

## Rapid, Simplified Method for Production and Purification of Tetanus Toxin

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**A rapid, simplified method for production and purification of tetanus toxin from bacterial extracts was described. The extracts were prepared by stirring young cells (ca. 45-h culture) of *Clostridium tetani* in 1 M NaCl-0.1 M sodium citrate, pH 7.5, overnight at 0 to 4°C. The toxin was purified by a combination of (i) ammonium sulfate fractionation (0 to 40% saturation), (ii) ultracentrifugation for removal of particulate materials, and (iii) gel filtration by high-pressure liquid chromatography on a TSK G3000 SW-type column. This method required 6 days as follows: (i) overnight incubation of the seed culture, (ii) 2 days for growing the bacteria for toxin production, (iii) overnight extraction of the toxin from the bacteria, (iv) overnight precipitation of the toxin with ammonium sulfate, (v) 2 h for ultracentrifugation of the ammonium sulfate concentrate of the bacterial extract, and (vi) 1 h for high-pressure liquid chromatography. The minimum lethal dose of the purified toxin preparations for mice was  $1.4 \times 10^7$  to  $1.5 \times 10^7$  per mg of protein and they showed 360 to 390 Lf (flocculating activity) per mg protein and a 280/260 nm absorbance ratio of 2.0 to 2.1. The final recovery of the toxin from bacterial extracts was 90 to 93%. The purified preparations gave a single band of toxin protein with a molecular weight of  $150,000 \pm 5,000$  on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. On crossed immunoelectrophoresis, the purified toxin preparations gave a single precipitation arc against anti-crude toxin serum.**

Tetanus toxin is produced by *Clostridium tetani* and is one of the most powerful toxins known. It has a molecular weight of approximately 150,000 and is produced within organisms and discharged into the medium as they autolyze (1).

Previously the toxin was prepared from culture filtrates after autolysis of the organisms. However, since Raynaud developed a method for extracting the toxin from organisms before they autolyzed (9), several groups have used bacterial extracts as starting material for purifying the toxin (1, 10). This method has the advantage that the toxin is obtained in a more concentrated and purer form than that from culture filtrates (9, 10).

In this study we used bacterial extracts as starting material and developed a simple, rapid method for purification of the toxin which included high-pressure liquid chromatography (HPLC). We first determined the optimal time for harvesting the organisms to extract the toxin reproducibly in maximal yield. For determining the optimal conditions for extraction, we studied the kinetics of release of the toxin from cells in hypertonic solution. Then we purified the toxin by techniques which included gel permeation chromatography by HPLC, a method which has recently become available and has the advantages of being rapid and giving high resolution.

### MATERIALS AND METHODS

**Bacterial strain.** A Biken substrain of the Harvard A47 strain of *Clostridium tetani* was used for toxin production. The strain was subcultured at least once a month in modified Tarozzi liver-liver broth (13); the Tarozzi medium was modified by replacing guinea pig liver with bovine liver. The culture was stored at 4°C after overnight growth at 35°C. Samples (0.1 ml) of an overnight culture were inoculated into

15-ml volumes of liver-liver broth, incubated overnight, and used as the seed culture for toxin production.

**Culture for production of tetanus toxin.** Samples (5.0 ml) of the seed culture were inoculated into 450-ml volumes of modified Latham medium (3, 8) in large test tubes (6 by 38 cm) covered with loose cotton plugs and incubated at 35°C in a water bath. Routinely, 18 tubes were incubated at a time. The optical density of the culture was determined at 590 nm in a Spectronic 20 spectrophotometer (Bausch & Lomb, Shimazu, Japan).

**Extraction of the toxin from bacterial cells.** At the end of the incubation period, the culture was chilled in an ice-water bath. The bacteria from 8.1-liter (18 tubes) of culture were harvested by centrifugation at  $10,000 \times g$  for 25 min at 4°C and washed twice with chilled 0.145 M NaCl by centrifugation. The washed cells were suspended in 270 ml (1/30 volume of the original culture) of chilled 1 M NaCl-0.1 M sodium citrate (pH 7.5) and incubated at 0 to 4°C for the periods indicated, with stirring (only to keep cells in suspension) unless otherwise described. After the incubation, the bacteria and debris were precipitated by centrifugation at  $10,000 \times g$  for 30 min at 4°C, and the supernatant was used as the bacterial extract.

