PCU per mg)	Purification factor ($\times$)
van Heyningen (1941 <i>b</i>)	141	54	43	3.3	33
Bangham and Dawson (1962)	146	56	302	0.5	5
Roth and Pillemer (1953)	35	14	33	1.1	11
Shemanova <i>et al.</i> (1965)	37	14	79	0.5	5

* The 2.6 g of starting material contained a total of 262 phospholipase-C (PCU) units. Specific activity = 0.1 PCU per mg of protein.

TABLE II

Yield of toxins on fractional ammonium-sulphate precipitation of culture supernatant fluids of strain BP6K

Percentage saturation with (NH ₄) ₂ SO ₄	Yield and percentage recovery of the stated activity*							
	α -Toxin		θ -Toxin		Collagenase		Hyaluronidase	
	ETU $\times 10^3$	Percentage recovery	HU $\times 10^6$	Percentage recovery	AU $\times 10^3$	Percentage recovery	IU $\times 10^3$	Percentage recovery
0	47.5	...	1.28	...	120	...	630	...
0-30	0.8	1.7	0.02	1.9	1.3	1.1	10	1.8
30-40	4.7	9.7	0.18	14.4	6.1	5.0	7	1.3
40-50	21.1	44.5	0.23	18.3	22.7	18.9	31	4.5
50-60	0.3	0.7	0.65	50.4	10.1	8.3	426	67.7
60-70	0	0	0.14	11.0	3.6	3.0	112	17.7
Total recovery	26.9	56.6	1.22	95.0	43.8	36.5	586	93.0

* See footnote to fig. 1.

the 75% saturation recommended by Bangham and Dawson (1962) yielded material of low specific activity (table I), culture-supernatant fluids were fractionated in narrow steps with increasing amounts of ammonium sulphate and each fractional precipitate was assayed for α -toxin, θ -toxin, collagenase and hyaluronidase. The total activity recorded in each fractional precipitate and the corresponding percentage recoveries are given in table II. Whereas most of the α -toxin and the collagenase were precipitated between 40 and 50% saturation, the major fractions of θ -toxin and hyaluronidase activity did not precipitate until a saturation value of more than 50% was obtained. Further

investigations at percentage-saturation values of between 30 to 50 were made to determine the best combination of recovery and purification. The results of one investigation are shown in table III. A fractional ammonium sulphate precipitate between 35 to 50% saturation was optimal and was used in all preparative-scale electrofocusing experiments.

Electrofocusing of S107 and BP6K α -toxin preparations

Fig. 2 illustrates the separation of α -toxin from θ -toxin, in particular, and from collagenase and hyaluronidase; a broad pH gradient of 3–10 was used. Table IV summarises the pI and recovery in each case. Similar results were obtained with toxin from strain S107. The peak fraction of α -toxin was separated from the bulk of collagenase and hyaluronidase activity, but the pooling of

TABLE III

Precipitation of α -toxin from culture supernatant fluids of strain BP6K with ammonium-sulphate saturation between 30 and 50%

Percentage saturation (NH ₄) ₂ SO ₄	Total activity per fraction (ETU)*	Total protein per fraction (mg)	Specific activity (ETU per mg)	Percentage recovery	Purification factor (×)
0	25,000	15,100	1.6	...	...
30–45	7,000	410	17	28	10
30–50	14,300	490	29	57	18
35–50	13,500	450	30	54	19

* ETU = egg-yolk turbidity units.

fractions with significant α -toxin activity resulted in contamination with these factors. The α -toxin and θ -toxins were separated, but some 10% of the total θ -toxin recovered was found in fractions containing α -toxin. In broad pH gradients the α -toxin was eluted as a single peak of activity with a pI around 5.2. Yields of α -toxin were usually lower than that achieved in the experiment shown, being of the order of 5 to 15%. Indeed, on some occasions with toxin prepared by the procedures of van Heyningen (1941*b*) and Bangham and Dawson (1962) no α -toxin activity was recovered after electrofocusing. The method of toxin preparation did not affect the general elution pattern of these toxins or their pIs.

