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Isolation and Purification of Two Antigenically Active, "Complementary" Polypeptide Fragments of Tetanus Neurotoxin

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Tetanus neurotoxin (molecular weight approximately 160,000) was purified from bacterial extracts (intracellular toxin) and mildly trypsinized and from culture filtrates (extracellular toxin). Both purified preparations could be dissociated reversibly into two polypeptide chains, with molecular weights of 53,000 (fragment α) and 107,000 (fragment β), by treatment with 100 mM dithiothreitol (DTT) and 4 M urea with concomitant loss of toxicity. Upon removal of DTT and urea from the dissociated toxin preparation by dialysis, these fragments reassociated, forming the whole toxin. The two fragments were isolated and purified from the dissociated toxin by gel filtration on an Ultrogel AcA 44 column equilibrated with buffer containing 2 M urea and 1 mM DTT. The preparation of fragment α was nontoxic whereas that of fragment β was slightly toxic. Immunodiffusion analyses, using horse antitoxin, showed that the antigenicities of fragment α and fragment β were distinct from each other but were partially identical with that of undissociated toxin. The abilities of these fragments to precipitate antitoxin were lost on heating at 60 C for 5 min. The molecular substructure of tetanus neurotoxin is discussed on the basis of these findings.

Recently much attention has been focused on the molecular structure of tetanus neurotoxin (20) and structural studies on it have been made in several laboratories (1, 3, 5, 6, 10, 14, 16). Treatment of the toxin with thiol reducing agents and various denaturants resulted in dissociation of the toxin into fragments, indicating that the tetanus toxin molecule has a subchain or subunit structure (3, 6, 14). On the other hand, antigenically active materials have been obtained from the tetanus toxin by digestion with papain (1, 10), freezing (1, 5), or prolonged storage (16).

Our previous study (14), partially confirming the results of Craven and Dawson (6), showed that either extracellular or tryspsin (EC 3.4.4.4)-treated intracellular toxin was dissociable into two polypeptide fragments by treatment with dithiothreitol (DTT) and sodium dodecyl sulfate (SDS). These fragments were complementary for the whole toxin in terms of molecular weight. However, the procedures and conditions employed for dissociation yielded fragments without any activity. Accordingly we tried to isolate biologically active fragments and found that under certain conditions urea could dissociate DTT-reduced toxin reversibly into two antigenically active fragments which were complementary for the whole toxin.

This paper (part of which was presented at the 47th Annual Meeting of the Japanese Society for Bacteriology, Kyoto, Japan, 2–4 April 1974) reports the reversible dissociation of the toxin into two antigenically active polypeptide fragments and the isolation and purification of these fragments.

MATERIALS AND METHODS

Tetanus toxin. A substrain (Biken) of the Harvard A47 strain of *Clostridium tetani* was used for toxin production in a modified Latham medium. The Latham medium (12) was modified by replacing 25 g of casein digest by 20 g of polypeptone (Daigo Eiyokagaku Co.) and 10 g of whale heart extract (Nissui Seiyaku Co.) and by adding 100 μg of folic acid per liter. An overnight culture of the organism in liver-liver broth was inoculated into 60-ml volumes of the modified Latham medium in large test tubes (2.8 by 20 cm) and incubated at 35 C in a water bath for toxin production. Extracellular toxin was prepared from culture filtrates after incubation of the organism for 4 to 5 days. Intracellular toxin was obtained from bacterial extracts of cells grown for 42 to 45 h. The extracts were prepared by the method of Raynaud et al. (2, 18) by overnight extraction at 0 C with gentle stirring. The toxin in the filtrates or extracts was isolated and purified as described previously (14). Purified preparations of the toxin contained 365 to 390 flocculating units/mg of protein and had 0.8 to 1.6×10^7 minimum lethal doses (MLD)/mg of protein

and a 280 nm/260 nm absorbance ratio of 2.0 to 2.1. They each formed a single precipitation band against crude horse antitoxin in Ouchterlony plates and migrated as a single protein band upon polyacrylamide gel electrophoresis (Fig. 1A). Unless otherwise stated, intracellular toxin was employed after mild trypsin treatment (14).