**Ammonium sulfate precipitation.** A saturated solution (at 25°C) of ammonium sulfate in distilled water, pH 7.0, was added to the bacterial extract in an ice-water bath to give 40% saturation at 25°C. The mixture was kept cold overnight and then centrifuged at  $15,000 \times g$  for 30 min at 4°C. The precipitate was washed once with chilled 40% saturated ammonium sulfate in 0.06 M sodium-potassium phosphate buffer, pH 7.5.

**Removal of particulate material by ultracentrifugation.** The ammonium sulfate precipitate was dissolved in 20 ml of 0.1 M sodium-potassium phosphate buffer, pH 7.5, at 4°C and subjected to ultracentrifugation at  $100,000 \times g$  for 2 h at 4°C. Then, the supernatant was filtered through a membrane filter

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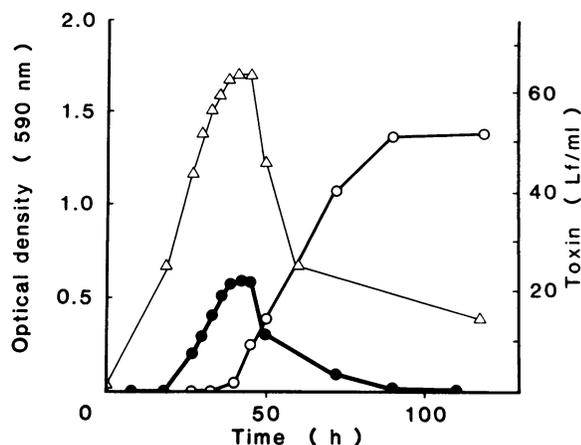


FIG. 1. Kinetics of tetanus toxin production by *Clostridium tetani* in modified Latham medium at 35°C. Symbols: ●, toxin extracted from cells with 1 M NaCl-0.1 M sodium citrate at 0 to 4°C for 24 h with stirring (Lf per milliliter of the culture); ○, toxin released into the culture medium (Lf per milliliter of the culture); and △, optical density at 590 nm of the culture.

(Acrodisc [pore size, 0.2  $\mu$ m]; Gelman), and used as the ammonium sulfate concentrate of the bacterial extract.

**HPLC.** For a final step of purification, sample (40  $\mu$ l to 0.4 ml) of the ammonium sulfate concentrate of the bacterial extract dissolved in 0.1 M sodium-potassium phosphate buffer (pH 7.5) were applied to a TSK G3000 SW column (0.75 by 60 cm; Toyo Soda Co., Tokyo) equilibrated with 0.1 M sodium-potassium phosphate buffer (pH 6.8). Elution was carried out with the latter buffer at a flow rate of 0.6 ml/min, by an HPLC pump (model HLC-803A; Toyo Soda, Co.) at room temperature, and fractions in peaks of absorbance at 280 nm were collected.

**Gel filtration by Ultrogel Aca 34.** To examine the effectiveness of HPLC in purification of tetanus toxin, we prepared the purified toxin preparation from the ammonium sulfate concentrate of the bacterial extract by using an Ultrogel column which has been previously used for the final step of purification. A 4.5-ml sample of the ammonium sulfate concentrate of the bacterial extract was applied to a column (2.5 by 100 cm) of Ultrogel Aca 34 (LKB, Bromma, Sweden) equilibrated with 0.1 M sodium-potassium phosphate buffer, pH 7.5. Material was eluted at a flow rate of 9 ml/h at 4°C, and 4.5-ml fractions of eluate were collected.

**Analysis of the toxin preparation.** Toxicity was determined as the minimum lethal dose (MLD) from the dose (log of toxin dose)-response (survival time) relationship as described previously (6). Samples (0.5 ml) of toxin preparations diluted in phosphate-buffered saline, pH 7.0, containing 0.2% gelatin were injected subcutaneously into OF1 mice weighing 20 to 25 g. Flocculating activity (Lf) was determined by a single radial immunodiffusion method (5), using 1.5% agarose gel in 50 mM Tris-0.6 M glycine buffer, pH 8.5, containing 1 mM EDTA as a preservative (TGE buffer) and a reference toxin of known Lf units. Horse antitoxin serum from which nonspecific flocculating antibodies had been removed (2) was used at 1.25 U/ml of gel for the single radial immunodiffusion method. Horse anti-crude toxin serum (lot no. B139-P) was a gift from the Kanonji Institute, The Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa, Japan. Crossed immunoelectrophoresis was carried out as described previously (2).