It seemed likely that better resolution of these four factors could be achieved by using a narrow pH gradient of pH 4–6, because they were all acidic proteins focusing around this pH range. Fig. 2 shows that most of the protein in crude preparations of toxin focused at or near the anode compartment. Accordingly, toxin for electrofocusing was applied when possible in the dense solution of the sucrose gradient to facilitate equilibration of the pH gradient and to minimise any possibility of the separated components being distributed by settling of such anodic precipitates. The suitability of a gradient of pH 4–6 for the preparation of highly purified α -toxin is demonstrated by the elution profile of one such experiment (fig. 3); a high resolution was achieved. Most of the recovered

FIG. 2.—Electrofocusing of α -toxin, θ -toxin, collagenase and hyaluronidase from *C. perfringens* strain BP6K in pH 3–10 gradient; 47 mg of toxin harvested from M-II medium by acetone precipitation were applied to an 8101 column. The two parts of this figure illustrate the elution profile of a single experiment. Duration of run was 67 hours = pH; ●—● = extinction at 280 nm; $\triangle$ — $\triangle$ = collagenase activity; $\square$ — $\square$ = α -toxin activity; ■—■ = hyaluronidase activity; ○—○ = θ -toxin activity.

α -toxin resided in fractions from pH 5.1 to 5.6 with the peak of activity at a *pI* of 5.50. A pronounced shoulder on the acidic side of the α -toxin-elution profile from pH 5.1 to pH 5.4 should be noted; in other electrofocusing experiments in the same pH gradient a second α -toxin peak of activity was clearly resolved in this region of the gradient. Fractions 38 to 47 (fig. 3) were pooled and concentrated by dialysis against 70% saturated ammonium sulphate. The resulting precipitate was harvested, dissolved in distilled water, dialysed against 1% (w/v) glycine and refocused in a pH gradient of pH 5–8. The elution profile is shown in fig. 4. Two peaks of hot-cold haemolytic activity were detected with *pIs* of 5.25 and 5.49. In contrast to earlier experience with broad pH-gradient experiments, yields from both of these electrofocusing runs were as high as 80 and 84% respectively.

TABLE IV

Results of electrofocusing of Clostridium perfringens BP6K toxin concentrate in a broad pH gradient of 3–10

Extracellular product (units*)	<i>pI</i>	Total units of activity applied	Total units of activity recovered	Percentage recovery
α -Toxin (ETU)	5.20	380	120	32
θ -Toxin (HU)†	6.54	2.20×10^5	1.47×10^5	66
Collagenase (AU)‡	4.61	5400	2200	40
Hyaluronidase (IU)§	4.72	2.23×10^4	1.62×10^4	73

* See footnote to fig. 1.

† *pI* = 6.56 ± 0.13 ($\pm$ SD) with average recovery of 72% from eight determinations with toxin from strains S107 and BP6K, and toxin AGX 1846, in pH 3–10 gradients.

‡ *pI* = 4.54 ± 0.14 ($\pm$ SD) with average recovery of 57% from seven determinations with toxin from strains S107 and BP6K, and toxin AGX 1846, in pH 3–10, 3–6 and 4–6 gradients.

§ *pI* = 4.73 ± 0.05 ($\pm$ SD) with average recovery of 56% from eight determinations with toxins from strains S107 and BP6K, and toxin AGX 1846, in pH 3–10, 3–6 and 4–6 gradients.

Toxin concentrates prepared by harvesting the 35 to 50% saturation ammonium-sulphate precipitate from culture-supernatant fluids were focused on a preparative scale in the 440-ml column. Up to 3.87 g of such preparations was applied to a single column; both the heavy and the light solutions were used for the application of a large volume of sample—up to 310 ml. The average recovery of α -toxin was 60% of the activity applied. During these preparative-scale electrofocusing experiments, large amounts of precipitate formed at the anode but these did not interfere with the drainage of columns nor, with one exception, did they cause trailing of this acidic material into fractions containing α -toxin. Although the anodic precipitates did not normally stick to the sides of the separation chamber, it is recommended that columns be siliconised. These anodic precipitates were harvested by centrifugation at 38,000*g* from one such preparative-scale experiment and analysed for the presence of protein, DNA and RNA; only protein was detected. The nature of these components was not further investigated. The major electrophoretic component of α -toxin had a *pI* of 5.49 ± 0.06 ($\pm$ SD), the average of 10 determinations in narrow

pH gradients. The minor peak of α -toxin activity had an average pI of 5.25 and accounted for some 2–3% of the recovered activity in any one experiment.