Thiol reduction and urea treatment of the toxin. Thiol reduction of the toxin [1.0 to 7.4 mg of protein/ml in 50 mM tris(hydroxymethyl)aminomethane-hydrochloride, 1 mM ethylenediaminetetraacetate, pH 8.2] was carried out with DTT (final concentration, 100 mM) at 25 C for 60 min. Urea treatment of the toxin was performed by adding 100 μl of the application buffer containing 4.8 M urea to 20 μl of thiol-reduced toxin preparation for urea-polyacrylamide gel electrophoresis, or by adding solid urea to the reduced toxin to a final concentration of 4 M for reversible dissociation of the toxin and for gel filtration.

Polyacrylamide gel electrophoresis. Conventional polyacrylamide gel electrophoresis using 7.5% gel and urea-polyacrylamide gel electrophoresis using 7% gel containing 2 M urea were carried out with 10 mM tris(hydroxymethyl)aminomethane-77 mM glycine buffer, pH 8.6, as the electrode buffer after the methods originally described by Davis (7) and by Jovin et al. (11), respectively. SDS-polyacrylamide gel electrophoresis was carried out employing 5% gel as described by Weber and Osborn (21). Gels were stained using Coomassie brilliant blue, destained by diffusion, and scanned at 550 nm in a Beckman spectrophotometer-Gilford multiple sample absorbance recorder equipped with a linear transport system. Molecular weight was estimated from the relative mobility which was determined from scans of SDS-gels by measuring the distance from the top to the position of maximum peak height, employing standard protein markers as references.

Separation of fragments α and β. Fragments α and β were separated from urea-treated DTT-reduced toxin at 4 C by passing 2.2 ml of the treated toxin (originally containing approximately 4,500 flocculating units of toxin) through a column (1.5 by 90 cm) of Ultrogel AcA 44 (void volume, about 48 ml) equilibrated with 2 M urea containing 50 mM tris(hydroxymethyl)aminomethane, 0.6 M glycine, 1 mM ethylenediaminetetraacetate, and 1 mM DTT at pH 8.5. Elution was carried out at a flow rate of 5

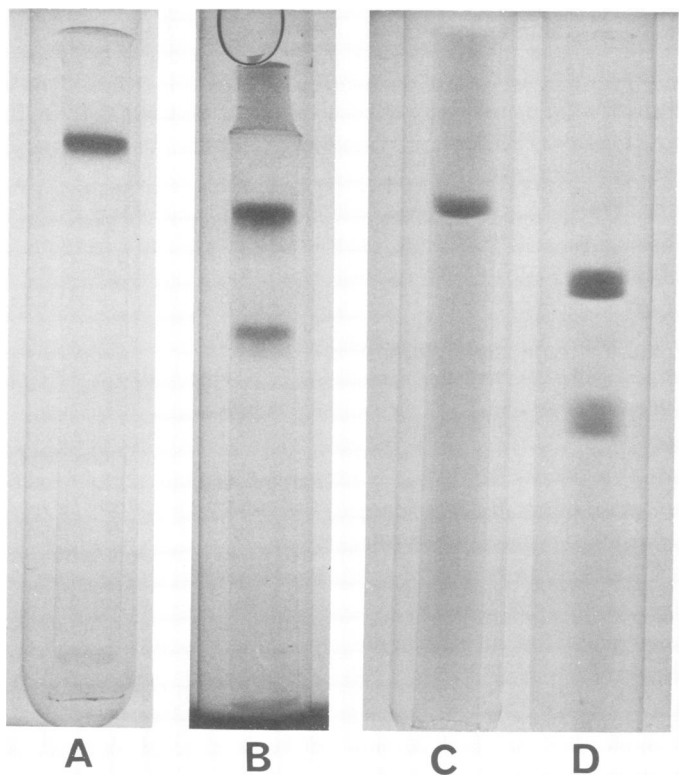

FIG. 1. *(A) Conventional, (B) 2 M urea-, and (C and D) SDS-polyacrylamide gel electrophoresis of (A and C) intact toxin and (B and D) urea-treated DTT-reduced toxin. Approximately 10 μg of protein was applied on each gel for (A and C) and 20 μg of protein was applied for (B and D). Electrophoresis was carried out at a constant current of 2 mA per gel at 4 C for 160 min (A and B), and of 8 mA per gel at room temperature for 4 h (C and D). Migration was from top to bottom.*

ml/h. Fractions of 1.2 ml were collected and the eluate was continuously monitored at 280 nm with an LKB Uvicord II.

Flocculation test. The flocculation test was carried out by Ramon method using tetanus antitoxin serum from which nonspecific flocculating antibodies had been removed (19).

Immunodiffusion test. The double diffusion precipitation method originally described by Ouchterlony (17) was employed using 1% agarose containing 0.5 M glycine in buffered saline.