Briefly, the samples were initially subjected to electrophoresis on a 5.5-by-5.5-cm glass plate in agarose gel (1%) with the same buffer as electrode buffer (first-dimension electrophoresis). Then, the electric field was applied at right angles, and electrophoresis was continued into agarose gel containing horse anti-crude toxin serum (5 U/ml of gel), by applying a voltage of 2 V/cm for 20 h at room temperature (second-dimension electrophoresis). Gels were stained with Coomassie brilliant blue and destained in ethanol-acetic acid-water (4.5:1:4.5, by volume). Sodium dodecyl sulfate-polyacrylamide gel (5% gel) electrophoresis was carried out at a constant current of 8 mA per gel at room temperature for 5 h, as described originally by Weber and Osborn (12). Gels were stained with Coomassie brilliant blue and destained in ethanol-acetic acid-water. Protein was measured by the method of Lowry et al. (4).

## RESULTS

**Kinetics of tetanus toxin production.** To determine the optimal conditions for preparing bacterial extract as starting material for purification of the toxin, we studied the kinetics of toxin production by *C. tetani* in modified Latham medium at 35°C. Figure 1 shows toxin production in relation to bacterial growth and the incubation time. As the organism grew, toxin became detectable in the bacterial extract but not in the culture medium. The amount of toxin extracted from the bacterial cell (intracellular toxin) with stirring increased with the time, reaching a plateau at about 45 h just before autolysis of the organisms. Next, there was a sharp decrease in the optical density of the culture and concomitant appearance of the toxin in the culture medium (Fig. 1). In smears of the cells, increasing numbers of elongated cells were observed up to about 45 h, and then the cells autolyzed and bacterial debris became more and more obvious. Therefore, to obtain the maximum yield of intracellular toxin, we harvested the culture at about 45 h, using the optical density of the culture and the appearance of smears to determine the appropriate time.

**Rapid extraction of tetanus toxin from bacterial cells.** The cells harvested at about 45 h (before cell autolysis) were

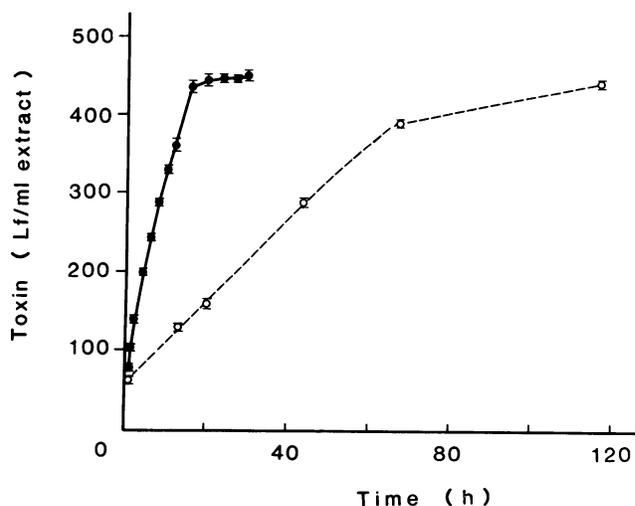


FIG. 2. Kinetics of discharge of tetanus toxin into 1 M NaCl-0.1 M sodium citrate at 0 to 4°C with (●) or without (○) stirring. The points and bars represent means  $\pm$  standard deviations for four measurements.

washed to free them of extracellular materials and then extracted with 1 M NaCl-0.1 M sodium citrate (hypertonic solution) with or without stirring. Figure 2 shows the kinetics of the release of toxin at 0 to 4°C from cells in hypertonic solution. With stirring, the toxin was rapidly released; more than 50% of the amount extracted overnight was released within the first few hours (Fig. 2). At about 16 h, the concentration of toxin in the extract reached a plateau (450 Lf units per ml) (Fig. 2). Without stirring, about 5 days were required for the concentration of the toxin in the extract to reach a plateau (Fig. 2). Reextraction of the cells overnight with stirring gave less than a quarter of the amount of the toxin obtained in the first extraction, and the second extract contained more nontoxic protein than the first. Thus, in the following experiments we used bacterial extracts prepared by a single, overnight extraction with stirring.