FIG. 3.—Separation of α -toxin, θ -toxin, collagenase and hyaluronidase from *C. perfringens* strain BP6K by electrofocusing in pH 4–6 gradient. 650 mg of precipitate obtained at 35–50% ammonium-sulphate saturation harvested from M-II medium were applied to an 8101 column. Duration of run was 70 hours. ●—● = extinction at 280 nm; △—△ = collagenase activity; ■—■ = hyaluronidase activity; □—□ = α -toxin activity; ○—○ = θ -toxin activity.

The major component was designated α_A and the minor α_B . From 20–30% of the total recovered α -toxin activity was usually obtained in a single α_A peak

4-ml fraction from preparative experiments. This represents 10–15% of the α -toxin activity detected in the original culture supernatant fluids precipitated by fractional ammonium sulphate saturation.

Purity and properties of purified α -toxin

Examination for the presence of other extracellular products of C. perfringens. Collagenase, hyaluronidase and θ -toxin were not detected in α_A preparations obtained from single peak fractions from pH 4–6 gradients. These preparations were also free from neuraminidase activity (Collee, Fraser, Smyth and Arbuthnott, unpublished result). Deoxyribonuclease activity could not be detected in

FIG. 4.—Refocusing of α -toxin from a narrow pH gradient in pH 5–8 gradient. Duration of run was 49 hours. = pH; ●—● = extinction at 280 nm; □—□ = α -toxin activity.

partially purified preparations assayed by the methods of Kunitz (1950) and Wadström (1967). Insufficient α_B was obtained in these studies for comparable analyses.

Serological tests for homogeneity. Immunoelectrophoresis of α_A and α_B preparations obtained from peak activity fractions from preparative electrofocusing experiments indicated that such preparations were homogeneous as gauged by the use of standard commercial antitoxin. Only one precipitin arc was observed anodic to the origin (fig. 5). Antistreptolysin O did not give rise to a precipitin arc in either case. α_A and α_B showed a single line of identity in Ouchterlony double-diffusion tests.

SDS-polyacrylamide disk-gel electrophoresis. Disk-gel electrophoresis of α_A in polyacrylamide containing SDS revealed one major protein band with two minor bands (fig. 6). The protein concentration of α_A applied to the gels was high so as to reveal the presence of trace amounts of impurities. The

approximate molecular weight of α_A was determined as $53,800 \pm 1400$ ($\pm$ SE). Electrophoresis of α_B under identical conditions revealed two bands lying close together, the lower of which corresponded to that seen in α_A preparations. The second band had a molecular weight of $60,400 \pm 1000$ ($\pm$ SE). A number of other components were also readily visible. A gel showing electrophoresis of crude toxin is shown for comparison. Twelve components can be seen in the photograph although many more were detected in the original gel.

Substrate specificity of α_A and α_B . The phospholipase activities of α_A and α_B were tested with lecithin and sphingomyelin as substrates. The results obtained for phosphate release by two preparations of α_A and α_B from Wellcome AGX 1846

TABLE V

Release of water-soluble phosphorus from emulsions of lecithin and sphingomyelin by preparations of α_A and α_B

α -Toxin preparation	Phosphorus released* (μ g) from	
	lecithin	sphingomyelin
α_B (preparation 1)	4	14
α_A (preparation 1)	13	23
α_B (preparation 2)	6	19
α_A (preparation 2)	12	21
AGX 1846†	34	49
Control emulsion phosphorus content	43	51

* After incubation at 37°C for 30 min.

† Used at a concentration of 100 μ g in assay.

toxin are shown in table V. Moreover, these results could be correlated with the picture obtained by TLC of chloroform extracts of duplicate assays. Under the conditions used, no detectable difference was noted between α_A and α_B . Both preparations hydrolysed lecithin and sphingomyelin.