Toxicity test. Toxicity measured as the MLD in OF1 mice was estimated from the log of the dose-survival time relationship as described by Murata et al. (15).

Protein determination. Protein was estimated by the method of Lowry et al. (13).

Tetanus antitoxin serum. Horse antitoxin serum (lot no. B215, 900 antitoxin units/ml) which had been pepsinized and partially purified by ammonium sulfate fractionation was a gift from the Kanonji Institute, The Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa.

Chemicals. DTT was obtained from Sigma Chemical Co., SDS from Pierce Chemical Co., Ultrogel AcA 44 from LKB, and urea (for biochemistry) from E. Merck, Darmstadt. Human gamma globulin, ovalbumin (Schwarz/Mann Co.), bovine serum albumin (Armour Pharmaceutical Co.), pepsin (2× crystallized), and trypsin (Worthington Biochemical Corp.) were used as standard molecular weight markers.

RESULTS

Reversible dissociation of thiol-reduced toxin with urea. The purified preparation of toxin migrated as a single protein band on conventional polyacrylamide gel electrophoresis (Fig. 1A). On SDS-polyacrylamide gel electrophoresis it moved as a single protein component with a molecular weight of approximately 160,000 (Fig. 1C). However, when the toxin was reduced with DTT and then treated with urea (final concentration, 4 M) and subjected to conventional polyacrylamide gel electrophoresis, almost all the preparation was found to be dissociated into two protein bands. Further investigation showed that the urea-treated DTT-reduced toxin gave the sharpest bands upon electrophoresis on gel containing 2 M urea (Fig. 1B). On SDS-gel electrophoresis, the urea-treated DTT-reduced toxin gave two protein bands (Fig. 1D), the slower band having the mobility of fragment β (molecular weight approximately 107,000 [14]) and the faster band having the mobility of fragment α (molecular weight approximately 53,000 [14]). The ratio of the amount of the slower moving component to that of the faster moving component, estimated from the areas under the densitometer scan curves of the stained urea gels, was about 2.0, indicating that the two components exist in an equimolar ratio in the toxin molecule.

After treatment with DTT and urea, the toxin lost its toxicity greatly (Table 1). However, when the DTT and urea were removed from the urea-treated DTT-reduced toxin preparation by dialysis against 0.1 M potassium sodium phosphate buffer, pH 7.5, at 4 C for 48 h, about 30 to 50% of the original toxicity was restored (Table 1). On conventional gel electrophoresis, the dialyzed material gave essentially a single protein band (Fig. 2B) at a position corresponding to that of the untreated toxin (Fig. 2C). Toxicity tests on eluates from slices of the unstained gel of the dialyzed material showed that the restored toxicity was in eluates from a few slices in a position corresponding to that of the single protein band in the stained gel (Fig. 2A). The dialyzed material formed a single precipitation band which fused completely with that formed between antitoxin and untreated toxin on immunodiffusion.

Separation of the fragments from urea-treated DTT-reduced toxin by gel filtration. When the urea-treated DTT-reduced toxin was subjected to gel filtration on Ultrogel AcA 44 in the presence of 2 M urea and 1 mM DTT, two protein peaks (Fig. 3A and B) were eluted from the column. These two protein fractions, A and B, were identified as fragment α and fragment β, respectively, in subsequent experiments. A peak eluted in an eluate volume of about 140 ml was due to DTT present in the sample applied to the column.

Properties of the two purified fragments. The purities of the materials in the two protein peaks obtained from the urea-treated DTT-reduced toxin by gel filtration were checked by

TABLE 1. *Change in toxicity on treatment[a] with DTT and urea*

Determinants	Expt 1	Expt 2
	Toxicity (MLD/mg of protein)[b]	Toxicity (MLD/mg of protein)[b]
Toxin before treatment	8.2×10^6 (100)	1.1×10^7 (100)
Toxin after treatment	1.5×10^4 (0.18)	3.2×10^5 (2.9)
Treated toxin after removal of DTT and urea	2.2×10^6 (27)	5.4×10^6 (49)

[a] Toxin preparations at concentrations of 1.1 and 7.4 mg of protein/ml were treated with DTT and urea for experiments 1 and 2, respectively.

[b] Calculated taking the toxicity of each toxin preparation before treatment as 100. Parentheses indicate percentage.