**Ammonium sulfate fractionation and ultracentrifugation.** Unlike the culture filtrate (7), the bacterial extract prepared by a single, overnight extraction with stirring was not precipitated appreciably at 20% saturation of ammonium sulfate. However, the toxin in the extract was precipitated

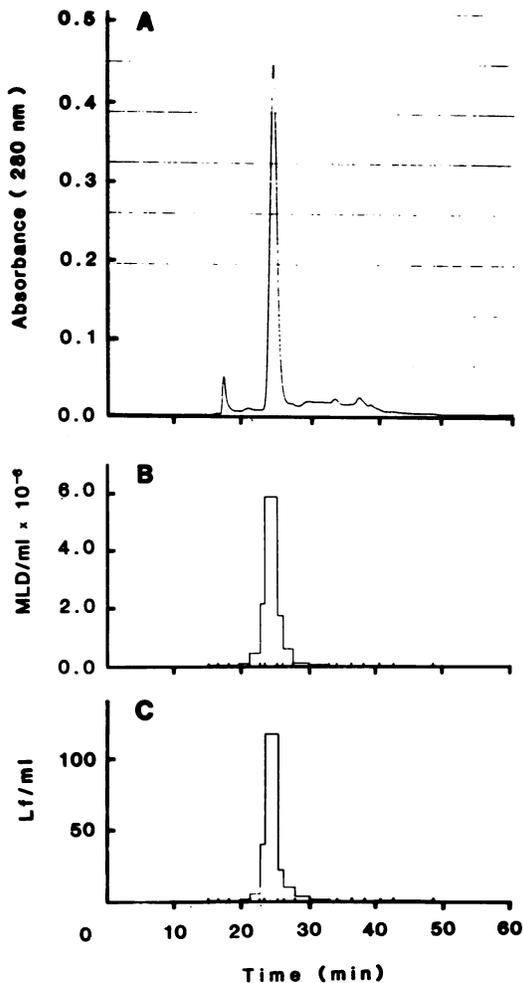


FIG. 3. Elution profile on HPLC of the ammonium sulfate concentrate of the bacterial extract. Flow rate, 0.6 ml/min. (A) Absorbance at 280 nm, (B) toxicity in mouse MLD per milliliter, and (C) Lf per ml. Sample injected: volume, 0.04 ml; amount, 1 mg of protein.

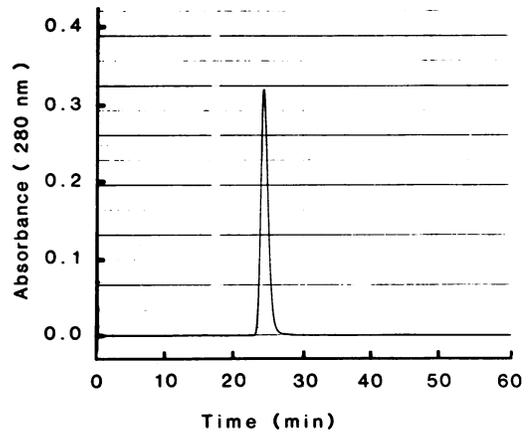


FIG. 4. Rechromatography on an HPLC column of the purified toxin preparation obtained by the present method. Flow rate, 0.6 ml/min. Sample injected: volume, 0.2 ml; amount, 0.43 mg of protein.

completely at 40% saturation of ammonium sulfate. The precipitate obtained at 40% ammonium sulfate saturation of the bacterial extracts was dissolved in 20 ml of 0.1 M sodium-potassium phosphate buffer (pH 7.5) and subjected to ultracentrifugation to remove particulate materials which were otherwise eluted as a large peak near the void volume before the toxin peak in the subsequent step of gel permeation chromatography. The toxin preparation obtained at this stage had specific activities of 240 to 260 Lf and  $0.8 \times 10^7$  to  $1.1 \times 10^7$  mouse MLD per mg of protein and a 280/260 nm absorbance ratio of 1.5 to 1.6.

**Gel permeation chromatography of the ammonium sulfate fraction of the bacterial extract by HPLC.** Figure 3 shows the elution profile on HPLC on a G3000 SW-type column of the ammonium sulfate concentrate of the bacterial extract after ultracentrifugation. About 50 min was required for this HPLC step at a flow rate of 0.6 ml/min. The elution profile showed a single sharp major peak of absorbance at 24 min (Fig. 3A). The positions of the peaks of toxicity (Fig. 3B) and

