A Purification and some Properties of an Insecticidal Exotoxin from *Bacillus thuringiensis* Berliner

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An insecticidal exotoxin from *Bacillus thuringiensis* var. *thuringiensis* (Berliner) has been purified. The efficiency of each stage of the purification has been ascertained and the yield of toxic material estimated by means of a quantitative bioassay. It is shown that the exotoxin is an adenine derivative substituted at position 9 and having a molecular weight of approximately 825. It can be dephosphorylated enzymically or chemically under conditions that define the exotoxin as a phosphomonoester. This results in loss of toxicity, both to insects and to mice. Spectroscopic and kinetic data are presented which suggest that a β -ribofuranosyl moiety may be attached to the adenine. Glucose and allomucic acid have been positively identified as hydrolysis fragments from the exotoxin. These results are discussed and compared with the results of others on similar (or possibly identical) compounds.

McConnell & Richards (1959) demonstrated by injection into several species of insects that the autoclaved supernatant from liquid cultures of the insect pathogen *Bacillus thuringiensis* was toxic. Although this discovery has since stimulated study in many laboratories, it is still difficult to decide exactly which strains of *B. thuringiensis* are active, which species are susceptible and by what route, and whether more than one toxin is involved.

To resolve these problems a detailed account of a purification that is subject to careful quantitative control and an investigation of the chemistry of the exotoxin(s) are essential. Farkas *et al.* (1969) have made a notable contribution to the chemistry of an exotoxin and we present here our own results.

EXPERIMENTAL

Materials. AnalaR grade chemicals were used except where otherwise specified. Barium acetate and activated charcoal (reagent grade) were products of British Drug Houses Ltd. (Poole, Dorset). The charcoal was heated at 100° for 1 hr. with *m*-HCl and then washed on a Buchner funnel first with water and then with a large volume of ethanol containing 1% of aq. NH₃ (sp.gr. 0.880). The washed charcoal was then dried and stored until required.

Analytical grades of Dowex 1, AG 1 (X2; Cl⁻ form; 50–100 mesh) and Dowex 50 AG 50 (X2; H⁺ form; 50–100 mesh) were purchased from V. A. Howe Ltd. (London W.11) and washed with deionized water before use. De-Acidite FF-IP (SRA 70; 52–100 mesh) and Zeo-Karb 225 (1% divinylbenzene) were products of The Permutit Co. Ltd. (London W.4) and were used similarly.

The Sephadex grades G-10 and G-25, obtained from

Pharmacia Ltd. (London W.13), were hydrated according to the manufacturer's directions. Cellulose for t.l.c. was Whatman CC41, obtained from W. and R. Balston Ltd. (Maidstone, Kent) and MN 300 from Macherey, Nagel and Co. (Düren, Germany). Silica gel HF₂₅₄ was a product of E. Merck A.-G. (Darmstadt, Germany). Ion-exchange t.l.c. materials used were DE41 and MN 300 (ECTEOLA-cellulose) from W. and R. Balston Ltd. and Macherey, Nagel and Co. respectively.

For ion-exchange cellulose column chromatography, Whatman DE 52 and DE 11 were used. Both materials are products of W. and R. Balston Ltd. and were pretreated where necessary according to the manufacturer's instructions. The solvent systems used for paper chromatography and t.l.c. were: system A, ethyl acetate–pyridine–water (10:4:3, by vol.); system B, butan-1-ol–acetic acid–water (4:1:1, by vol.); system C, propan-1-ol–aq. NH₃ soln. (sp.gr. 0.880)–water (13:2:5, by vol.); system D, methanol–formic acid–water (16:3:1, by vol.). Wheat-germ acid phosphatase and alkaline phosphatase from calf intestinal mucosa were obtained from British Drug Houses Ltd. Phosphatase activity was assayed with *p*-nitrophenyl phosphate supplied by Koch–Light Laboratories Ltd. (Colnbrook, Bucks.). Glucose oxidase was purchased as Fermcozyme from Hughes and Hughes Ltd. (London E.C.3).

Spectroscopic methods. Ultraviolet spectra were obtained on a Cary 14 spectrophotometer (Applied Physics Corp., Monrovia, Calif., U.S.A.).

Infrared spectra were run on a Perkin–Elmer Infracord spectrophotometer and, for more accurate determinations, on a Grubb–Parsons Spectromaster. Proton n.m.r. spectra were obtained on Varian A-60 and Varian HA-100 machines. Spectra in D₂O used 3-(trimethylsilyl)propanesulphonic acid (sodium salt) from E. Merck A.-G. as internal standard. The ³¹P n.m.r. spectra were obtained through the courtesy of Dr K. Dillon at the University of Warwick on a Perkin–Elmer R.10 instrument at 24.29 × 10⁶ c/c. sec. with H₃PO₄

and P_4O_6 as external references. Optical-rotatory-dispersion curves were measured on a Bellingham-Stanley/Bendix Polarmatic 62 with cells of 0.1 mm. path length.

Titration curve. The titration curves were measured with a Radiometer TTT1C automatic titrator fitted with the SBU1a burette and the SBR2c Titrigraph, and also manually by using an EIL pH-meter and a Pye-Ingold glass electrode.

Periodate oxidations were followed by the method of Fleury & Lange as described by Dyer (1956) and, on a micro-scale, by the method of Schiffman, Kabat & Leskowitz (1962).

Culture. The Berliner strain of *Bacillus thuringiensis* (serotype I, Milstead Culture Collection 9) was grown in liquid culture on a phosphate-buffered peptone-Lablemco broth in a 125 l. stainless-steel fermentor. Full details are given by Cooksey (1968). The supernatant was collected after 36 hr. when sporulation was complete.

Semi-quantitative bioassay. A nutrient medium was prepared by heating a mixture of powdered agar (10 g.), fresh yeast (7 g.) and fresh milk (500 ml.). A 10 ml. sample was then transferred to a disposable Petri dish (9 cm. diam.) in which 250 μ l. of test solution had already been placed. When the gel had set, 20 eggs of *Musca domestica* were added. The lid of the Petri dish was lightly greased with petroleum jelly to prevent condensation, which otherwise tended to trap and drown any larvae that emerged. A filter paper was then placed under the lid, which was secured with an elastic band. The dishes were set aside in an incubator at 30° for 48 hr. The following criteria were applied to assess activity: high activity, little or no development beyond first-instar larval stage and very little disturbance of the gel; moderate activity, the majority of larvae in the second instar with some disturbance of the gel; no activity, the majority of larvae in third instar and extensive disturbance of the gel.