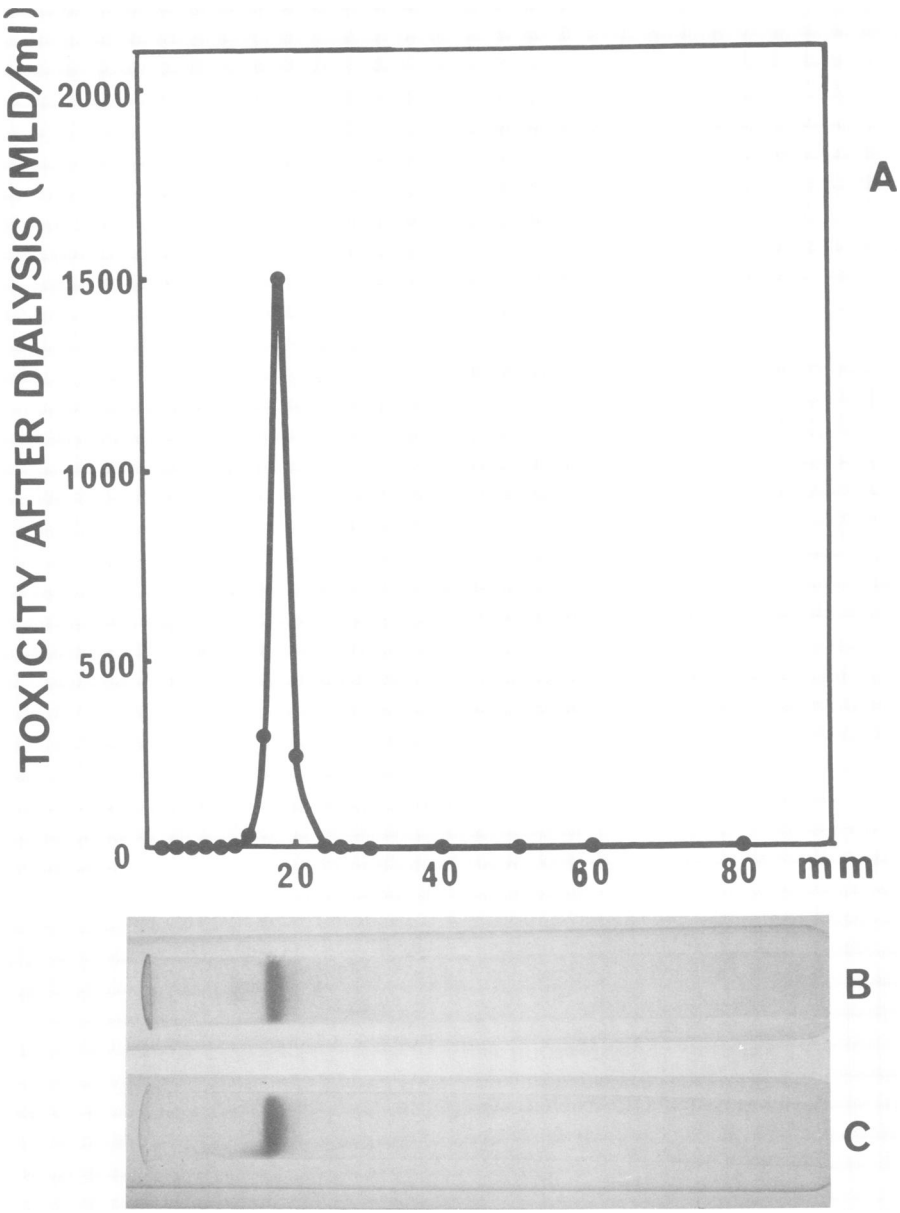

FIG. 2. *Toxicity (A) and electrophoretic patterns on conventional polyacrylamide gel (B) of urea-treated DTT-reduced toxin after dialysis. Purified toxin (1.1 mg/ml) was reduced with DTT and treated with urea as described. A sample of 20 μl of the treated toxin was analyzed by urea-polyacrylamide gel electrophoresis. The mixture of toxin, DTT, and urea was then dialyzed to remove DTT and urea. After dialysis the mixture was analyzed by conventional polyacrylamide gel electrophoresis in duplicate using 10- and 30-μl samples. One of the gels (10-μl sample) was stained (B), and the other (30-μl sample) was analyzed for toxicity (A) after overnight elution of 2-mm slices with 0.8 ml of the modified Latham medium at 4 C. (C) Electrophoretic pattern on conventional polyacrylamide gel of intact toxin (10 μg of protein).*

SDS-gel electrophoresis. The materials in peak A and peak B, respectively, moved virtually as single protein bands (Fig. 4), with molecular weights of 53,000 and 107,000 (Fig. 5). These values agree well with those of the fragments of the SDS-treated DTT-reduced toxin originally designated as fragment α and fragment β (14).

