cini and Angeletti (*21*) demonstrated that fluorescent forms of the antibodies are fixed in the cytoplasm of neurons. Sabatini and his co-workers (*22*), who used the electron microscope, describe "considerable damage to most of the neurons while neurolemma and endothelial cells remain intact" a few hours after injection of the antiserum. These findings are interpreted as cytotoxic antigen-antibody reactions in sympathetic neurons. The findings seem to require that NGF or some closely related hapten be present in the neurons. However, the antibodies produced against Cohen NGF in this laboratory or by Abbott (*23*) are complex as judged by the several precipitin lines that appear on Ouchterlony plates or by immunoelectrophoresis. We do not know whether there is an antibody to NGFB among these.

We are uncertain whether NGFA and NGFB are entirely different substances or differently bound forms of the same biologically active substance. However, it seems clear that the response by neurons is all-or-none, and that a single molecule may affect several neurons.

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Tetanus Toxin: Fine Structure Localization of Binding Sites in Striated Muscle

Abstract. *When striated muscle from mice poisoned with purified tetanus neurotoxin was incubated with tetanus antitoxin labeled with horseradish peroxidase and stained for peroxidase activity, an electron-opaque reaction product was found in the transverse and terminal sacs of the longitudinal elements of the sarcotubular system. Demonstration of toxin in this location suggests that it acts by interfering with contraction coupling or mechanisms of contraction-relaxation.*

Although many data (*1*) indicate that the central nervous system is a major site of toxin action, tetanus toxin binds to skeletal muscle and produces abnormalities of electrolyte flux within it (*2, 3*). Studies in which toxin labeled with fluorescein was used have shown marked selectivity for the central nervous system and striated muscle. To increase the resolution of binding studies, we used the technique of labeling proteins with horseradish peroxidase (*4*).

Mice poisoned with a minimum saturation dose (9 μg) of purified tetanus toxin (*5*) were killed when signs of generalized intoxication were well developed. Pieces of intercostal and leg muscles were excised, fixed in 10 percent phosphate-buffered formalin for 4 hours, and washed overnight in 0.7*M* phosphate buffer (*p*H 7.6) containing 5 percent sucrose. Frozen sections (40 μm) were cut in a cryostat and incubated 30 minutes in tetanus antitoxin labeled with horseradish peroxidase according to the method of Nakane and Pierce (*4*). The sections were washed with agitation for 30 minutes in phosphate-buffered saline, fixed with 4 percent glutaraldehyde, washed, and incubated for 10 minutes in 0.05*M* tris-HCl buffer (*p*H 7.6) containing 3′,3′-diaminobenzidine and 0.001 percent H_2O_2. After additional washing, the sections were treated for 90 minutes in 1.3 percent OsO_4 in buffered saline (*p*H 7.6) containing 5 percent sucrose. The sections were dehydrated and embedded in Epon 812. The embedded material was sectioned with a Porter-Blum microtome and examined in an RCA EMU-3F electron microscope. Unstained sections and sections stained in 1 percent uranyl acetate in ethanol were examined. Staining increased the contrast of muscle structures but left the histochemical reaction product unaltered. Control preparations consisted of normal skeletal muscle incubated with tetanus antitoxin labeled with peroxidase. Immunoelectrophoresis of the labeled antitoxin was also performed to ascertain whether the antitoxin was labeled and whether it retained its ability to react with the toxin. The precipitin line was found in its normal position, and staining with 3′,3′-diaminobenzidine revealed that the line contained peroxidase activity. Despite the rigorous treatment of the tissues, the major ultrastructural components of skeletal muscle were still recognizable. Myofibrillar fine structure was less disrupted than elements of the sarcotubular system. However, transverse and longitudinal components were easily identified.

In muscle from intoxicated mice, frequent but not ubiquitous accumulations of electron-opaque amorphous material characteristic of the histochemical reaction product were found free within the lumen and on the lumenal

surface of the T-tubules (Figs. 1–3). Some sections revealed this product in the space between the T-tubule and the terminal sacs of the longitudinal component and within the lumen of the terminal sacs. Reaction product was rarely found in the longitudinal components at a distance from the terminal sacs. The reaction product present as granules or amorphous masses was considerably more opaque than the granular material recognized as a normal component within the sarcotubular system (*6*). Similarly, the moderately electron-opaque material (*7*) visible in the interface of T-tubules and terminal sacs of the normal triad after formalin fixation was considerably less opaque than the histochemical reaction product. No reaction product was observed within motor endplates. Control preparations were devoid of reaction product within the sarcotubular system or elsewhere (Fig. 4).

Both of the sites where tetanus toxin was localized are involved in the relaxation-contraction cycle of muscle. The T-system appears to function in conducting the wave of depolarization into the muscle; calcium ions are released as an initial event in contraction (*8*) and relaxation is also accompanied by a movement of calcium in which the sarcotubular system participates (*9*). The electrolyte flux of muscle poisoned with tetanus toxin is abnormal relative to that of normal muscle (*3*), and our results indicate that calcium metabolism of such muscles is abnormal (*10*). In addition, Ranson (*11*) has shown by direct measurement that the relaxation time of muscle is increased in cats poisoned with tetanus toxin. Thus the localization of the toxin which we have observed is consistent with independent evidence on its physiological action on muscle.