Quantitative bioassay. The aqueous test solution (2 ml.) was placed in a disposable waxed paper carton (6 cm. mean diam., 6 cm. high, supplied by Lillieup, Liverpool). A portion (2 ml.) of a 10% (w/v) suspension of yeast in fresh milk was then pipetted in. A single paper tissue (Kleenex medical wipe, 21 cm. $\times$ 21 cm.) was added to absorb the liquid. Twenty *M. domestica* larvae (third instar) were introduced and the carton was covered with polythene film, punctured with four pin-holes and secured with an elastic band. The number of adult flies that had emerged was counted after 7 days' incubation at 30°. A separate record of deformed (usually wingless) adults was made at the same time. To facilitate counting, the flies were first killed either by injecting approx. 0.5 ml. of acetone into each carton or by keeping the cartons at -18° for several hours. For accurate assessment of activity, six concentrations were chosen from pilot experiments to give between 5 and 95% emergence of adults.

The mean percentage emergence at each concentration was calculated and the data were used to determine the quantity of exotoxin in the aqueous test solution required for 50% inhibition of emergence, which was selected as the unit of activity. This was done either by fitting a line by eye to the data plotted on log dose-probit paper and reading the answer directly or, for full analysis, by fitting a log dose-logit response relationship by using the Rothamsted Experimental Station Library Program for parallel logits.

Specific activity was calculated as the number of units of activity/mg.

Purification procedure

Barium precipitation. 2.45 M-Barium acetate (2.5 l.) was added, at room temperature, to 110 l. of the bacterial supernatant, which was agitated with a stream of N_2 . The precipitate was allowed to settle at 5° overnight and was then separated from the supernatant by decantation and filtration, washed with water and acetone and finally dried in a stream of air.

Charcoal adsorption. The barium precipitate from 110 l. of bacterial supernatant was stirred in 4 l. of ice-cold M-HCl for 5 min. The insoluble material, obtained after centrifuging at 600 g for 5 min., was resuspended in a further 2 l. of acid and centrifuged again. The combined dark supernatants were diluted with approx. 20 l. of water and 55 g. of activated charcoal was added. After 1 hr. at 4°, the charcoal was collected on a bed of washed Celite and washed with 2 l. of water. It was then eluted with four separate 1 l. portions of aq. ethanol (1:1, v/v) containing 1% of aq. NH_3 soln. (sp.gr. 0.880) and the combined eluates were sterilized by passage through a 0.22 μ m. bacteriological Millipore filter.

Chromatography on Dowex 1. The aqueous ethanolic eluate from the previous stage was percolated through a column (3 cm. $\times$ 50 cm.) of Dowex 1 AG 1 (X2; Cl^- form; 50-100 mesh). The column was washed with 2 l. of 0.1 M-NaCl and then eluted with a solution of 0.1 M-HCl in 0.1 M-NaCl. The effluent, like that from all other columns used in this work, was monitored for ultraviolet absorption at 254 nm. with an LKB 4701A Uvicord absorptiometer manufactured by LKB Produkter A.B., Stockholm, Sweden.

Gel filtration. The active eluates from the Dowex 1 chromatography were combined, neutralized with dilute NH_3 soln. and concentrated to approx. 40 ml. under reduced pressure at 50°. The concentrate was divided into four equal fractions and each was applied to a 3 cm. $\times$ 75 cm. column of Sephadex G-10 which was then eluted with distilled water. The active fractions were combined and evaporated to dryness under reduced pressure at 50°. The residual glassy solid was triturated with ethanol and the amorphous product collected and dried.

Preparative thin-layer chromatography. The material obtained from the Sephadex step was dissolved in water and applied in a line to the cellulose plates (100 cm. $\times$ 20 cm.; 1 mm. coating), 100 mg. to each plate. These were equilibrated over propan-1-ol-formic acid (7:3, v/v) in a stainless steel Chromatank (Shandon Scientific Co. Ltd., London N.W.10; catalogue no. 2978) for 1 hr. and then developed in the same system. The active fraction was recovered by removing the relevant band of cellulose and eluting it with aq. 0.01 M- NH_3 soln.

The recovered material was applied in a line to silica-gel plates (100 cm. $\times$ 20 cm.; 1 mm. coating), 50 mg. to each plate. These were equilibrated for 1 hr. over system C and developed in the same system. Recovery of the active fraction was again achieved by elution with aq. 0.01 M- NH_3 soln. Materials that absorbed ultraviolet light at 254 nm. were located on chromatograms either by direct observation of the extinction or by quenching of the fluorescence of the plates with an ultraviolet lamp fitted with suitable filters.

Analytical chromatography

DEAE-cellulose chromatography. DEAE-cellulose (DE 52; CO_3^{2-} form) was suspended in 0.1 M-ammonium carbonate

buffer, pH 8.9, and freed from 'fines' by settling in the usual way. It was then packed into a column (1.5 cm. $\times$ 35 cm.), fitted with a low dead-volume outlet, with a buffer flow of approx. 250 ml./hr. while packing.

Another jacketed column (0.5 cm. $\times$ 100 cm.), maintained at 55°, was packed with DE 11 and operated as described by Appar, Everett & Holley (1966).

The effluents were monitored for ultraviolet absorption at 254 nm.

Thin-layer and paper chromatography. DEAE-cellulose plates were prepared by using a mixture of Whatman DE 41 and CC 41 thin-layer Chromedia (1:1, w/w) formed into a slurry in water-methanol (1:4, v/v). After spreading, the plates were allowed to dry at room temperature.

ECTEOA-cellulose plates were prepared with ECTEOA-cellulose powder MN 300 formed into a slurry in water-methanol (1:4, v/v), spread in the usual way and dried at room temperature.

For t.l.c. with cellulose, MN 300 was used.

For paper chromatography, acid-washed Whatman no. 1 paper and the downward elution technique were used unless otherwise specified.

Electrophoresis. High-voltage electrophoresis runs were performed on a machine manufactured by Locarte Co. (London W.14) with Whatman 3MM paper at 60 v/cm. For the results recorded in Table 6, the buffers were made up according to Miller & Golder (1950).

Other buffers used were: system E1, pyridine-acetic acid, pH 6.1 (Efron, 1960), and system E2, sodium diethylbarbiturate-HCl buffer, pH 7.0 (0.04 M) (Dawson & Elliott, 1959).

Phosphate analysis. Compounds containing phosphate groups were located on chromatograms by the method of Hanes & Isherwood (1949). Total phosphate was estimated quantitatively by the method of Sumner (1944) or by the method of Fiske & Subbarow (1925).

Structural studies

Isolation of adenine. (i) A sample of purified exotoxin (3 mg.) was dissolved in M-HCl (30 ml.) and heated for 1 hr. at 100°. A part of the solution (24 ml.) was concentrated and applied to a column of DEAE-cellulose DE 52 (CO_3^{2-} form; 1.5 cm. $\times$ 30 cm.). A single peak of material absorbing light at 254 nm. was eluted on the solvent front with water; this was concentrated and examined by t.l.c., ultraviolet spectrophotometry and finally by mass spectrometry, by using an AEI MS 9 and a direct-insertion probe.