The two fragments formed single precipitation bands which crossed each other against horse antitoxin (Fig. 6). These precipitation

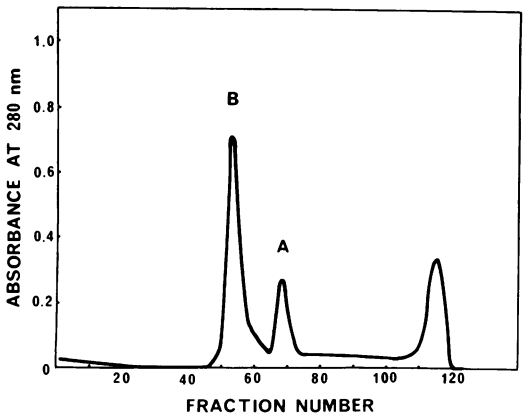

FIG. 3. *Gel filtration of the urea-treated DTT-reduced toxin on an Ultrogel AcA 44 column in the presence of 2 M urea and 1 mM DTT. Peak A, fragment α; peak B, fragment β.*

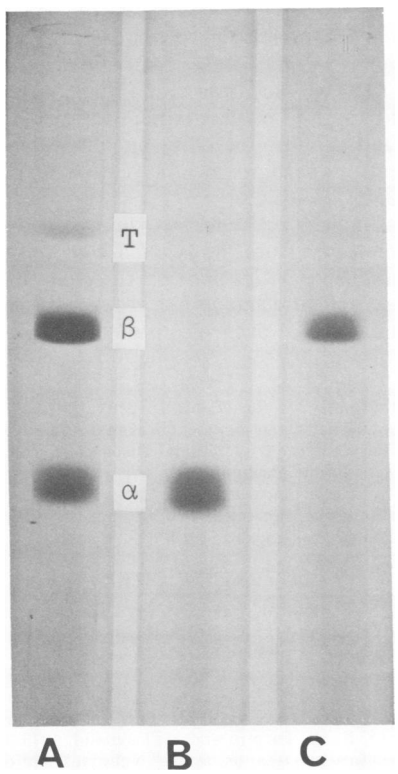

FIG. 4. *SDS-polyacrylamide gel electrophoresis of urea-treated DTT-reduced toxin (A), purified fragment α (B), and purified fragment β (C). Approximately 10 μg of protein was applied on each gel for (B and C) and 20 μg of protein was applied for (A). T, Residual intact toxin in the urea-treated DTT-reduced toxin preparation; α, fragment α; β, fragment β. Electrophoresis was carried out at a constant current of 8 mA per gel for 5 h. Migration was from top to bottom.*

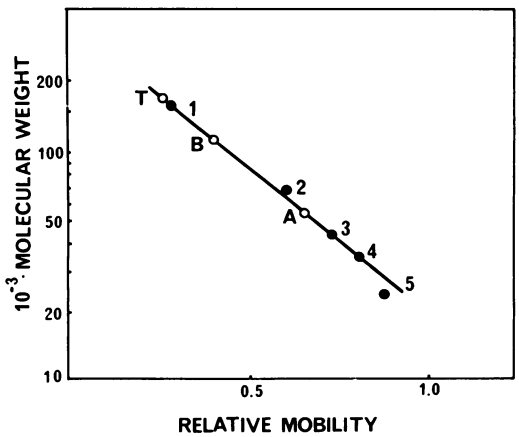

FIG. 5. *Calibration of the dodecyl sulfate gels with marker proteins. T, Intact toxin; A, fragment α; B, fragment β. Protein markers: 1, human gamma globulin; 2, bovine serum albumin; 3, ovalbumin; 4, pepsin; 5, trypsin.*