Zinc content of α -toxin preparations. Two preparations obtained by pooling fractions from single electrofocusing experiments containing 3.50 mg and 0.85 mg of protein respectively were examined by atomic emission spectroscopy for the presence of zinc. In neither were the characteristic emission lines of zinc detected. The limit of sensitivity was 0.2 μ g of zinc per sample.

Electrofocusing studies with Wellcome AGX 1846 toxin

During our preliminary studies with S107 and BP6K toxin in broad pH gradients, the observations of Bernheimer *et al.* (1968) came to our attention. A lyophilised culture filtrate, designated AGX 1846, similar to that used by these authors was obtained from Wellcome Research Laboratories. Because of the limited amount of material available, only a small number of experiments could be carried out.

Material AGX 1846 was focused under conditions similar to those used by Bernheimer *et al.* (1968). An elution profile identical to that obtained by these

FIG. 7

FIG. 8

FIG. 7.—Elution profile of α -toxin on electrofocusing of Wellcome type-A toxin AGX 1846 (10 mg applied in 8101 column) in a pH 5–8 gradient. The three parts of this figure illustrate different measures of α -toxin activity from one experiment: top—egg-yolk-turbidity activity; middle—hot-cold-haemolytic activity; bottom—lethal activity. Duration of the run was 40 hours; = pH; $\square$ — $\square$ = α -toxin activity (ETU, HU, LU); $\bullet$ — $\bullet$ = extinction at 280 nm.

FIG. 8.—Elution profile of α -toxin on electrofocusing of Wellcome type-A toxin AGX 1846 (20 mg applied in 8101 column) in a pH 5–8 gradient in the presence of 6M urea. The three parts of this figure illustrate different measures of α -toxin activity from one experiment: top—egg-yolk-turbidity activity; middle—hot-cold-haemolytic activity; bottom—lethal activity. Duration of run was 46 hours; = pH; $\square$ — $\square$ = α -toxin activity (ETU, HU, LU); $\bullet$ — $\bullet$ = extinction at 280 nm.

authors was observed, with two peaks of α -toxin activity having pI s of 5.26 and 5.55. However, recovery of activity was limited to 31%.

Further experiments were performed to determine whether these two forms of α -toxin were reproducible and whether they possessed hot-cold haemolytic, phospholipase-C and lethal activities. The results of these tests are shown in fig. 7. For clarity, only fractions in which α -toxin activity was detected are shown. Fractions were assayed first for phospholipase-C and hot-cold haemolytic activities and only those containing these activities were further tested for lethal activity in mice. The total recoveries of α -toxin activity by each assay are shown in table VI. These findings demonstrate that (a) all three assays revealed the presence of two peaks of α -toxin activity after electrofocusing, (b) the peaks of activity detected by each assay were coincident, (c) the recoveries of α -toxin activity detected by each assay were of the same

TABLE VI

Recovery of α -toxin electrofocused in narrow pH gradient of 5-8 from preparation AGX1846

Activity	Recovery in terms of			Ratio of activities ETU:HU:LU
	ETU	HU $\times 10^3$	LU	
Total recovered	270	11.6	670	1:43:3
Total applied	1230	37.2	2420	1:40:2
Percentage recovery	22	31	28	...

ETU, HU, see footnote to fig. 1; LU = lethal units.

order, (d) the ratios of the three activities of α -toxin were of the same order, and (e) these ratios compared well with those found for α -toxin in the material applied to the electrofocusing columns; this indicated simultaneous purification of the phospholipase-C, hot-cold haemolytic and lethal activities. The average pI s of these two forms of α -toxin were 5.57 ± 0.04 and 5.23 ± 0.09 ($\pm SE$) corresponding to α_A and α_B respectively.

