**References and Notes**

1. E. S. Russell and S. E. Bernstein, in *Biology of the Laboratory Mouse*, E. L. Green, Ed. (Dover, New York, ed. 2, 1976), pp. 351-372; J. B. Whitney, Jr., *Cell* **12**, 863 (1977); B. P. Alter and S. C. Goff, in preparation.
2. C. Friend, M. C. Patuleia, E. deHarven, *Natl. Cancer Inst. Monogr.* **22**, 505 (1966); M. C. Patuleia and C. Friend, *Cancer Res.* **27**, 716 (1967); C. Friend, W. Scher, J. G. Holland, T. Sato, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 378 (1971).
3. D. Kabat, C. C. Sherton, L. H. Evans, *Cell* **5**, 331 (1975); U. Nudel, J. E. Salmon, M. Terada, A. Bank, R. A. Rifkind, P. A. Marks, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1100 (1977); B. P. Alter and S. C. Goff, *Blood* **50**, 867 (1977); *ibid.* **52**, 1047 (1978); G. Rovera, J. Vartikar, G. R. Connolly, C. Magarian, T. W. Dolby, *J. Biol. Chem.* **253**, 7588 (1978)
4. Line 9M of line 745 was the gift of Dr. A. Deisseroth.
5. G. Rovera, C. Magarian, T. W. Borun, *Anal. Biochem.* **85**, 506 (1978).
6. W. M. Bonner and R. A. Laskey, *Eur. J. Biochem.* **46**, 83 (1974); R. A. Laskey and A. D. Mills, *ibid.* **56**, 335 (1975).
7. J. Gibbons, *Non-Parametric Statistical Inference* (McGraw-Hill, New York, 1971), pp. 146, 183.
8. Th. Papayannopoulou, M. Brice, G. Stamatoyannopoulos, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2923 (1977); E. J. Benz, Jr., J. E. Barker, J. E. Pierce, P. A. Turner, A. W. Nienhuis, *Cell* **14**, 733 (1978).
9. D. J. Weatherall, J. B. Clegg, W. G. Wood, *Lancet* **1976-II**, 660 (1976).
10. E. C. Abraham, A. Reese, M. Stallings, T. H. J. Huisman, *Hemoglobin* **1**, 27 (1976-77).


11. We thank Dr. David Nathan for encouragement and helpful discussions, Dr. Stuart Orkin for his advice regarding cloning, and Dr. Kenneth Stanley for the statistical analyses. Supported by NSF grant PCM 76-09302, NF/MOD grant 1-513, and NIH research career development award HL-00177 (B.P.A.)


* Present address: Division of Hematology, Mount Sinai School of Medicine, 1 Gustave Levy Place, New York 10029.


9 July 1979; revised 7 November 1979


# Postsynaptic Transmission Block Can Cause Terminal Sprouting of a Motor Nerve


Abstract. *Sprouting of mouse soleus motor nerve terminals can be evoked by daily intramuscular injections of purified α-bungarotoxin. This finding supports the hypothesis that an important stimulus to terminal sprouting in partial denervation and presynaptic nerve blockade is a product of inactive muscle fibers.*


The factor or factors responsible for controlling new growth from adult motor nerve terminals are still unknown. That terminal sprouting can be evoked by presynaptic blockade of transmission, by either botulinum toxin (*1*) or tetrodotoxin (*2*), has led to the suggestion that the sprouting signal arises from inactivated muscle fibers, which are, of course, also present in partially denervated muscles. However, it is also claimed that blockade of axoplasmic transport without interference in neuromuscular transmission leads to sprouting (*3*). Presynaptic blockers were thus suggested to cause sprouting by preventing the release of a neuronally transported antisprouting agent (*3*). That directly activating the paralyzed muscles inhibits the expected sprouting produced by botulinum toxin paralysis (*4*) militates against this hypothesis. We now present further evidence to support an important role for inactive muscle in producing terminal sprouting. By giving repeated injections of a postsynaptic blocker—purified α-bungarotoxin (α-bgt) (a highly specific noncompetitive blocker of the acetylcholine receptor that is unlikely to have any direct effects on the nerve)—we have, in contrast to previous reports (*5*, *6*), produced significant terminal sprouting.