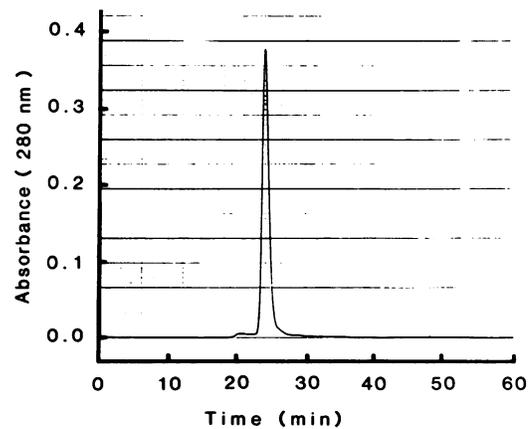


FIG. 5. HPLC analysis of the toxin preparation obtained by gel filtration of the ammonium sulfate concentrate of the bacterial extract through an Ultrogel AcA 34 column at the final step of purification. Flow rate, 0.6 ml/min. Sample injected: volume, 0.2 ml; amount, 0.5 mg of protein.

TABLE 1. Lf, MLD, and protein contents of tetanus toxin preparations at each step of purification

Preparation	Total Lf (recovery %)	LF/mg of protein	Total MLD	MLD/mg of protein	Protein (mg)	Vol (ml)
Bacterial extract <sup>a</sup>	121,500 (100) <sup>b</sup>	ND <sup>c</sup>	ND	ND	ND	270 <sup>d</sup>
Ammonium sulfate concentrate of the bacterial extract <sup>e</sup>	118,000 (97.1)	243	$3.9 \times 10^9$	$0.81 \times 10^7$	485	20
HPLC-purified preparation	110,600 (90.6)	366	$4.2 \times 10^9$	$1.4 \times 10^7$	302	140

<sup>a</sup> Starting material for the purification.

<sup>b</sup> Calculated as a percentage of that in the bacterial extract.

<sup>c</sup> ND, Not determined.

<sup>d</sup> Derived from 8.1 liters of original culture medium.

<sup>e</sup> After ultracentrifugation and filtration.

of flocculating activity (Fig. 3C) corresponded to that of this major peak of absorbance. Similar elution profiles were obtained by increasing the amount of sample to 10 mg of protein. On rechromatography on HPLC, the fraction from the major peak of the purified toxin preparation gave only a single peak (Fig. 4). HPLC analysis of the toxin preparation obtained by gel filtration of the ammonium sulfate concentrate of the bacterial extract through an Ultrogel AcA 34 column, instead of HPLC, at the final step of the purification, gave minor broad peaks in addition to one major peak of the toxin (Fig. 5).

#### Purity and properties of the purified toxin preparations.

The specific activities and recoveries of the toxin preparations at each step of purification are summarized in Table 1. The final recovery of toxin as a percentage of that of the bacterial extract was 90 to 93%. The specific activity in terms of both Lf and MLD was increased about 1.5 times by the final HPLC step. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis the purified toxin preparations obtained by the present method gave a single protein band which migrated with the same mobility to the protein band of

the toxin (molecular weight,  $150,000 \pm 5,000$ ) purified on an Ultrogel AcA 34 column at the final step of purification (Fig. 6). On crossed immunoelectrophoresis the purified toxin preparations obtained by the present method gave a single precipitation arc against anti-crude toxin serum (Fig. 7A), unlike the ammonium sulfate concentrate of the bacterial extract (Fig. 7B). The purified preparations of the toxin contained 360 to 390 Lf and  $1.4 \times 10^7$  to  $1.5 \times 10^7$  mouse MLD per mg of protein and had a 280/260 nm absorbance ratio of 2.0 to 2.1.

#### DISCUSSION

The present results showed that the method described here is more rapid and simpler than the methods reported previously (1, 7) for preparation and purification of tetanus toxin. By this method, highly purified tetanus toxin was obtained within 6 days, which was at least 7 days less than the time required in previously reported methods (1, 7). In this study, the optimal time of bacterial culture for maximum yield of the extracted toxin was determined on the basis of the kinetics of toxin production for reproducible yields. Extraction of the toxin from the bacteria with stirring not only shortened the time needed to prepare the bacterial extract but also reduced the amount of nontoxin materials in the extract. We used ultracentrifugation to remove particulate components which did not contain toxin from the ammonium sulfate concentrate of the bacterial extract. This step facilitated the subsequent step of filtration through a membrane filter required before applying the sample to an HPLC column. Thus the toxin preparation at this step was composed almost entirely of toxin protein.