(ii) The exotoxin (50 mg.) was dissolved in 2.5 ml. of 0.75 M- H_2SO_4 and heated at 100° for 3 hr. The cooled solution was treated with $\text{Ba}(\text{OH})_2$ until it was just alkaline to pH paper. Carbon dioxide was bubbled into the solution until no further precipitation occurred and the barium salts were removed by centrifugation. The supernatant was evaporated to dryness under reduced pressure, redissolved in a minimum of water, centrifuged again and again evaporated to dryness. The residue (26 mg.) was lined on to a 20 cm. $\times$ 20 cm. cellulose t.l.c. plate, which was developed in water made to pH 10 with aq. NH_3 . The band absorbing light at 254 nm. was scraped off and eluted with aq. 1% NH_3 soln. This filtrate was evaporated to dryness, redissolved in a minimum of water and refiltered.

This final filtrate was treated with an aq.-ethanolic solution of picric acid. There was an immediate precipitation of a yellow solid. After three recrystallizations from water, this

compound melted with decomposition at 294°, undepressed by authentic adenine picrate.

Hydrolysis with alkaline phosphatase from calf intestinal mucosa. The exotoxin (as the ammonium salt, 20 mg.) was dissolved in 1 ml. of water. To this solution was added 20 mg. of alkaline phosphatase in 1 ml. of 0.2 M-ammonium carbonate buffer, pH 8.9. This mixture was incubated at 37° for 65 hr. The incubation was applied to a column (2.5 cm. $\times$ 35 cm.) of Whatman DE 11 cellulose in the CO_3^{2-} form and eluted with 0.1 M-ammonium carbonate. The column effluent was monitored at 254 nm. Two peaks were observed, one eluted between 210 and 300 ml. and one between 300 and 500 ml. The first material eluted, which did not contain any phosphate, had R_F 0.25 on silica gel in system C and R_{AMP} 0.73 on electrophoresis in buffer system E2. The second peak had R_F 0.1 and R_{AMP} 0.93 in the same two systems and was unchanged exotoxin. The contents of both peaks were isolated by evaporation under reduced pressure and excess of ammonium carbonate was removed by repeated re-evaporations with water. In all, 8.5 mg. of the dephosphorylated material was isolated. The ultraviolet spectra in acid, basic and neutral aqueous media were indistinguishable from those of exotoxin except that the $E_{1\text{cm}}^{1\%}$ at pH 7 and 260 nm. was 2×10^2 . In the second peak there was 12.0 mg. of unchanged exotoxin.

Hydrolysis with wheat-germ acid phosphatase. Exotoxin (ammonium salt, 20 mg.) was dissolved in 1 ml. of water and to this solution was added a solution of 20 mg. of acid phosphatase in 0.2 M-sodium acetate, pH 5. The reaction mixture was incubated at 37° for 65 hr. and the products were analysed exactly as for the alkaline phosphatase experiment. An almost identical elution pattern was observed, with the dephosphorylated material eluting between 210 and 300 ml. and unchanged exotoxin between 300 and 500 ml. The material from the first peak (7.2 mg.) had all the properties described for the similar material from the alkaline phosphatase experiment. The second peak was again unchanged exotoxin (13.9 mg.).

Hydrolysis at pH 4. (i) AMP. AMP (7.5 mg.) was dissolved in 1 ml. of 0.2 M-sodium acetate buffer, pH 4. A 100 μl . sample was withdrawn and analysed for inorganic phosphate. The solution was heated on a water bath at 100° and samples were withdrawn at intervals and analysed for phosphate. A total phosphate estimation was taken as an 'infinity' reading.

(ii) Exotoxin. Exotoxin (3.2 mg.) was dissolved in 0.3 ml. of 0.2 M-sodium acetate buffer, pH 4. To conserve material, this solution was divided into three portions before reaction and these were sealed in ampoules. The ampoules were heated in the water bath, removed at various times, opened and analysed for phosphate. A separate sample was analysed for total phosphate.

The first-order rate constant for the hydrolysis of AMP was calculated as $9 \times 10^{-4} \text{ min.}^{-1}$ and that for exotoxin as $14 \times 10^{-4} \text{ min.}^{-1}$. The rate constant for the hydrolysis of glycerol 1-phosphate is $8 \times 10^{-4} \text{ min.}^{-1}$ under similar conditions (Vernon, 1957).

Analysis by t.l.c. on DEAE-cellulose plates of the products of AMP hydrolysis after 24 hr. indicated that the main product was adenosine with a trace of adenine and hypoxanthine.

The products from the hydrolysis of exotoxin were analysed after 60 hr. on a 2.5 cm. $\times$ 35 cm. column of Whatman DE 52 cellulose eluted with 0.025 M-ammonium

carbonate. Four sharp peaks absorbing light at 254 nm. were eluted between 160 and 240 ml., 240 and 330 ml., 480 and 560 ml. and 640 and 700 ml. Between 1200 and 1690 ml., which represents the approximate elution volume of the dephosphorylated material produced by enzymic dephosphorylation (see above), a broad and highly asymmetric peak appeared. Rechromatography of the central portion of this on a 1 cm. $\times$ 45 cm. column of DE 52 cellulose and elution with 0.025 M-ammonium carbonate gave two peaks whose maxima eluted at 330 and 370 ml. The elution volume of the second peak corresponds to that of the product of enzymic dephosphorylation. When the contents of this peak, after removal of ammonium carbonate, were analysed by t.l.c. on silica gel in system C a single spot with R_F 0.25 was observed. This co-chromatographed with the product of enzymic dephosphorylation. Estimation of peak areas indicated that this product represented approx. 34% of the total ultraviolet-absorbing material eluted from the column.

Rate of production of a reducing group on acid hydrolysis of the exotoxin. Exotoxin (10 mg.) was dissolved in 4 ml. of 0.5 M- H_2SO_4 and the solution heated under reflux in an oil bath at 100°. Portions (0.3 ml.) were removed at appropriate times and were diluted to 2 ml. with water. A portion (1.2 ml.) of the diluted sample was then assayed for reducing function by the Somogyi & Nelson procedure (Hodge & Hofreiter, 1962); this gave a value of $1.9 \times 10^{-3} \text{ sec.}^{-1}$ for the first-order rate constant. From the remainder of the diluted sample, 80 μ l. portions were taken and assayed for reducing function by the ferricyanide procedure (Park & Johnson, 1949). In this case, the value for the rate constant was $0.6 \times 10^{-3} \text{ sec.}^{-1}$.

In both cases, the infinity reading corresponded approximately to 1 mole of reducing function/mole of adenine when the assay procedures were calibrated against glucose.

Rate of production of adenine on acid hydrolysis of AMP and the exotoxin. AMP (10 mg.) and exotoxin (ammonium salt, 10 mg.) were each dissolved in 4 ml. of 0.5 M- H_2SO_4 . The two solutions were heated at 100° under efficient reflux. Samples (100 μ l.) were withdrawn at appropriate intervals. Each sample was neutralized with aq. M- NH_3 soln. and applied to a small column (1.5 cm. $\times$ 0.5 cm.) of Zeo-Karb 225 (H^+ form), which was eluted with water. The eluates were collected in 3 ml. portions and the extinction of these solutions was measured at 258 nm. When the eluate had become transparent in this region, elution of the columns with M-NaOH was begun; portions (3 ml.) of the eluate were examined by ultraviolet spectrophotometry. In this instance, the spectrum of each portion was examined to see if any non-acidic adenines substituted at position 9 were present. If they were not, E at 258 nm. was measured. The elution with base was continued until no further material absorbing ultraviolet light was eluted.