The experiments were carried out on the soleus muscles of female Olac mice (20 to 35 g). The technique was essentially as described elsewhere (*7*). Several tests allowed us to be sure that we were dealing with α-bgt (*8*). It is important that pure toxin be used; commercially available "purified" α-bgt can cause degeneration of motor nerve terminals and a long-lasting paralysis after just one injection. The toxin we used causes a temporary paralysis with no degeneration, which is why repeated injections were required.

The toxin was dissolved in mammalian Ringer solution to which had been added 2 mg of bovine serum albumin (BSA) (fraction V, BDH biochemicals) per milliliter of solution to give a final toxin concentration of 1 mg/ml. The BSA was necessary to prevent the adsorption of the toxin to glass. The mice were sedated with 3.5 percent chloral hydrate (0.1 ml per 10 g of body weight) injected intraperitoneally before each injection of toxin, in an attempt to improve the localization of the toxin. The toxin was injected with a 10-μl Hamilton syringe directly into the back of the leg just above the Achilles tendon. The mice received 1 μg daily or 0.5 μg twice daily. Control mice were treated identically, but received only BSA in Ringer solution. Botulinum toxin (crude type A) was injected in the same way; one dose of 0.03 μg in 3 μl produced a profound, local, long-lasting paralysis. The physiologic experiment took place between 4 and 8 days after starting the injections, times at which sprouting would be expected to be well under way (*2*). The mice were anesthetized (0.2 ml of 3.5 percent chloral hydrate per 10 g of body weight), and the right soleus muscle and nerve were dissected free. The muscle was studied in vitro; the isometric nerve and directly evoked twitch tensions and the 50-Hz tetanic tensions were measured. The muscle's acetylcholine sensitivity was assessed by comparing its contracture in $5 \times 10^{-4}$ g per milliliter of acetylcholine perchlorate with the maximum 50-Hz direct tetanic tension. Finally, each muscle was pinned out on a Sylgard-bottomed dish and stained for 6 hours in zinc iodide and osmium tetroxide. The muscles were teased apart with fine forceps, dehydrated in ethanol, cleared in xylene, and mounted in Permount. The observer examined all slides without knowing to which group it belonged and noted for each end plate examined its length in the long axis of the muscle and the corresponding length of any sprouting of that end plate. Fifty or more randomly chosen end plates were examined in each muscle. The amount of sprouting was expressed as the percentage of the end plates examined that had terminal sprouts. Normal solei have, on average,

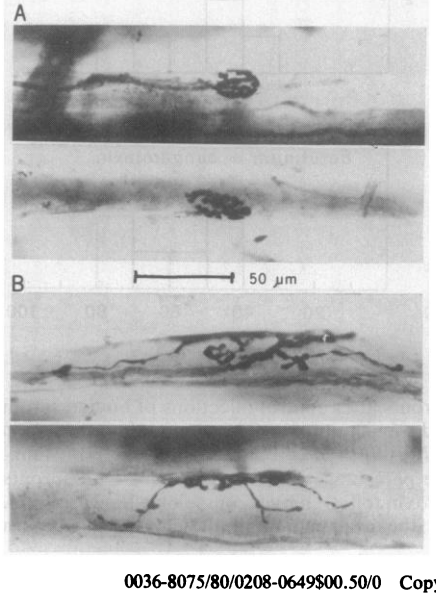


Fig. 1. End plates, stained with zinc iodide and osmium tetroxide, in mouse soleus muscles. (A) Two end plates of normal appearance from a muscle treated for 6 days with a daily injection of 1 μl of Ringer solution containing 2 μg of bovine serum albumin. (B) Two end plates with fine terminal sprouts from a muscle treated for 7 days with a single daily injection of 1 μl of Ringer solution containing 1 μg of purified bungarotoxin and 2 μg of bovine serum albumin. The virtue of the zinc iodide-osmium technique is that it stains the unmyelinated portions of the nerves in their entirety.



