New Method for the Large-Scale Preparation of Diphtheria Toxoid: Purification of Toxin

JOHN C. COX
Commonwealth Serum Laboratories, Parkville, Victoria 3052, Australia

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Diphtheria toxin of high purity was prepared in batch cultures of 1 to 40 liters by procedures capable of processing 200-liter batches without modification. The procedure incorporates preliminary purification of the growth medium and, after deep fermentation or surface culture of Corynebacterium diphtheriae, both concentration and partial purification of the toxin by membrane ultrafiltration. Final purification is achieved by Sephadex G-100 gel filtration. Purities of 2,000 to 2,500 flocculation units per mg of protein nitrogen (260 to 410 flocculation units per unit of absorbance at 280 nm) were routinely obtained with only a 10% loss of toxin. The toxin appeared pure on immunoelectrophoresis and ultracentrifugation, and only minor amounts of lower-molecular-weight impurities were revealed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Significant advantages of the procedure are its rapidity and reproducibility and the fact that all stages are performed at 4°C in neutral isotonic buffer.

The purification of diphtheria toxin or toxoid is usually achieved by methods largely dependent upon chemical precipitation using dipotassium hydrogen phosphate (8) or ammonium sulfate (2, 6, 8). Purities obtained by these procedures are good, but extension of the methods to large-scale purifications is time consuming and losses in excess of 30% are usually encountered. A purification based on physical techniques would overcome both these problems, and this became possible with the advent of membrane ultrafiltration technology (Diaflo, Amicon Corp., Lexington, Mass.). Membranes with a suitable range of pore sizes are now available with commercial-scale membrane holders. This paper describes a purification procedure for diphtheria toxin requiring only two ultrafiltration steps and one Sephadex gel filtration to yield a toxoid of high purity with minimal loss.

MATERIALS AND METHODS

Growth medium. The growth medium used was based on a medium incorporating casein hydrolysate or Casamino Acids (5) modified to have a total nitrogen content of 0.2% (wt/vol) and containing 1.5% (wt/vol) maltose (6). This medium was sterilized by ultrafiltration with a Diaflo PM30 membrane. Although polypeptides in this medium were found to have a molecular weight generally less than 15,000 to 20,000, this ultrafiltration step positively eliminated any molecules of molecular weight above 30,000 from the growth medium in which toxin would be formed. Preliminary experiments in shake flasks had shown that bacterial growth and toxin formation were unaffected by this ultrafiltration.

Toxin production. Corynebacterium diphtheriae (Park Williams strain 8) was used in all experiments. Seed was grown as a 24-h surface culture in tubes and was inoculated into 250-ml volumes of medium in Roux bottles or 500-ml Erlenmeyer flasks. Shake flasks were incubated at 35°C for 3 days on a table rotating at 200 rpm with an amplitude of vibration of 2.5 cm; surface cultures were incubated at 35°C for 4 days. Clarification of toxin was achieved by centrifugation for pilot batches and Seitz DO filtration for production batches. Complete removal of bacteria from production batches was found to be unnecessary at this stage. Thimerosal (0.01%, wt/vol) was added to prevent growth of bacterial contaminants.

Toxin purification. Initial purification was obtained by concentration at 4°C on a Diaflo XM 50 membrane to 1% of the initial volume and then washing at this volume with half the original volume of phosphate-buffered saline (0.01 M sodium phosphate, 0.145 M sodium chloride, pH 7.1). This was performed on a large scale by using an Amicon TCID thin-channel membrane holder, with approximately 80 lb/in² air pressure on the double-diaphragm pump and the filtration vessel unpresurized. Under these conditions a single membrane of 136-cm² surface area maintained a flow rate of 1.0 liter/h throughout the concentration and washing procedure. Application of pressure (nitrogen at 5 to 20 lb/in²) to the filtration vessel gave only a temporary increase in flow rate, all...
advantage being lost after about 10 liters. Small-scale concentrations were achieved by using an Amicon TCF10 thin-channel membrane holder. A UM10 membrane was used to concentrate some pilot batches so that lower-molecular-weight impurities could be determined by Sephadex gel filtration.