From the E values of the materials eluted in water from the columns, a rate constant was obtained by plotting $2.3 \log [(E_0 - E_\infty)/(E_t - E_\infty)]$ against time. Similarly, from the E values of the materials eluted with NaOH, a rate constant was obtained by plotting $2.3 \log [(E_\infty - E_0)/(E_\infty - E_t)]$ against time. The values so obtained for both AMP and exotoxin were $1 \times 10^{-3} \text{ sec.}^{-1}$.

Examination of neutral products from acid hydrolysis of exotoxin. Exotoxin (30 mg.) was dissolved in 0.5 M-HCl (6 ml.) and heated at 100° for 75 min. The solution was cooled and passed down a column (5 cm. $\times$ 0.5 cm.) of Dowex 1 (OH^- form; 50–100 mesh) to remove all acidic materials.

The neutralized solution was then passed down a similar column of Dowex 50 (H^+ form; 50–100 mesh) to remove both residual adenine and unhydrolysed non-acidic nucleosides. The solution eluted in water from this second column had no ultraviolet absorption at 260 nm. and contained no phosphate. The solution was decreased in volume and a sample analysed on paper in system A. A single spot (with R_{Glc} 0.89), responsive to the $AgNO_3$ -NaOH spray, was observed. The remaining solution was divided into two portions. One of these was evaporated to dryness and treated with 2 ml. of Trisil (Pierce Chemical Co., Rockford, Ill., U.S.A.) trisilylating reagent and mechanically shaken overnight. A residual 'rim' of undissolved material was removed by gentle heating and scratching with a spatula. The resulting solution was partitioned between light petroleum (b.p. 60–80°) and water. The light-petroleum solution was washed three times with water and twice with saturated brine and was then evaporated by a stream of N_2 and analysed by g.l.c. on a 5 ft. $\times$ 0.25 in. glass column of QF-1 on Chromosorb W (60–80 mesh) (that had been treated with hexamethyldisilazane) by using a programme of 4 min. isothermal at 180° and 8°/min. to 230°. The first materials eluted were a pattern of hydrocarbons from C_{20} to C_{26} . The final hydrocarbon peak emerged at 6.5 min. At approx. 12 min., a complex of six peaks emerged, of which the second and most abundant was shown by mass spectroscopy of the collected material to be a silyl derivative. The peak of highest m/e indicated an approximate mol.wt. of 800. Both the g.l.c. retention data and the approximate mol.wt. of the trimethylsilyl derivative indicate that this fragment has the dimensions of a disaccharide. On the same column, with a programme of 2 min. at 100° and 8°/min. to 230°, the trimethylsilyl derivative of sucrose appeared as an asymmetric peak after 21 min. The final hydrocarbon peak emerged after 16½ min. The hydrocarbon series are presumed to come from the light petroleum. They were identified by mass spectroscopy.

A quantity of the second portion of the above hydrolysate corresponding to approx. 1.2 mg. was placed in a line on Whatman 3MM paper, which was developed with solvent system A overnight. Carbohydrate zones were located by means of the $AgNO_3$ -NaOH spray and the appropriate strips were excised and eluted with water. The material with R_{Glc} approx. 0.89 noted in the analytical chromatogram was recovered and hydrolysed with 0.5 M- H_2SO_4 for 3 hr. This treatment produced no change in the chromatographic pattern.

Production of glucose on acid hydrolysis. Dephosphorylated exotoxin (20 mg.) was dissolved in 4 ml. of water and, to this solution, approx. 4 ml. wet vol. of Zeo-Karb 225 (H^+ form) ion-exchange resin was added. This suspension was vigorously stirred and heated on a steam bath at 100° for 15 hr. The stirring was stopped, the flask removed from heat and the resin allowed to settle. The supernatant was drawn off and percolated down a column (5 cm. $\times$ 0.5 cm.) also of Zeo-Karb 225 (H^+ form). The hydrolysis resin was washed twice with water and the washings were treated similarly. The resulting effluent from the column was collected; it had no ultraviolet absorption above 215 nm. This solution was now, in its turn, passed down a column (7 cm. $\times$ 1 cm.) of De-Acidite G (free base) and the column washed with water (100 ml.). When this aqueous solution was evaporated to dryness, there was a residue weighing 10 mg. This residue, on analysis by paper chromatography, had the same mobility as glucose

in systems A and B. Its identity as glucose was confirmed by reaction with glucose oxidase by using the method of Marks (1959), which showed that in all 2.2 mg. of glucose was present.

RESULTS

Bioassay. The assay with housefly eggs suffered from relatively poor reproducibility and a variable and sometimes high control response. However, it proved to be useful as a comparatively quick method for distinguishing between active and inactive fractions.

In contrast, the mean percentage control response in the longer assay, based on the inhibition of development of third-instar larvae to the adult stage, was only 2% and not greater than 5% in any individual assay. The results given in Table 1 indicate the reproducibility of the method. The two solutions A and B, which were from different stages of the purification, were assayed together, with three replicates of each, on four separate occasions. The maximum variation in the 50% effective dose

for either solution was twofold, and as expected the variation in relative potency (i.e. A/B) was even less, particularly when the results were calculated from the total number of adults to emerge at each dose (i.e. including deformed adults).

The number of replicates (each of six doses with 20 larvae/dose) required to make various potency ratios significantly different when the assays were done at the same time are shown in Table 2.

There was no evidence, in any assay, of heterogeneity about the log dose-logit response line.

Stability studies of the crude bacterial exoculture. Initial stability studies showed that the biological activity of the supernatant was not detectably affected by heating for 4 hr. at 110°. More extensive heating resulted in considerable loss of activity; 24 hr. heating, for example, destroyed about 80% of activity. No difference in activity was detectable when small samples of autoclaved supernatant (120° for 15 min.) were compared with sterile filtered material.

When the supernatant was made 1 M with respect to sodium hydroxide, only slight decomposition was detected after heating for 1 hr. at 100°, but heating with the same concentration of hydrochloric acid for 15 min. at 100° resulted in complete loss of activity. Activity was unaffected after 24 hr. in M-hydrochloric acid at room temperature; nevertheless, all procedures requiring acid were carried out in the cold and as rapidly as possible.

Preliminary purification experiments. Approx. 0.1 ml. of sterile supernatant/carton was sufficient to give 50% inhibition of adult emergence in the larval assay. However, after treatment with a slight excess of barium acetate and removal of the precipitate, no activity could be detected at 2 ml./carton. The activity of the supernatant must therefore have been decreased by at least 95%. The maximum recovery of activity (and of ultraviolet-absorbing material) was achieved by washing the precipitate with M-hydrochloric acid.