0036-8075/80/0208-0649$00.50/0

fewer than 10 percent of such sprouts on this index (*2*). The size of the sprouts expressed as the mean length of the sprouts per sprouting end plate takes no account of the numbers of sprouts (*9*).

Examples of end plates from control and α-bgt-poisoned muscles are shown in Fig. 1, and the amounts of sprouting in Fig. 2A.

In the mice treated with α-bgt, 33 ± 3 percent ($N = 11$) of terminals had sprouts, compared with a control mean of 15 ± 8 percent ($N = 9$) (Mann-Whitney U test, $P < .01$). The actual size of the sprouts themselves was increased from 15.6 ± 7.3 (controls) to 26.4 ± 3.8 μm (α-bgt animals), $P < .05$. In the α-bgt-blocked preparations, the sprouting end plates (excluding the sprouts) were not significantly larger than the nonsprouting and BSA control end plates. Most of the growth of the sprouting end plates must therefore occur as fine outgrowths (Fig. 1) rather than as a generalized increase in end plate size and complexity. The lack of sprouting seen by Pestronk and Drachman (*6*) may well be due to a failure to stain these outgrowths.

The relatively high mean value for the controls is due to one muscle in which 76 percent of the terminals had sprouts. This muscle was in fact partly denervated, as the nerve-evoked tension was less than the direct tension, and degenerating axons could be seen histologically. No such degeneration was seen in any of the other control or experimental muscles; in this case it was probably caused by too proximal an insertion of the injection needle. Damage and inflammation are dangers of repeated injections, but they cannot explain the differences between the experimental and control muscles, since they received exactly the same number of injections.

Previous attempts at producing sprouting by postsynaptic blockade have not been successful (*5*, *6*). One possible reason is that although the α toxin binds essentially irreversibly to acetylcholine receptors, the turnover of receptors is fast enough for sufficient numbers to appear within 24 hours to restore transmission (*10*). As even small amounts of activity suppress the development of acetylcholine sensitivity (*11*), and presumably also any putative sprouting stimulus, the absence of sprouting in previous research and the relatively small amount in our experiments may result from this difficulty in obtaining a lasting block. The degree of blocking (100 percent minus nerve-evoked twitch tension as a percentage of the directly evoked twitch tensions) in our solei averaged only 13 percent when the muscles were examined 24 hours after 1 μg or 12 hours after 0.5 μg of α-bgt, but if examined within 2 hours of the last toxin injection the degree of blocking averaged 94 percent. In fact, complete blockade of junctional and extrajunctional receptors with α-bgt requires constant infusion (*12*, *13*). This necessitates anesthesia and artificial respiration since the toxin spreads rapidly through the animal, giving only a relatively greater block at the site of injection than elsewhere. A regime of daily injections of the purified toxin is also difficult, requiring as it does sufficient toxin to at least partially paralyze the lower leg for 24 hours yet not so much that it kills the animal. Empirically, 1 μg per day turned out to be the maximum tolerated by our mice.

Alternatively, Pestronk and Drachman (*6*) suggested that α-bgt actively inhibits sprouting by "covering up" the extrajunctional acetylcholine receptors, which they believe are the stimulus to terminal sprouting. A major part of their evidence is that they were able to inhibit the expected terminal sprouting produced by botulinum toxin (a long-lasting, presynaptic neuromuscular blocker) by giving repeated doses of α-bgt.

On repeating Pestronk and Drachman's experiment of administering α-bgt to muscles paralyzed by botulinum toxin, no suppression of sprouting occurred, nor were the sprouts any smaller, whether the α-bgt was delivered once daily (as Pestronk and Drachman did) or even twice daily. The mean percentage of terminals with sprouts after botulinum toxin alone was 50 ± 6 ($N = 6$); in muscles treated with α-bgt as well, it rose to 58 ± 4 ($N = 6$) (Fig. 2B). The lengths of the sprouts grew from 40 ± 11 μm to 59 ± 8 μm. Even with twice-daily injections of α-bgt, however, we could not achieve a lasting block of the acetylcholine receptors; within 12 hours of an injection the muscles became substantially sensitive to acetylcholine (contractures > 50 percent of the 50-Hz direct tetanus). On theoretical grounds, single daily injections of α-bgt cannot be expected to block all the acetylcholine receptors permanently, since the average lifetime of an extrajunctional receptor is less than 20 hours (*10*).