Final purification was obtained by Sephadex G-100 gel filtration, applying a sample of volume up to 7% of the column volume and eluting with phosphate-buffered saline at 4 C. Eluant was monitored spectrophotometrically at 280 nm.

Production of antiserum. Rabbits were dosed intramuscularly in each hind leg with protein from peak I and, after detoxification, peak II (see Results) emulsified with an equal volume of Freund complete adjuvant. A dosing rate of 0.5 mg of antigen/kg of body weight was used (4). At 6 weeks the rabbits were redosed as above and bled out 3 weeks later.

Testing procedures. Optical density scans from 230 to 500 nm were obtained on a Cary continuous recording spectrophotometer.

Polyacrylamide gels (7%) were run in tris(hydroxymethyl)aminomethane-glycine buffer, pH 8.3 (3), and stained with Coomassie blue G250. Sodium dodecyl sulfate (SDS)-gels (0.1% SDS, 10% polyacrylamide) were run in sodium phosphate buffer (12).

Immunoelectrophoresis was performed on microscope slides in 1.5% ion agar, using sodium barbitone buffer, pH 8.2 (9). Results were recorded photographically after 24 h of diffusion at 20 C.

The acute toxicity of protein fractions was determined by injecting guinea pigs subcutaneously with a dilution of the fraction contained in 0.5 ml of 0.145 M NaCl and observing the animals for death over 4 days. The toxicity of electrophoretically purified proteins was determined as follows. Purified diphtheria toxin (20 mg, i.e., about 330 times the mean lethal dose) was subjected to electrophoresis in 7% polyacrylamide in tris(hydroxymethyl)aminomethane-glycine buffer, pH 8.3 (3). The relevant 30 mm of this gel was sliced on a gel cutter into segments (20 by 1.5 mm). One-third of each segment was stained with Coomassie blue G250, and the remainder was held in a sealed tube. The unstained segments containing the major protein bands as detected in the stained segments were again subjected to electrophoresis as described above. This time the relevant 18 mm was sliced and stained as above, and the unstained portions corresponding to the isolated protein bands were emulsified in saline and injected subcutaneously into guinea pigs.

The adenosine diphosphate ribosylation activity of fragment A of diphtheria toxin was determined in vitro by using radioactive oxidized nicotinamide adenine dinucleotide (2). Results are expressed as a percentage of the activity obtained for the same molar equivalent of purified electrophoretically homogeneous fragment A.

Flocculation tests were performed at 37 C in Wasserman tubes by using glycerinated horse anti-diphtheria toxoid serum with an initial potency of 950 flocculation units (Lf)/ml.

Svedberg constants for various protein fractions were determined on a Spinco model E ultracentrifuge.

Protein nitrogen levels were estimated upon trichloroacetic acid-precipitable material by the micro-Kjeldahl procedure.

RESULTS

Purity of diphtheria toxoid. Purities of around 1,500 to 1,800 Lf per mg of protein nitrogen were obtained for concentrated toxoid after washing on a Diaflo XM 50 membrane. Decrease in purity upon toxoiding was found to be small enough that purity at this stage should have generally exceeded specified limits (1).

The purity results for pilot and large-scale batches of Sephadex-purified diphtheria toxoid are shown in Table 1. Purities are expressed in terms of Lf per optical density (OD) unit (280 nm), a procedure that gave highly reproducible results and permitted an estimate of purity when there was insufficient sample to enable accurate protein nitrogen assays to be performed. The values reported here compare very favorably with a figure of 270 Lf/OD unit reported previously (7). In five cases, nitrogen determinations were made that permitted a correlation between the two terminologies for expressing purity.