There was no apparent loss of activity when a fraction (5 ml.) of sterile supernatant (pH 7.8) was treated with 100 mg. of purified charcoal, nor was there any loss of activity if the pH was decreased to 6.5 before charcoal treatment by addition of a few drops of hydrochloric acid. However, on further acidification to pH 4.0, no activity was detectable in the supernatant, at 2 ml./carton, after the charcoal had been removed and the solution brought to pH 7. Further experiments at pH 4.0 showed that 500 mg. of charcoal/l. of supernatant decreased the activity by at least 95%. Activity could be recovered by eluting the charcoal with ammoniacal ethanol.

The precipitation and adsorption steps were therefore conveniently combined in the manner described in the Experimental section.

Table 1. *Potency ratios and their confidence limits for the quantitative bioassay*

A and B are solutions from different stages of the purification. They were assayed together, with three replicates of each, on four separate occasions. Assays under (a) differ from those under (b) in that deformed adults were omitted from consideration in the latter.

Occasion	Dose for 50% inhibition ($\mu\text{g.}$)		Potency ratio A/B	95% confidence limits for A/B
	<i>A</i>	<i>B</i>		
(a) Based on all adults to emerge				
1	2336	5.3	443	344-569
2	1501	4.8	313	220-447
3	1128	2.9	386	270-550
4	1206	3.3	361	253-514
(b) Based on normal adults only				
1	1382	2.9	476	344-659
2	821	3.0	278	176-440
3	876	2.0	447	282-706
4	808	2.3	349	221-552

Table 2. *Number of replicates required for a given level of significance of potency differences*

Each assay is of six doses, 20 larvae/dose being used.

Potency ratios	All larvae			Normal larvae only		
	1.1	1.2	1.3	1.1	1.2	1.3
Two-sided test	29	8	4	47	13	6
One-sided test	20	6	3	34	9	4

Main purification. From a 110l. batch of supernatant exoculture, which contained 17g. of solids/l., 1.1kg. of barium precipitate containing the active fraction was recovered. After washing with M-hydrochloric acid, 6l. of active material was obtained but this was immediately diluted to minimize exposure to high concentrations of acid during the charcoal-adsorption step (adjusting the pH to 4 with ammonia caused precipitation and a consequent loss of activity from the supernatant). After elution from charcoal, the volume of the active fraction was 4l. Evaporation of a sample gave a brown gum (equivalent to a total of 8.5g.), which on quantitative bioassay showed a 127-fold increase in specific activity compared with the crude solid obtained by concentrating the supernatant exoculture. This represented a 57% overall recovery of the biological activity. No significant increase in recovery could be achieved by further washing of the barium precipitate or the charcoal.

When pure material was available (see below) a control experiment was run to determine the recovery by ultraviolet absorption for comparison with the bioassay result. The purified toxin was made up in the culture medium used for the fermentation to 50mg./l., i.e. to approximately the same concentration of toxicity units as normal exoculture. The recovery from barium precipitation and charcoal adsorption in this case was estimated to be 50%.

When the ammoniacal ethanolic eluate from charcoal was percolated through the Dowex 1 (Cl⁻ form) column, which was re-equilibrated by washing with 0.1M-sodium chloride, both the recovered ammoniacal ethanol and the sodium chloride washings were inactive. When the column was eluted with 0.1M-hydrochloric acid in 0.1M-sodium chloride a single active fraction was recovered in the first 200ml. after the eluate became amber-coloured. Bioassay and spectrophotometry of subfractions indicated that activity and ultraviolet absorption at 260nm. ran together.

When one-quarter of the neutralized concentrate

from Dowex 1 chromatography was applied to the Sephadex G-10 column and eluted with distilled water, an amber-coloured inactive fraction that had only end-absorption was obtained in the first 20ml. of eluate after collection of the void volume (185ml.). The pale-coloured active fraction was eluted between 20 and 80ml. after the void and was cleanly separated from inorganic material, which began to emerge 110ml. after the void volume. Similar treatment of all the material from Dowex 1 chromatography yielded a total of 4g. of an amorphous solid. This had $\lambda_{\max}$, 260nm., $E_{1\text{cm.}}^{1\%}$, 1.58×10^2 , and a specific activity of 260, representing a 1.7-fold increase in activity from the charcoal eluate and 79% recovery from that stage.

Examination by t.l.c., with subsequent bioassay of subfractions (using the systems defined for preparative layer chromatography), revealed a single active zone and a number that were inactive. Removal of these on a larger scale was achieved by using preparative layer chromatography. On cellulose, the only active fraction corresponded with a single band, R_F approx. 0.1, when the plate was eluted with propan-1-ol-formic acid (7:3, v/v). It could be located either by its ultraviolet absorption at 260nm. or by spraying the edges of the plate with the Hanes & Isherwood (1949) molybdate spray. This reagent also revealed a second major phosphate-positive band, R_F approx. 0.5, which was non-toxic and did not absorb ultraviolet radiation. A third band, R_F 0.55–0.60, otherwise similar to the band of R_F 0.5, was also observed. Its intensity tended, however, to vary considerably from batch to batch.

When the active fractions from the cellulose plates were subjected to further purification by preparative layer chromatography on silica-gel plates with system C, three bands could be observed by their strong quenching of the fluorescence of the silica gel. The two leading bands, with R_F approx. 0.2, which were not always completely separated from one another, were inactive. Toxicity was associated only with a band of low R_F (approx. 0.1).

Table 3. *Summary of the data for the purification of the exotoxin of B. thuringiensis*

Stage	Volume (l.)	Wt. of solid fraction (g.)	ED ₅₀ and 95% limits (μg.)	Specific activity	10 ⁻³ × Total activity (units)	Overall recovery (and 95% confidence limits) (%)	10 ⁻² E _{1cm.} ^{1%} (λ _{max} , 260nm.)
Exoculture	110	1870	804 (679–951)	1.23	2300	—	End-absorption, slight shoulder at 260nm.
Barium precipitate	—	1100	—	—	—	—	—
Charcoal eluate	4	8.5	6.4 (5.4–7.6)	156.2	1328	57 (46–71)	0.9
Dowex 1/Sephadex G-10	0.2/0.06	4.0	3.8 (3.2–4.5)	263	1052	46 (37–56)	1.58
Pure	—	2.5	3.0 (2.5–3.5)	333	820	36 (29–44)	1.66

After elution with 0.01M-ammonia and freeze-drying, approx. 25mg. of toxic material was obtained/l. of original exoculture supernatant. This had a specific activity of 330 and $E_{1\text{cm}}^{1\%}$ at 260nm. of 1.66×10^2 . The overall recovery of activity from both preparative layer chromatography stages was approx. 80%.

The freeze-dried material was a white powder. The details of the purification are summarized in Table 3. Analysis of the purified material gave the values: C, 34.9; H, 4.7; N, 8.9; P, 4.2%.