Since there is no known connection of the longitudinal component of the sarcotubular system to the exterior (*12*) and all the reaction product was found in either the T-system or the longitudinal components of the sarcotubular system, it seems likely that toxin enters the muscle via the T-system and traverses the gap separating it from the longitudinal system in the triadic area where there is membrane specialization. Recently, Luft (*13*) demonstrated apparent penetration of ruthenium red into terminal sacs of the longitudinal system via T-tubules.

We suggest that the peripheral action of tetanus neurotoxin lies in its interference with electrolyte flux in the sarcotubular system (*14*).

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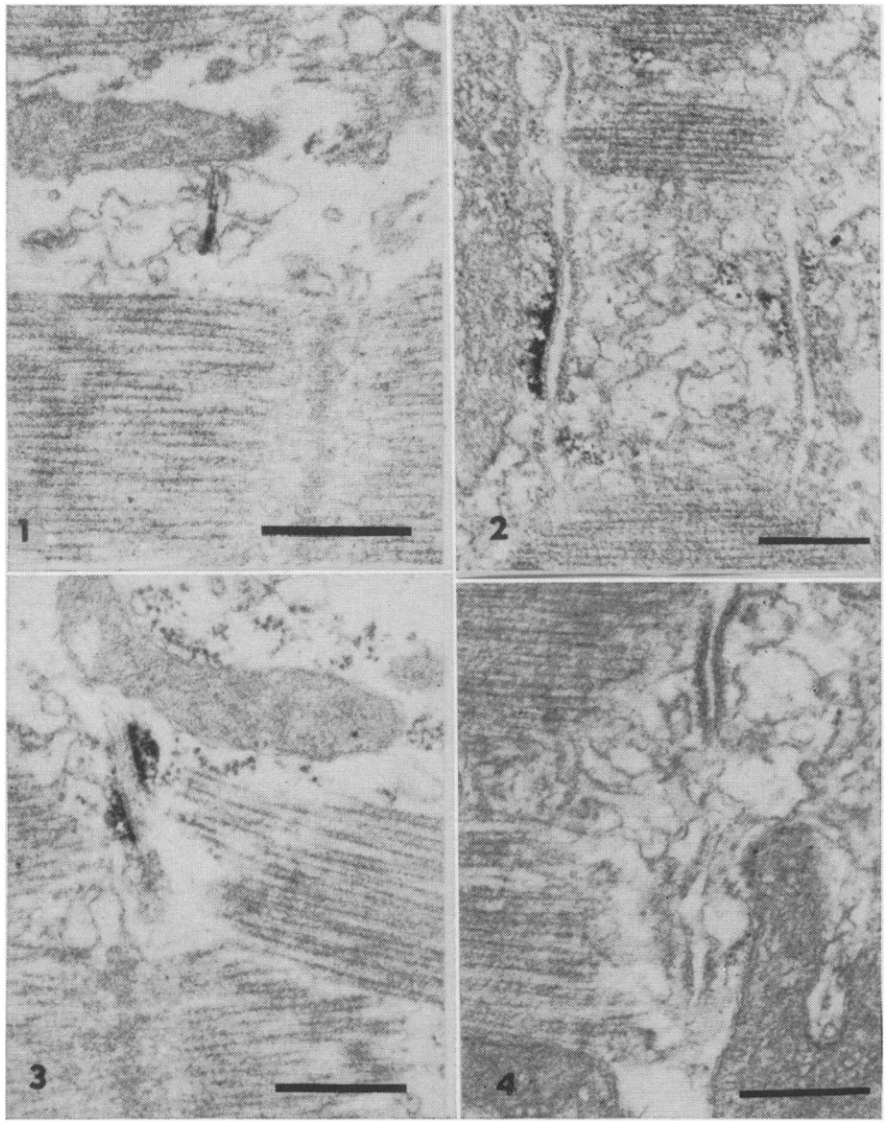

Fig. 1. Mouse skeletal muscle poisoned with tetanus toxin and incubated with peroxidase-labeled tetanus antitoxin. Electron-opaque amorphous and particulate histochemical reaction product partially fills the lumen of a T-tubule. Dilated terminal sacs of the longitudinal system forming a triad are also present. In all figures the line indicates 0.5 μ. Fig. 2. Electron-opaque histochemical reaction product at the sites of toxin binding in the region between T-tubules and longitudinal components and within terminal sacs. Fig. 3. Granules of histochemical reaction product demonstrating localization of tetanus toxin within a terminal sac of a longitudinal element of the sarcoplasmic reticulum. Fig. 4. Appearance of normal mouse skeletal muscle incubated with tetanus antitoxin labeled with peroxidase and stained to demonstrate peroxidase activity. The sarcoplasmic reticulum is devoid of reaction product.

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