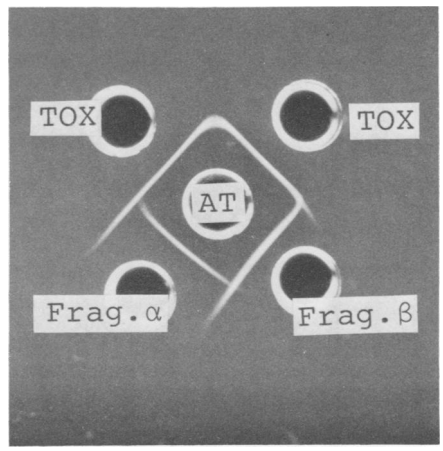

FIG. 6. *Immunodiffusion pattern of toxin, fragment α, and fragment β against horse antitoxin. TOX, Intact toxin (2.0 mg/ml); AT, antitoxin (900 U/ml); Frag. α, fragment α (0.3 mg/ml); Frag. β, fragment β (0.8 mg/ml).*

bands each fused partially with the precipitation band of the undissociated toxin (Fig. 6). The antitoxin precipitating abilities of these fragments were lost on heating at 60 C for 5 min.

A purified preparation of fragment α showed no detectable toxicity in mice, even when injected at a dose of as much as 4 μg of protein. Purified preparations of fragment β were slightly toxic (2.2 to 89 MLD/μg of protein). It can be calculated that 2.2 to 89 MLD are equivalent to 0.14 to 11 ng of undissociated toxin, which corresponds to less than 1.2% of the protein content of fragment β preparations.

DISCUSSION

Previous investigations, using SDS-gel electrophoresis (3, 6, 14) or gel filtration in the presence of 6 M guanidine (3, 6) or 7 M urea (6), demonstrated that reduced tetanus toxin can be dissociated into polypeptide fragments of different molecular sizes. However, these fragments were devoid of biological activity, and in fact, our preliminary experiments showed that treatment of tetanus toxin with SDS or guanidine resulted in irreversible loss of toxicity and immunochemical activity.

The results presented in this paper show that tetanus toxin (either extracellular or trypsin-treated intracellular toxin) can be dissociated reversibly into two antigenically active polypeptide fragments which are complementary for the whole toxin in terms of molecular size, toxicity, and antigenicity. Our success in reversible dissociation of the toxin is chiefly due to use of urea at an appropriate concentration (4 M) as a denaturant in the presence of a thiol reducing agent. We found that use of urea at a concentration higher than 4 M yielded increasing amounts of aggregated material with no immunochemical reactivity, whereas its use at a lower concentration resulted in incomplete dissociation.

After this reversible dissociation, the antigenically active fragments were successfully isolated by gel filtration in the presence of 2 M urea and it was found that 2 M urea was optimal for the separation of these fragments on gel electrophoresis (Fig. 1B). The reversible nature of the dissociation in terms of toxicity and antigenicity proves that the fragments retained functional activities. Several attempts have been made to elucidate the antigenic structure of tetanus toxin (1, 4, 5, 10, 16) and at least four antigenic determinant groups have been distinguished in the tetanus toxin molecule by isolating three antigenic materials from the toxin after its degradation by freezing or prolonged storage (5, 16). However, no antigenically active fragments which are complementary for the whole tetanus toxin molecule have previously been obtained, so the antigenic structure of tetanus toxin could not be analyzed precisely in relation to its molecular substructure. Studies are in progress on whether the antibodies against fragment α and fragment β are equally effective in neutralizing toxin and how they contribute to protection against tetanus in vivo. The isolation of the antigenically active, complementary fragments α and β as described above will provide a basis for elucidation of the structure-function relationship of the tetanus toxin molecule.

Bizzini et al. (3) proposed that the tetanus toxin molecule was a dimer of two identical subunits (molecular weight 73,000) associated by noncovalent bonds. However, in contrast with their view, the present findings give definitive evidence for the existence of the following molecular states of tetanus toxin: (i) intracellular tetanus toxin consists of a single polypeptide chain (intact toxin) with a molecular weight of about 160,000 (6, 14). (ii) Most extracellular toxin molecules are composed of two polypeptide chains (fragment α, molecular weight 53,000, and fragment β, molecular weight 107,000, respectively) which have distinct antigenicities and which are linked by at least one disulfide bridge and associated by noncovalent bonds dissociable with denaturants such as SDS or urea. (iii) The intracellular toxin can be converted to the nicked form (8, 9) as extracellular toxin (14) by mild trypsin treatment.

The slight toxicity associated with fragment β is probably due to undissociated toxin contaminating the preparation, though the possibility that the toxicity is an intrinsic activity of fragment β has not yet been excluded. Further purification of fragment β is required to clarifiy this point.

Experiments on reconstitution of the whole toxin molecule from the two isolated fragments and further investigations on the chemical, immunological, and biological properties of these fragments are in progress in our laboratory.

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