A typical Sephadex G-100 purification elution profile using Diaflo UM10-concentrated toxin is shown in Fig. 1. Three distinct peaks, labeled I, II, and III in order of decreasing molecular weight, were observed. The use of Sephadex G-75 did not permit a satisfactory separation of peaks I and II since the latter, being diphtheria toxoid with a molecular weight of 62,000, was eluted fairly close to the void volume of this Sephadex grade. Purification runs for XM 50-concentrated toxin were similar

| Lf/ml | Grown in: | Lf/mg of protein nitrogen | Lf/OD unit |
|-------|-----------|--------------------------|------------|
| 55    | Flask     | 2,200                    | 275        |
| 36    | Flask     | 2,500                    | 380        |
| 41    | Flask     | 2,350                    | 380        |
| 60    | Flask     | ND*                      | 410        |
| 50    | Flask     | ND                       | 380        |
| 64*   | Roux bottle | ND                      | 290        |
| 43*   | Roux bottle | 2,050                   | 265        |
| 42*   | Bottle    | 2,200                    | 260        |

* Large-scale.

* ND, Not determined.
is summarized in Table 2. It can be clearly seen that the two proteins varied in chemical and immunological behavior. Figure 2c shows the immunoelectrophoresis pattern of concentrated, washed toxin. Several lines additional to

except that peak III was reduced to less than one-tenth the size shown here.

**Recovery of diphtheria toxin.** The percentage recovery of diphtheria toxin was monitored at all stages of purification. Losses averaged 7% on concentration, 2% on washing, and 20% on Sephadex filtration. This loss on Sephadex filtration varied from 10 to 30% and was determined by the purity of separate fractions in terms of Lf/OD unit. In the large-scale experiments, all fractions with purities above 200 were included in the final toxin pool, whereas all those with lesser purity but of significant toxin level were collected for reprocessing at the concentration stage of a subsequent batch. Thus, overall percentage losses for all stages of purification were held at about 10%.

**Comparison of peaks I and II.** Various physical and immunochemical properties of peaks I and II were determined because an initial observation that the protein entity in both peaks was capable of forming floccules with anti-diphtheria toxoid serum suggested that peak I may simply be polymerized diphtheria toxin. The comparison of these properties

![Fig. 1. Elution profile of UM10-concentrated diphtheria toxin on Sephadex G-100.](image)

**TABLE 2. Comparison of physical and immunochemical properties of peaks I and II**

| Property                                   | Peak I                      | Peak II                      |
|--------------------------------------------|-----------------------------|------------------------------|
| Ultraviolet absorption maxima              | 257 nm                      | 276 nm                      |
| Flocculation with anti-diphtheria toxoid serum | Fine floccules in 10-12 min | Coarse floccules in 2 min   |
| Immuno-electrophoresis against anti-diphtheria toxoid serum (Fig. 2) | Single straight diffuse line | Single curved, sharp line   |
| Polyacrylamide gel electrophoresis         | Several high-molecular-weight bands | Two major and two minor bands |
| 7% Gel without SDS (Fig. 3a, b)            | Variable from 11 to 21S     | Single broad band           |
| 7% Gel with SDS (Fig. 3c)                  | No deaths at 0.5 Lf         | Mainly 4.2S                 |
| Molecular weight by ultracentrifugation    | 1%                          | LD₅₀ = 0.02 Lf              |
| Toxicity in guinea pigs                    | Reacts on immunoelectrophoresis with peak I only | Reacts on immunoelectrophoresis with peak II only |
| ADP* ribosylation activity                 |                             |                             |
| Immunization of rabbits                    |                             |                             |

*Adenosine diphosphate.

*Mean lethal dose.
those of peaks I and II are visible (Fig. 2a, b), one of which was located during chromatography on the lower-molecular-weight edge of peak II. Polyacrylamide gel electrophoresis patterns of peaks I and II without SDS and peak II with SDS are shown in Fig. 3a, b, and c, respectively. Apart from a few minor lower-molecular-weight bands, peak II appeared electrophoretically homogeneous on SDS-gel but was revealed as two major and two minor bands in the absence of SDS. The two major bands were found to be toxic, but the two minor bands appeared nontoxic, as judged by injection of the twice electrophoretically purified bands into guinea pigs.