We conclude that postsynaptic blockade with α-bgt will, if muscle inactivity can be maintained long enough, cause terminal sprouting from mammalian alpha motoneurons. Our results support a direct role for inactive muscle fiber in producing terminal sprouting, as the specificity of action of α-bgt makes it improbable that it could cause sprouting in any other way. It does not interfere with axonal transport, for example (*12*).

Unfortunately, although we are unable to confirm Pestronk and Drachman's finding (*6*) that α-bgt delivered daily to a muscle paralyzed with botulinum prevents sprouting, we cannot claim to have

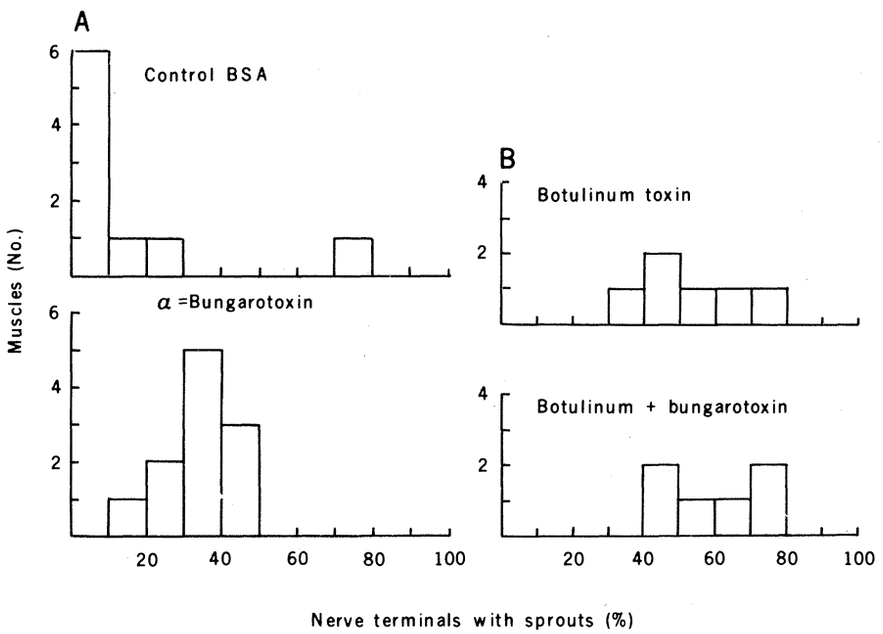


Fig. 2. (A) Percentage of nerve terminals with sprouts after control injections of bovine serum albumin (*BSA*) Ringer or BSA Ringer and purified α-bungarotoxin. The pure toxin increases in the sprouting from a mean of 15 ± 7.9 percent ($N = 9$) to 33 ± 2.6 percent ($N = 11$) (Mann-Whitney U test, $P < .01$). (B) Histograms of the percentage of nerve terminals with sprouts after botulinum toxin alone, and after botulinum toxin followed by a course of α-bungarotoxin. The α-bungarotoxin does not inhibit the sprouting; the mean percentage of nerve terminals with sprouts rises from 50 ± 6.3 to 58 ± 4.5 ($N = 6$).

ruled out a role for the acetylcholine receptor in inducing or aiding sprouting, as single daily or twice daily injections of α-bgt are not sufficient to bind with all the extrajunctional receptors that appear within 12 or 24 hours. However, consideration of other research is relevant. Crushed motor axons will regenerate perfectly to end plates completely blocked by continuously infused α-bgt (*13*). In the light of the recent research of McMahan *et al.* (*14*), this finding should not be too surprising, as it appears that the factor recognized by nerves lies within the basement membrane separating the nerve terminal from the lipid plasma membrane. Presumably any local stimulus to nerve growth, if such exists, may lie here too, although its positioning in the basement membrane might initially depend on underlying acetylcholine receptors. The appearance of the newly formed presynaptic nerve endings in Jansen and Van Essen's (*13*) fully paralyzed preparations, however, shows that the axons not only fill out the old synaptic site but also sprout beyond it over the muscle surface into regions where presumably no underlying acetylcholine receptors remained unblocked.