In subsequent work, several minor modifications in procedure have been found to give similar results. The Dowex 1 resin can be replaced with the equivalent Permutit (De-Acidite FF-IP) resin without affecting the purification of the exotoxin.

The silica-gel preparative layer chromatography procedure can be replaced by anion-exchange chromatography on DEAE-cellulose (CO_3^{2-} form) by using elution with 0.075M-ammonium carbonate.

Analytical chromatography. (a) DEAE-cellulose column chromatography. A portion (12mg.) of purified material was eluted as a single active peak when chromatographed on a DEAE-cellulose (DE 52; CO_3^{2-} form) column (1.5cm. $\times$ 30cm.) by using a linear gradient from 0.05 to 0.15M-ammonium carbonate with 1l. of eluent in each reservoir. This peak was eluted between 440 and 545ml., with a maximum at 475ml. AMP, ADP and ATP (5mg. of each) used to calibrate the column were eluted between 120 and 160, 290 and 350 and 440 and 520ml. respectively.

A single peak was also obtained under similar conditions with DEAE-cellulose (formate form) and a linear gradient (0.1–0.3M) of ammonium formate, pH 6.4.

In a similar manner, the purified material, on being chromatographed at 53° on DE 11 by using 0.02M-tris chloride, pH 8, in 7M-urea as eluent, gave only one active peak, absorbing in ultraviolet light at 254nm., and which was eluted between 42 and 55ml. All of the exotoxin and ATP peaks were slightly asymmetric (Anderson, Green, Barber & Ladd, 1963).

(b) DEAE-cellulose t.l.c. The purified exotoxin gave a single spot, R_F 0.23, on 20cm. $\times$ 20cm. plates eluted with 0.1M-ammonium carbonate. On the same plate the R_F values of adenosine, AMP and ATP were 0.57, 0.35 and 0.25 respectively. On ECTEOLA-cellulose t.l.c. in the system saturated ammonium sulphate–M-trisodium citrate–propan-2-ol (40:9:1, by vol.) (M. H. Rogoff, personal communication), the purified exotoxin ran as a single spot with R_F 0.56.

On t.l.c. with MN 300 cellulose and a solvent system ethanol–M-acetic acid (1:1, v/v), buffered to pH 3.5 with ammonia, the purified exotoxin was apparently resolved into two bands separated by a

streak. The two bands and the streak all quenched the fluorescence of the cellulose. The leading band had an R_F value very close to that of AMP (0.63), and the trailing band had not moved from the starting line. The separation was repeated with 8mg. of purified exotoxin lined on to a 20cm. $\times$ 20cm. $\times$ 0.03cm. plate and the three regions (leading band, streak and trailing band) were eluted from the cellulose with 0.01M-ammonia. The three solutions were bioassayed by the semi-quantitative procedure. All were active. A sample of the band of high R_F , recovered from the large plate, was re-chromatographed in the same system. Precisely the same pattern, of two bands separated by a streak, was observed.

Isolation of adenine. The single ultraviolet-absorbing fraction recovered after hydrolysis of pure exotoxin with M-hydrochloric acid at 100° for 1hr. and chromatography on DEAE-cellulose had an ultraviolet spectrum identical with that of an authentic sample of adenine in acid, base and neutral solution. Its mass spectrum had m/e 135 and a cracking pattern identical with that of authentic adenine.

Adenine:phosphate ratio. When the adenine content of a sample of purified exotoxin was estimated by its absorption at 259nm. and total phosphate by Sumner's (1944) method, it was found that the adenine:phosphate ratio was 1:0.98.

Spectroscopic results. The ultraviolet-spectral characteristics of the exotoxin are recorded in Table 4.

There have now been recorded a sufficient number of the ultraviolet spectra of variously acylated (Ralph & Khorana, 1961; Hall, 1964) and alkylated (Leonard, Carraway & Helgeson, 1965; Shaw, Smallwood & Wilson, 1967; Littlefield & Dunn, 1958) adenine samples to enable the position of substitution to be assigned with confidence (Townsend, Robin, Leoppy & Leonard, 1964) on the basis of the ultraviolet spectrum and its variation as a function of pH. Since the presence of adenine in this exotoxin has been firmly established, both the exotoxin and its dephosphorylation product are defined by the data of Table 4 as adenine compounds substituted in the 9-position. A molecular weight can be calculated for the exotoxin by assuming that the molar extinction has the value already known to be common to other adenine compounds substituted in the 9-position (Morell & Bock, 1954; Beaven, Holiday & Johnson, 1955). The $E_{1\text{cm}}^{1\%}$ at 259nm. for the free acid of exotoxin in buffer at pH 7 (1.82×10^3) and for the dephosphorylated exotoxin (2.0×10^2) lead to calculated molecular weights of 850 and 770 respectively for ϵ at 259nm. of 15.4×10^3 (Morell & Bock, 1954). An ϵ_{259} of 15.0×10^3 (Beaven *et al.* 1955) gives molecular weights of 825 and 750 for the same two compounds.

Table 4. *Ultraviolet-spectral data for exotoxin*

Spectra were recorded in 1cm. cells with solutions containing approx. 0.1mg. of the ammonium salt/ml.

Solvent	$\lambda_{\max.}$ (nm.)	$10^{-2}E_{1\text{cm.}}^{1\%}$	$\lambda_{\min.}$ (nm.)	$E_{1\text{cm.}}^{1\%}$	$\frac{E_{250}}{E_{260}}$	$\frac{E_{280}}{E_{260}}$	$\frac{E_{290}}{E_{260}}$
0.01M-NaOH	259	1.7	227	26	0.77	0.188	0.0118
0.01M-HCl	257.4	1.64	229.5	34	0.83	0.24	0.051
0.05M-Phosphate buffer, pH 7	259	1.68	226.5	28	0.77	0.21	0.023

Table 5. *Proton nuclear-magnetic-resonance spectrum of the dephosphorylated exotoxin*

Spectra were run at 100×10^6 cyc./sec. with a concentration of 195mg. of dephosphorylated exotoxin in 0.4ml. of D_2O . Chemical shifts are in p.p.m. with respect to the methyl signals of 3-(trimethylsilyl)propanesulphonic acid. s: singlet; d: doublet; t: triplet. Coupling constants are quoted in cyc./sec.

Chemical shift	Multiplicity	Assignment
8.4 (1)	s	Adenine ring
8.18 (1)	s	Adenine ring
6.13 (1)	d (approx. 4.5)	Anomeric proton
5.17 (1)	d (approx. 3)	Anomeric proton
4.95 (4?)	?	
4.68 (1)	t	
4.49		
4.17		
4.09		
3.95		
3.76		

Our own estimate of the ϵ_{259} for adenosine agrees with the latter value. A molecular weight, which is independent of the absolute value of ϵ , can be calculated from these same two $E_{1\text{cm.}}^{1\%}$ values on the assumptions (i) that the molecular weights of the exotoxin and the dephosphorylated product differ by 80 (i.e. HPO_3) and (ii) that the two $\epsilon_{\max.}$ values are the same. This gives the value 845 for the free acid of the exotoxin.