Electrofocusing of AGX 1846 in 6M urea. The presence of these two forms of α -toxin raised the question of the relationship between α_A and α_B . It seemed possible that they could be related as conformers (molecules with the same amino-acid sequence, but different three-dimensional structures and/or charge) or as polymers or aggregates (molecules from a different number of sub-units). Electrofocusing in the presence of 6M urea was used to determine whether any change in the isoelectric points of these forms of α -toxin occurred. This technique has also been used successfully by McNiven *et al.* (1972) with staphylococcus α -toxin. Furthermore, Ispolatovskaya and Klimacheva (1966) had reported that α -toxin was not inactivated by exposure to urea. The elution profile is shown in fig. 8; only fractions in which α -toxin activity was detected are shown. In contrast to the results obtained in the absence of urea, only one peak of α -toxin activity was observed. Again the peak fractions of α -toxin detected by each assay were coincident, but by contrast with the experiments carried out in the absence of urea, recoveries of α -toxin as detected by the

turbidity assay were considerably lower than those detected by hot-cold haemolysis of lethality assays (table VII). The main conclusion from these experiments was that this form of α -toxin, α_{urea} , possessed all three activities

FIG. 9.—Refocusing of α_{urea} prepared from toxin AGX 1846 by electrofocusing in pH 5–8 gradient (fig. 8). Duration of run was 49 hours; pH gradient 5–8. = pH; $\square$ — $\square$ = α -toxin activity.

associated with α -toxin and an average pI of 5.52 ± 0.16 ($\pm SE$). It thus corresponded to the α_A form found by electrofocusing experiments on toxin from strains S107 and BP6K, and on AGX 1846 toxin, in the absence of urea.

TABLE VII

Recovery of α -toxin AGX1846 electrofocused in a narrow pH gradient of 5–8 in the presence of 6M urea

Activity	Recovery in terms of			Ratio of activities ETU:HU:LU
	ETU	HU $\times 10^4$	LU	
Total recovered	560	6.8	2050	1:120:4
Total applied	1570	12.5	3150	1:80:2
Percentage recovery	36	54	65	...

ETU, HU, see footnote to fig. 1; LU = lethal units.

Refocusing of α_{urea} in the absence of urea. It was of interest to investigate whether the α_B component reappeared on removal of the urea. All fractions containing α -toxin activity from an electrofocusing experiment in 6M urea were pooled, dialysed exhaustively against several changes of 1% glycine for 48 hours at 4°C and refocused in a pH 5–8 gradient. The elution profile of this refocused α -toxin is shown in fig. 9 as assayed by hot-cold haemolysis. The elution

profile of α -toxin detected by the egg-yolk turbidity assay was identical. Insufficient material was available for lethality assays in mice. A major peak corresponding to α_A was detected with a pI of 5.48 together with a minor peak corresponding to α_B with a pI of 5.23. The lack of AGX 1846 toxin prevented further investigation of this observation. Identical results were obtained when α -toxin from strain BP6K was electrofocused in urea and then refocused in the absence of urea.

DISCUSSION

Under appropriate conditions, milligram quantities of highly purified *C. perfringens* α -toxin can be prepared by a simple procedure comprising fractional ammonium-sulphate precipitation of culture-supernatant fluids followed by isoelectric focusing in a pH 4–6 gradient. The technique of electrofocusing offers some advantages over the previously employed electrophoretic techniques of Habermann (1959), Stephen (1961), Bangham and Dawson (1962), and Bernheimer *et al.* (1968); intrinsically it separates and concentrates proteins. In addition, the method removes the necessity for multistage purification-procedures, such as those of Shemanova *et al.* (1968) or Diner (1970), with their low yields. The fewer the number of manipulations needed to obtain highly purified toxin the less likelihood there is of altering its molecular structure.

It is of interest that five of the major extracellular toxins and enzymes of *C. perfringens* type A are acidic proteins with pI s between 4.50–6.50 viz. collagenase, hyaluronidase, α -toxin, neuraminidase (Collee, Fraser, Smyth and Arbuthnott, unpublished result) and θ -toxin. This observation may account for some of the difficulties experienced by previous authors in applying other electrophoretic techniques and ion-exchange chromatography to their resolution. In addition, electrofocusing of α -toxin has revealed the presence of two forms of this toxin with differing pI s. These have been identified by electrofocusing of crude toxin prepared from strains BP6K and S107 and of a Wellcome toxin preparation (AGX 1846) in narrow pH gradients. The two components have been designated α_A and α_B with pI s of 5.50 and 5.25 respectively. That α_A was the main component of α -toxin was evidenced by (a) the observation that in freshly prepared toxin this form constituted 80–90% of the total α -toxin activity recovered, and (b) that α_A was the only form of the toxin observed in electrofocusing experiments carried out in the presence of 6M urea. For this reason it was given alphabetical precedence. This is consistent with the terminology adopted by McNiven *et al.* (1972) for the nomenclature of the multiple forms of staphylococcus α -toxin.