For further purification of the toxin preparation, previously we used an Ultrogel column (7). The Ultrogel-purified toxin had 365 to 390 Lf units per mg of protein and  $0.8 \times 10^7$  to  $1.5 \times 10^7$  MLD per mg of protein. However, since we obtained on HPLC analysis of the Ultrogel purified toxin preparation additional minor protein peaks in addition to a major protein peak of toxin, we chose HPLC, which not only gave purer preparation but also provided a rapid procedure as a final step of purification in place of an Ultrogel chromatography.

The molecular weight of the purified toxin preparation obtained by the present simple method was similar to that (140,000 to 160,000) reported previously (1). Our purified preparation had specific activities, in terms of flocculating units and mouse toxicity per mg of protein, similar to those (360 to 420 Lf per mg of protein and  $0.5 \times 10^7$  to  $3.2 \times 10^7$  MLD per mg of protein) reported for the preparations obtained in recent years by other methods (1, 7).

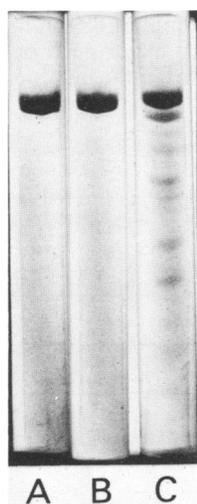


FIG. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the toxin preparation in 5% gel. (A) Toxin preparation purified on an Ultrogel AcA 34 column, instead of an HPLC column, at the final step of the purification; (B) purified toxin preparation obtained by the present method, and (C) ammonium sulfate concentrate of the bacterial extract. Samples (30  $\mu$ g) of protein were applied to each gel. Electrophoresis was performed at 8 mA per gel for 6 h at room temperature. Migration was from top to bottom.

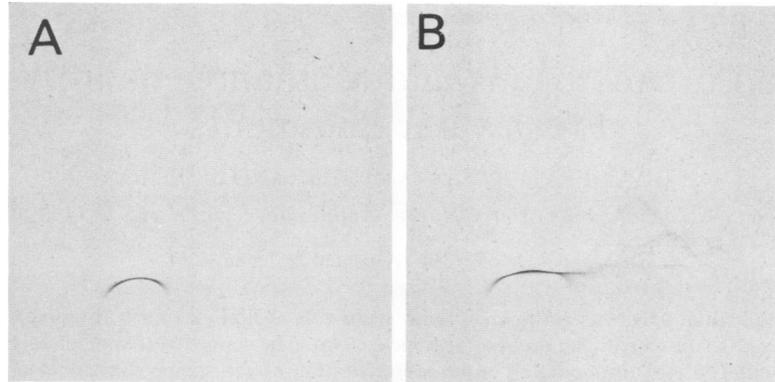


FIG. 7. Crossed immunoelectrophoresis of the tetanus toxin preparations. (A) Toxin purified by the present method (ca. 30  $\mu$ g of protein, 10 Lf). (B) Ammonium sulfate concentrate of the bacterial extract (ca. 40  $\mu$ g of protein, 10 Lf). In the first electrophoresis the anode was on the right. In the second dimension in gel containing horse anti-crude toxin serum (5 U/ml of gel), the anode was at the top.

In addition, the final preparation of tetanus toxin obtained by the present method was highly pure as shown by crossed immunoelectrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 6 and 7). Thus, additional purification procedures based on differences in electrical properties, such as ion-exchange chromatography or preparative gel electrophoresis, were not required.

These merits of the present method overcome the problem of a lower toxin content of the bacterial extract than that of the culture filtrate (about 30% of the latter). Apparently, in the bioassay the total MLD in the HPLC-purified preparation was by about 8% more than that in the ammonium sulfate concentrate. This indicates that the toxicity of the preparation was not decreased during the HPLC procedure.

For preparation of tetanus vaccine, tetanus toxoid has routinely been prepared throughout the world from partially purified toxin (30 to 70% purity) from culture filtrates containing larger amounts of nontoxigenic proteins than present in bacterial extracts. The adverse reactions of toxoid preparations are supposed to be due mainly to antigenic impurities in the preparations (11). In preliminary experiments, the toxoid prepared from the toxin obtained by the present method showed high immunogenicity. In our experiments, at the final step of the purification, about 10 mg of the toxin protein was purified within 1 h on an HPLC column (0.75 by 60 cm) in one cycle of this step. With a larger column (2.5 by 60 cm), as much as 100 mg of the toxin protein can be purified within 1 h at the HPLC step. Thus, the present method of preparing highly purified tetanus toxin will be useful not only for experimental but also for practical purposes.

#### ACKNOWLEDGMENT

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