Nuclear-magnetic-resonance spectra. The salient features of the proton n.m.r. spectrum of the dephosphorylated exotoxin are given in Table 5. The spectrum of the exotoxin was similar but not as well resolved. The lack of any signal at a field higher than 3.8δ indicates that all protons are attached to carbon atoms bearing electronegative substituents. This observation makes it probable that the unidentified fragment of the exotoxin is carbohydrate in nature, since routine methods showed that amino acids were not present.

The separation of the protons at positions 2 and 8 of the adenine is equivalent to 13.2cyc./sec. at 60×10^6 cyc./sec. and provides further evidence that the adenine is substituted at N-9 (Townsend *et al.* 1964). The lower-field region of the spectrum, en-

compassing the two aromatic protons and the doublet at 6.13δ , is very closely similar to that region of the spectrum of adenosine (Fromageot, Griffin, Reese, Sulston & Trentham, 1966). The signal at 6.13δ is therefore assigned to the anomeric proton of the sugar residue that is attached to the adenine. The shift of the anomeric proton can be interpreted, on the basis of the correlations noted by Nishimura & Shimizu (1965), to indicate that the $1',2'$ configuration is *trans*.

The coupling constant $J_{1,2'}$ has been shown to have little value in determining the configuration of the *N*-glycosidic bond (Nishimura & Shimizu, 1965; Lemieux & Lown, 1963).

The signal at 5.17δ is, because of its low-field position and its multiplicity, assigned as the anomeric proton of a glucosyl residue (see below). The position and coupling constant (3cyc./sec.) strongly suggest that the glucose is present in the α -D-glucopyranose configuration (van der Veen, 1963; Rao & Foster, 1963).

The ^{31}P n.m.r. spectrum of the magnesium salt, which was obtained with the aid of a computer of average transients, shows a shift of $+0.3$ p.p.m. with respect to 85% phosphoric acid as an external standard. The magnesium salt of AMP has a ^{31}P shift of -0.1 p.p.m. Data of ^{31}P shifts for phosphate esters are scanty (Clarke & Lyons, 1966). Such data as exist (Nielson, Pustinger & Strobel, 1964) show that there is little difference between the value given above and those for phosphodiester. Enzymic and chemical hydrolysis experiments define the exotoxin as a phosphomonoester, however, and the ^{31}P shift does not contradict these results. Unfortunately, the multiplicity could not be resolved.

Curve for optical rotatory dispersion of exotoxin. This curve for exotoxin [(c 0.6; 0.1M-hydrochloric acid; 20°) $[\phi]_{312.5} + 2000$; $[\phi]_{285.5} + 3000$ (peak); $[\phi]_{278} + 2220$; $[\phi]_{270} \pm 0$; $[\phi]_{261} - 1600$ (trough); $[\phi]_{256.5} - 690$; $[\phi]_{253.5} \pm 0$; $[\phi]_{247} + 2290$; $[\phi]_{236.5} + 3900$ (peak); $[\phi]_{230} + 1870$; $[\phi]_{225.5} \pm 0$; $[\phi]_{222} - 2440$] is very closely similar to, but displaced from in a positive sense, that of adenosine or AMP (Yang, Samejima & Sarkar, 1966). The curve defines the configuration of the anomeric linkage to the adenine to be β (Ulbricht, Jennings, Scopes &

Klyne, 1964). This conclusion can be drawn from both the sign of the Cotton effect and its amplitude (Shimizu, 1967). The latter effect reinforces the conclusion reached from the n.m.r. spectrum that the configuration of the groups about the 1', 2'-bond of the sugar attached to the adenine is *trans*.

Rates of hydrolysis of the exotoxin in acid. For the exotoxin, the rates of release of the reducing function and of adenine were identical, within the limits of experimental error, with each other and with the corresponding rates for AMP. The values agree with the corresponding rate constant for adenosine calculated by Venner (1964) and extrapolated to 100° by use of the Arrhenius equation and a value of 13 kcal. for the activation energy. Venner (1964) quotes the range 11–16 kcal. for the activation energy for this reaction.

High-voltage electrophoresis. The behaviour of the exotoxin on high-voltage electrophoresis is shown in Tables 6 and 7. From these data it appears that the exotoxin undergoes ionizations between pH 2.0 and 4.6 that AMP, ADP and ATP do not show. The exotoxin forms a complex with borate but the effect on its electrophoretic mobility is less than the effect on AMP.

Titration curve. The titration curve of the ammonium salt of exotoxin fully bears out the conclusions of the electrophoretic experiments. There

are three well-separated ionizations with pK values at 3.85, 6.95 and 9.8. The last is due to the ammonia originally present as the cation. The titre of the group at pK 3.85 and that of the group at 6.95 (which is the phosphate moiety) are in the ratio 3.15:1. We interpret this to indicate the presence of two carboxyl groups in the molecule. The correctness of this interpretation is borne out by the isolation of allomucic acid from exotoxin (Bond, 1969).

Periodic acid oxidation. The exotoxin consumed 2 moles of periodate within 30 min. and the dephosphorylated material consumed 5 moles within the same period. We were not able to separate the periodate consumption into two stages.

DISCUSSION

The early studies on the insecticidal properties of the thermostable ' β -exotoxin' of varieties of *B. thuringiensis* have been reviewed by Heimpel (1967). The first purification of the factor (or a factor) responsible for the insecticidal action of the autoclaved supernatant from *B. thuringiensis* var. *thuringiensis* was recorded by de Barjac & Dedonder (1965). They showed that the toxicity was associated in five paper-chromatographic systems with a zone that contained adenine, phosphate and ribose. The purification employed paper chromatography and was suitable for the purification of only small amounts of the exotoxin. More recently, de Barjac & Dedonder (1968) have outlined another purification that is similar to the earlier stages of the one described here. Their second purification stops after Dowex 1 fractionation, however, and they did not offer sufficient evidence for the homogeneity of their product to be assessed.

A purification, leading similarly to a compound containing adenine and phosphate, has been described by Sebesta, Horska & Vankova (1967, 1969a). In it, phenol extraction of the eluate from charcoal is used instead of metal precipitation to remove peptide contaminants. These authors disagreed with the findings of de Barjac & Dedonder (1965) in so far as they were unable to observe ribose as a product of acid hydrolysis of their exotoxin.

Benz (1966) has also described a purification procedure for the exotoxin, with alcohol precipitation, but he did not claim that his product was homogeneous. His results were in broad agreement with those of de Barjac & Dedonder (1965).