The observation of two forms of α -toxin poses the question of the relationship, if any, between α_A and α_B . Could α_B be an artefact of preparation? If so, how does it arise? Or are α_A and α_B unique molecular species of α -toxin? Possible causes of artefactual multiple forms revealed by the technique of isoelectric focusing are listed in table VIII.

None of these possible causes of multiple forms appears to be relevant to the observations described in this paper. Causes *i-v* result in irreversible alteration of the net charge of proteins. The fact that α_B disappears in the pre-

sence of 6M urea and reappears on the removal of the urea by dialysis indicates that the alteration of charge in this case is reversible. Also, treatments likely to induce deamidation, such as freezing and thawing, storage in the frozen state, and freeze-drying were avoided (Chilson, Costello and Kaplan, 1965; Berson and Yallow, 1966).

Because metal ions were not detected in highly purified toxin preparations examined by atomic emission spectroscopy, possibilities *iii* and *iv* can be excluded. Evidence that the technique of electrofocusing *per se* can cause charge alteration has been presented in only one instance, as described by Pejaudier *et al.* (1971) for ceruloplasmin. Because that protein contains both a carbohydrate moiety and copper ions, causes *ii*, *iii* and *iv* could have contributed to the effect.

TABLE VIII
Possible causes of artefactual multiple forms observed by electrofocusing

Possible cause	Proposed by authors
<i>i</i> Deamidation	Carlström (1966) Flatmark and Vesterberg (1966)
<i>ii</i> Loss of a carbohydrate moiety	Carlström (1966)
<i>iii</i> Alteration in charge of metal ion ligand	Vesterberg (1967)
<i>iv</i> Interaction with ampholines	Haglund (1971)
<i>v</i> Carbamylation	Gerding <i>et al.</i> (1971)
<i>vi</i> Electrofocusing <i>per se</i>	Pejaudier, Audran and Steinbuch (1971)
<i>vii</i> Dehydration in sucrose gradients	Vesterberg (1967)

The findings of Vesterberg (1967) on the behaviour of myoglobin on electrofocusing suggest that the high sucrose concentrations (*vii*) used in the stabilising gradient do not affect the *pI* and degree of heterogeneity.

In a consideration of the relationship of α_A and α_B , two distinct possibilities remain; that α_B is an aggregate of α_A or vice versa, or that α_B is a conformer of α_A . Although the discrepancies observed by various authors for the molecular weight of α -toxin suggest that under certain conditions the toxin can form aggregates, there is no evidence from the present work, or that of Bernheimer *et al.* (1968), that α_A and α_B have different molecular weights. On this basis it seems unlikely that the charge difference between α_A and α_B can be explained in terms of aggregation. The approximate molecular weight found in the current study for α_A and α_B was 53,800 and agrees well with the estimation of Shemanova *et al.* (1968) derived from sedimentation-velocity and diffusion data. However, the molecular weight determined by SDS disk-gel electrophoresis was 90,000 (Casu *et al.*, 1971), whereas values determined by gel filtration (Bernheimer and Grushoff, 1967; Teodorescu *et al.*, 1970) were around 30,000. These discrepancies cannot yet be explained, but they emphasise the need to employ several methods in molecular-weight determinations. The fact that different strains and different purification methods have been employed must also be taken into account.

The use of electrofocusing to detect the presence of conformers has been proposed by Ui (1971*a* and *b*). A reduction in heterogeneity in the presence of

6M urea was taken to suggest that multiple forms observed in the absence of urea were conformationally related. On this basis it is possible to speculate that α_B represents a conformer of α_A in which positive charges are shielded on folding of the molecule, but other physico-chemical techniques would have to be used to test this hypothesis.