Mammalian skeletal muscle may be unique in being so trophically dependent on activity for its normal biochemical constitution. Other muscles and tissues may depend more on ill-understood agents delivered by nerves. Thus it may not be possible in other tissues to cause sprouting by blocking impulse activity (*15*). The common factor in evoking sprouting in a wide variety of denervated tissues, whether the denervation primarily affects the target organ by withdrawing activity or by withdrawing nerve-borne chemical agents, seems likely to be the resulting biochemical change in the tissues that causes them to manufacture a sprouting agent or, alternatively, stops them from manufacturing a growth-inhibiting agent.

R. L. HOLLAND
M. C. BROWN
*University Laboratory of Physiology, Oxford OX1 3PT, England*

### References and Notes

1. L. W. Duchen and S. J. Strich, *Q. J. Exp. Physiol.* **53**, 84 (1968).
2. M. C. Brown and R. Ironton, *Nature (London)* **265**, 459 (1977).
3. J. Diamond, E. Cooper, C. Turner, C. Macintyre, *Science* **193**, 371 (1976).
4. M. C. Brown, G. M. Goodwin, R. Ironton, *J. Physiol. (London)* **267**, 42 (1977).
5. D. Tonge, *Q. J. Exp. Physiol.* **63**, 39 (1978).
6. A. Pestronk and D. B. Drachman, *Science* **199**, 1223 (1978).
7. C. Y. Lee, C. L. Chang, T. H. Chie, P. J. S. Chang, T. C. Tseng, S. Y. Lee, *Arch. Exp. Pathol. Pharmcol.* **259**, 360 (1968); J. P. Changeux, M. Kasal, C. Y. Lee, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 1241 (1970); D. K. Berg, R. B. Kelly, P. B. Sargent, P. Williamson, L. W. Hall, *ibid.* **69**, 147 (1972); H. B. Bosmann, *J. Biol. Chem.* **247**, 130 (1972); C. Y. Lee, S. L. Chang, S. T. Kan, S. H. Luh, *J. Chromatogr.* **72**, 71 (1972); M. J. Anderson and M. W. Cohen, *J. Physiol. (London)* **237**, 385 (1974). Crude venom of the snake *Bungarus multicinctus* was dissolved in 0.1*M* ammonium acetate, *p*H 5.0, and passed through a column of Sephadex G-50 (100 by 3 cm). After lyophilization, fraction 4 from this column was then applied to a column of carboxymethyl Sephadex C-25 (70 by 2.2 cm) which had been equilibrated with 0.075*M* ammonium acetate buffer, *p*H 5.0. The material was eluted with a linear gradient from 0.1*M* ammonium acetate, *p*H 5.0, to 1.0*M*, *p*H 7.0. The toxin was found in fraction 4-2.
8. (i) Under sodium dodecyl sulfate gel filtration it runs to the same point as bungarotoxin prepared elsewhere, having a molecular weight ~ 8000; (ii) it precipitates with $^{125}$I-labeled acetylcholine receptors isolated from *Electrophorus electricus*; (iii) it competes with bungarotoxin prepared elsewhere for rabbit antibody to α-toxin; (iv) it blocks the cholinergic synapse between the cercal nerve and the giant interneuron in the cockroach; and (v) it blocks the mouse soleus neuromuscular junction.
9. The measurement of the length of the sprouts from an end plate was the length of a straight line in the long axis of the muscle fiber whose ends encompassed the sprouting, minus the length in that axis of the end plate. This measure takes no account of the branching or curvature of the sprouts.
10. D. M. Fambrough, *Physiol. Rev.* **59**, 165 (1979).
11. T. Lømo and R. Westgaard, *Cold Spring Harbor Symp. Quant. Biol.* **40**, 263 (1976).
12. D. K. Berg and Z. W. Hall, *J. Physiol. (London)* **250**, 659 (1975).
13. J. K. S. Jansen and D. Van Essen, *ibid.*, p. 651.
14. U. J. McMahan, J. R. Sanes, L. M. Marshall, *J. Cell Biol.* **78**, 176 (1978).
15. S. Roper and G. P. Ko, *Science* **202**, 66 (1978).
16. We thank H. Meiri for preparing the purified α-bungarotoxin and D. Chiverton for help with some of the experiments.