The purification that we report here offers for the first time quantitative data on the efficiency of recovery at each stage and a yield for the overall purification. These data are obtained by the use of a quantitative bioassay, the potentialities and limitations of which have been fully examined. In an area in which there has been doubt about how many

Table 6. *Behaviour of exotoxin on high-voltage electrophoresis*

Buffers were prepared according to Miller & Golder (1950).

pH	Mobility relative to AMP		
	ADP	ATP	Exotoxin
2.0	5.35	7.05	1.0
4.6	1.77	1.89	1.54
8.6	1.02	0.91	0.725
12.0	0.94	0.91	0.72

Table 7. *Behaviour of exotoxin on high-voltage electrophoresis in buffer, pH 9, in the presence and absence of borate*

Run (a) was in a buffer at pH 9.0 prepared according to Miller & Golder (1950). In run (b) the same buffer was made 0.1M in sodium borate at the same pH.

Run	Compound	Mobility relative to AMP	Mobility relative to dAMP
		AMP	dAMP
(a)	Exotoxin	0.84	0.84
	dAMP	1.0	1.0
	AMP	1.0	1.0
(b)	Exotoxin	0.82	1.09
	dAMP	0.75	1.0
	AMP	1.0	1.34

'thermostable toxins' were involved in the insecticidal action of the autoclaved supernatant of *B. thuringiensis* (Anderson & Rogoff, 1966) we believe that this information is essential. Under the conditions of our purification we have found no evidence for the presence of other insecticidal materials.

The first two stages of this purification, barium precipitation and charcoal adsorption, remove inorganic salts, protein, polypeptide and most carbohydrate materials. In addition, they yield the exotoxin in a sufficiently small volume for sterilization by membrane filtration. We avoided autoclaving, particularly of the bulk supernatant, since it was our experience that the procedure required for autoclaving large batches (100l.), which involves heating at 120° for 8 hr., destroyed much of the toxicity, despite the fact that the exotoxin is usually referred to as the 'thermostable toxin' (McConnell & Richards, 1959). The term has some justification, however, as can be seen from the data on this point recorded in the Results section.

After removal of several impurities by gel filtration and preparative layer chromatography, the toxin fraction was shown to be an adenine nucleotide in so far as adenine could be identified as a hydrolysis product and was present in a ratio of 1:1 with inorganic phosphate. The techniques that were then used in an attempt to achieve further resolution were selected accordingly. Staehelin (1963) has achieved excellent separations of nucleotides on a pressure-packed column of DEAE-cellulose with linear gradients of ammonium hydrogen carbonate, pH 8.5, or of ammonium formate, pH 6.4. Apgar *et al.* (1966) have achieved equally impressive results with 7M-urea in the eluting buffer to eliminate the effects of hydrogen bonding. Our failure to achieve any observable resolution with these and with several paper chromatography and t.l.c. systems leads us to believe that the toxin is pure. The only doubt arises from the behaviour of the purified toxin on paper chromatography and t.l.c. with solvent systems at low pH. This is explained by the subsequent isolation of allomucic acid (Bond, 1969) as a hydrolysis product of the exotoxin. Tailing of polycarboxylic hydroxy acids on paper when ionization is not fully suppressed has been noted by Lugg & Overell (1947). Our experiments on the chemical nature of the exotoxin show that it contains adenine substituted at position 9. The results from n.m.r., optical rotatory dispersion and acid-hydrolysis rates are explained if that substitution is by a ribofuranose moiety in the β -configuration. However, more exotic possibilities cannot be rigorously excluded from consideration. The molecule contains, in addition, a glucose residue probably, on the basis of the n.m.r. spectrum, bound as an α -D-glycopyranose, and allomucic acid (Bond, 1969). The phosphate group is present as a phosphomonoester.

Fairly strong acid hydrolysis yields a reducing sugar with the molecular dimensions of a disaccharide but which is not affected by hydrolysis conditions that would be expected to cleave a disaccharide. The exotoxin contains two vicinal glycol groups.

All of these observations are in close agreement with the findings of Sebesta's group (Sebesta *et al.* 1969a; Farkas *et al.* 1969) and with those of de Barjac & Dedonder (1965, 1968). Farkas *et al.* (1969) have investigated a 'disaccharide' produced on acid methanolysis. They have suggested a structure for the 'disaccharide' and also a structure for the exotoxin (I) based largely on that of the 'disaccharide'. These studies enabled them to adduce more compelling evidence than we for the presence of ribose in their exotoxin. Thus they obtained a lactone whose optical-rotatory-dispersion curve was very similar to that of D-ribonolactone and the mass spectrum of the acetonide of the methanolysis product showed ions interpreted as being due to ribose fragments. This latter is excellent evidence provided that accurate mass measurements are available on the relevant ions.

The French workers have observed ribose as a product from their exotoxin under very mild hydrolytic conditions. The chemical results from the three laboratories suggest that all are dealing with the same compound or three very closely related compounds. There is agreement on the presence of adenine, phosphate and, probably, ribose. The Czechoslovak school and we agree that glucose

and allomucic acid are also present (Farkas *et al.* 1969; Bond, 1969). The only difficulty arising is that the isolation of free ribose would be very difficult to rationalize on the basis of the structure proposed by Farkas *et al.* (1969) (I). In this connexion, it is noteworthy that at no point do de Barjac & Dedonder (1965) claim that they obtain 1 mole of free ribose/mole of adenine. The orcinol reaction, which they use to determine the ribose, is known to work with substituted ribose (Albaum & Umbreit, 1947). To release 1 mole of reducing function from their exotoxin, de Barjac & Dedonder (1965) used stronger hydrolytic conditions than those used to release ribose for paper-chromatographic analysis. The presumption is that their ribose did not necessarily represent a major product.

We can offer at the moment no evidence on lactone derivatives of our exotoxin and its dephosphorylated product. The structure proposed by Farkas *et al.* (1969) (I) is based on the fact that one lactone ring is produced from their exotoxin and two from the dephosphorylated product. We find it strange that these lactones are reported to be eluted from Dowex 1 (formate form) after the corresponding hydroxy acid.

Infrared spectra of our exotoxin and the dephosphorylated material are very similar to those published by Sebesta *et al.* (1969a), but the compounds as we have isolated them exhibit no absorption around 1775cm^{-1} . Because of the uncertainties, noted by Farmer (1957), associated with potassium bromide disc infrared spectra of hydroxylic and carboxylic compounds we have not presented them in detailed evidence.

There is discrepancy in the various estimates of molecular weight. All values, except the lowest estimate of de Barjac & Dedonder (1968), are higher than required by the structure proposed by Farkas *et al.* (1969).

That we and de Barjac & Dedonder (1968) observe an uptake of 2 moles of periodate/mole of exotoxin whereas Sebesta *et al.* (1969a) give a value of 1 mole does not necessarily constitute a discrepancy. The elegant polarographic method of Zuman & Krupička (1958), used by the Czechoslovak group, permits measurement over a shorter period of time than do the methods employed by de Barjac & Dedonder (1968) or ourselves.

Our exotoxin, like that obtained by Sebesta & Horska (1968) and Sebesta, Horska & Vankova (1969b), is toxic to mice. Toxicity to both mammals and insects disappears on hydrolytic removal of the phosphate.

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