Fractions from electrofocusing columns were examined for phospholipase-C, haemolytic and lethal activities. Both α_A and α_B possessed all three properties. The elution profiles of these activities paralleled each other and peak fractions were coincident. Moreover, in 6M urea a similar parallelism was observed in the case of α_A . These findings confirm and extend the electrofocusing studies of Bernheimer *et al.* (1968) and Sugahara and Ohsaka (1970). It should be emphasised that both α_A and α_B hydrolysed lecithin and sphingomyelin and that neither component appeared to correspond to the specific sphingomyelinase described by Pastan *et al.* (1968).

In conclusion, several important points must be made with respect to the use of commercial or crude preparations of α -toxin (phospholipase C) derived from *C. perfringens* in studies of membrane structure and function. The use of phospholipase C as a "specific tool" is only justifiable when this reagent is of the highest purity. Recent studies by means of gel electrofocusing and disk-gel electrophoresis clearly indicate that most commercial preparations of this important enzyme are complex protein mixtures (Möllby, Nord and Wadström, 1973). Indeed, some of the morphological changes observed in membranes treated with commercial α -toxin have been shown to be attributable to contaminating θ -toxin (Smyth, Arbuthnott and Freer, 1972). In our opinion the present method provides a phospholipase C suitable for membrane studies.

SUMMARY

Clostridium perfringens type-A α -toxin (phospholipase C) was purified on a preparative scale by a simple, rapid, two-stage procedure involving precipitation of culture-supernatant fluids with ammonium sulphate at 35 to 50% saturation, followed by isoelectric focusing in a pH 4–6 gradient. Milligram yields of highly purified α -toxin with 10 to 15% recovery of activity were obtained in single fractions.

Two forms of α -toxin were identified by electrofocusing. The major peak of activity possessed a pI of 5.49 ± 0.06 (α_A) and the minor, a pI of 5.25 (α_B). The former was free from detectable collagenase (pI 4.54), hyaluronidase (pI 4.73), θ -toxin (pI 6.56) and neuraminidase. It gave a single precipitin arc on immunoelectrophoresis, but analysis by SDS polyacrylamide disk-gel electrophoresis at high protein-loading revealed a major protein component of molecular weight 53,800 and two minor protein bands. Atomic emission spectroscopy did not detect the presence of zinc in such preparations. The latter showed a single line of identity with α_A in Ouchterlony gel diffusion tests and contained a protein component with the same molecular weight as that of α_A .

Fractions α_A and α_B both possessed hot-cold haemolytic, phospholipase-C, and lethal activities. Both hydrolysed lecithin and sphingomyelin. Electrofocusing of α -toxin in the presence of 6M urea resulted in the detection of only

one component, α_{urea} , with a pI identical to α_A . It also possessed all three biological activities of α -toxin. Removal of the urea and refocusing was accompanied by the reappearance of α_B . The occurrence and formation of α_B could not be interpreted in terms of artefactual causes of multiple forms of proteins identified by isoelectric focusing. These studies provided evidence favouring the suggestion that α_A and α_B could be related as conformers, although aggregation or polymerisation could not be entirely excluded as possible alternative explanations.

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CLOSTRIDIUM PERFRINGENS α -TOXIN

FIG. 5.—Immunoelectrophoresis of α_A and α_B preparations of *C. perfringens* α -toxin. (The α_A and α_B preparations used in these studies were harvested from single fractions obtained on elution of a preparative-scale electrofocusing experiment with toxin precipitated by ammonium sulphate.) Anode to left. As = Standard type-A antiserum; 1 = α_B ($pI = 5.25$); 2 = α_A ($pI = 5.49$).

FIG. 6.—SDS polyacrylamide disk-gel electrophoresis of *C. perfringens* α -toxin (α_A and α_B preparations as for fig. 5). Separating gels contain 11.667% and 0.153% (w/v) acrylamide and methylene-bisacrylamide respectively. Stacking gels appear opaque.

A Marker standard proteins for MW determination: (i) artefact band, (ii) bovine-serum-albumin dimer, (iii) bovine-serum albumin, (iv) ovalbumin, (v) chymotrypsinogen, (vi) track-dye front; B α_B component (40 μg); C α_A component (96 μg); D blank control gel with artefact band (i); E crude BP6K toxin (103 μg)—precipitate obtained at 35–50% ammonium-sulphate saturation.

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