**DISCUSSION**

The procedures outlined in this report permit the rapid purification of diptheria toxin to commercially high purities with minimal loss of toxin. The reproducibility of the purification scheme is illustrated in Table 1, where purities of five separate batches lay between 2,050 and 2,500 Lf/mg of protein nitrogen (Lf/OD unit values for the other three batches suggest they would also fall in this range).

Preliminary ultrafiltration of growth medium by a Diaflo PM30 membrane is essential to ensure removal of peptides above 30,000 molecular weight from the growth medium. Evidence for their existence in papain digest of beef muscle (PDB) broth has been documented by Stainer (11), and a color variation between the diffusate and retentate of PM30-filtered Casamino Acid growth medium was sometimes observed in our experiments. The removal of these high-molecular-weight peptides before toxin formation not only is essential for success of subsequent purification procedures but also permits the safe use of PDB broth as a growth medium for *C. diphtheriae* if desired. This has hitherto not been possible due to the presence of antigenic material in the PDB broth that has been shown (10) to persist through to the purified toxoid and cause sensitization of guinea pigs to beef proteins.

The Sephadex G-100 purification step was not essential to raise purities to recommended levels (1). However, removal of the protein designated peak I (Fig. 1) not only results in a considerable improvement in purity, but eliminates from the final vaccine a high-molecular-weight protein of established antigenicity in rabbits (Table 2). The variable molecular weight of this protein probably indicates that it is a polymer of a considerably lower-molecular-weight protein.

The apparent heterogeneity of diptheria toxin has not previously been described. The results presented here show that two polyacrylamide bands are toxic and that another two bands are probably nontoxic. The heterogeneity can most easily be explained by assuming a proteolytic action upon the C terminal end of whole toxin by proteases of bacterial origin present in the growth medium, thus producing a modified diptheria toxin with a differing electrophoretic mobility.

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**LITERATURE CITED**

1. British Pharmacopoeia. 1973. Absorbed diptheria vaccine, p. 175. Her Majesty’s Stationary Office, London.
2. Collier, R. J., and J. Kandel. 1971. Structure and activity of diptheria toxin. 1. Thiol-dependent dissociation of a fraction of toxin into enzymically active and inactive fragments. J. Biol. Chem. 246:1496-1503.
3. Davis, B. J. 1964. Disc electrophoresis. 11. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121:404-427.
4. Ironside, P. N. J. 1968. Production of anti-human globulin in goats. Immunology 15:503-507.
5. Mueller, J. H., and P. A. Miller. 1941. Production of diptheric toxin of high potency (100 Lf) on a reproducible medium. J. Immunol. 40:21-32.
6. Norlin, G. 1965. Studies on the purification of diphtheria toxin. Br. J. Exp. Pathol. 36:599-606.
7. Pappenheimer, A. M., Jr., T. Uchida, and A. V. Harper. 1972. An immunological study of the diphtheria toxin molecule. Immunochemistry 9:891-906.
8. Pope, C. G., and M. F. Stevens. 1968. The purification of diphtheria toxin and the isolation of crystalline toxin-protein. Br. J. Exp. Pathol. 39:139-149.
9. Schiedegger, J. J. 1965. Une micro-methode de l'immuno-electrophorese. Int. Arch. Allergy Appl. Immunol. 7:103-110.
10. Stainer, D. W. 1967. Preparation and properties of diphtheria toxoids in submerged culture. I. Presence of bovine antigens. Can. J. Microbiol. 13:963-968.
11. Stainer, D. W. 1967. Separation of bovine sensitizing material from papain digest of beef broth. Can. J. Microbiol. 13:1001-1006.
12. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulphate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.