11 June 1979; revised 13 August 1979

## Angiotoxicity of Oxygenated Sterols and Possible Precursors

Abstract. *Cell death, inflammation, and repair in rabbits' aortas and pulmonary arteries were observed at 3-, 7-, and 10-day periods after the intravenous injection of oxygenated sterols. Thus, oxygenated sterols, not cholesterol, may play the primary role in arterial wall injury and lesion development.*

Atherosclerosis induced by diets containing cholesterol has been studied since the report by Anitschkow in 1913 (*1*). However, investigators conducting experiments on the effects of diet on the circulatory system have often not taken into consideration the ability of cholesterol to undergo spontaneous oxidation (*2*). In some experiments workers have increased the effectiveness of the diet by heating it in air, thereby causing oxidation of the cholesterol (*3*).

We reported previously that pure cholesterol was relatively ineffective in inducing cell death and lesions compared to the cholesterol prepared according to *U.S. Pharmacopeia* standards (*4*). We have now tested the effects of sterols known to be produced by autoxidation (*2*). We have also assayed two presumptive biogenetic precursors of 24- and 25-hydroxycholesterol. Kandutsch *et al.* (*5*) implicated oxygenated sterols as key regulators of sterol metabolism, and enzyme and isolation studies suggest that oxygenated sterols may occur in vivo (*6–10*).

In initial experiments the test material was administered orally (*4*), but this route led to uncertainties associated with biotransformation, retention, excretion, and absorption. We therefore used the intravenous route in subsequent experiments. A concentrate of impurities obtained from the recrystallization of impure cholesterol (*4*) was separated by thin-layer chromatography into three bands. The compounds in each band were eluted with ethanol and emulsified with physiological saline, and then injected into New Zealand White (NZW) rabbits. The aortas of the rabbits were examined 24 hours after the injection.

Only the components of the middle band, identified as sterol hydroperoxides, showed no angiotoxicity. Synthesis and bioassay of the components confirmed this result. We had in-

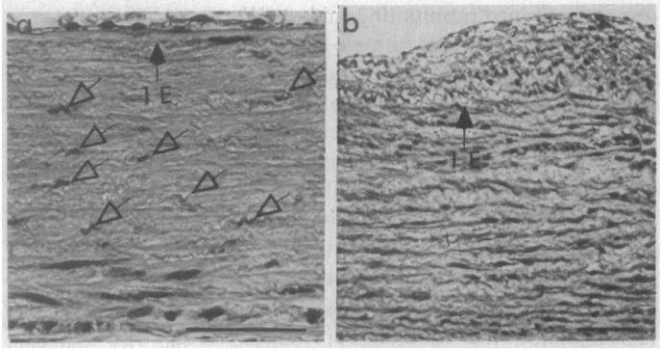


Fig. 1. (a) Transmural necrosis of the thoracic aorta of a JW-NIBS rabbit. A few viable smooth muscle cells are in the outer media. Hollow arrows indicate dead cells. Solid arrows point to the internal elastica (*IE*) demarcating the thin intima (×400). (b) Intimal plaque of thoracic aorta of a JW-NIBS rabbit injected intravenously with concentrate (40 mg/kg on each of 3 days) and killed 10 weeks after the third injection. The inner and mid media under the plaque are hypocellular (×200).


0036-8075/80/0208-0651$00.50/0 