usage, the child was given a ball to throw and a crayon with which to draw. In none of the 150 individuals was there any difficulty in assignment to the dextral or nondextral group. Data on race, age, and best-fitting shoe size were also recorded, but none of these variables showed any tendency to be associated with foot asymmetry, so subjects were collapsed over these categories.

Table 1 shows the number of dextral and nondextral male and female subjects having no foot-size asymmetry, or having a larger left or larger right foot. Among dextrals there is a significant association between sex and the direction of foot asymmetry [χ^2 (2) = 45.0, $P < .0001$]. Though the number of nondextrals is small, there is again an association between sex and foot asymmetry (*5*), but in the opposite direction. The mean asymmetry scores were 1.2 ± 0.2 for dextral males and -0.9 ± 0.2 for dextral females [t (125) = 8.4, $P < .0001$]. The mean scores for nondextrals were -1.1 ± 0.3 for males and 1.4 ± 0.2 for females [t (21) = 5.8, $P < .0001$]. The pattern of results in childen under age 6 was identical and, if anything, was even more extreme than for the rest of the sample; this result rules out the possibility that pubertal hormones induce asymmetric foot growth. The absolute asymmetry scores, ignoring sign, did not differ as a function of sex or handedness nor, for subjects having asymmetric feet, as a function of which foot was larger. For the group as a whole, the absolute asymmetry score was 1.3 ± 0.1, and for those with asymmetric feet (114 subjects) was 1.7 ± 0.1 (that is, somewhat more than a quarter shoe size).

The observations reported here offer strong evidence for the action of gene products of X- or Y-linked loci in promoting asymmetric development of the feet, the direction of the asymmetry within sexes being governed by the same factors that determine handedness. Thus, our hypothesis, based on the results of Reid (*3*) that the male genome is invariably associated with enhanced right-sided development and the female genome with enhanced left-sided development, was disconfirmed. Even in young children under the age of 6 the left foot was larger in females and the right foot larger in males only in dextral subjects, the reverse pattern being seen in nondextrals. Apparently, though there is a strong effect of sex on the development of both cerebral and pedal asymmetry, the effect is independent of handedness (and the nature of hemispheric specialization) in the former case, but directionally determined by handedness in the latter case. Although for both asymmetric brain maturation and foot growth the critical role of genes on the sex chromosomes seems certain, the mechanisms by which these genes realize their effects evidently differ for the two body regions.

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References and Notes

1. D. Kimura, *Can. J. Psychol.* **23**, 445 (1969); D. A. Lake and M. P. Bryden, *Brain Lang.* **3**, 266 (1976); H. Lansdell, *Nature (London)* **194**, 852 (1962); J. Levy and M. Reid, *Science* **194**, 337 (1976); J. McGlone and W. Davidson, *Neuropsychologia* **11**, 105 (1973); T. Marcel and P. Rajan, *ibid.* **13**, 489 (1975).
2. S. F. Witelson, *Science* **193**, 425 (1976).
3. M. Reid, in preparation.
4. J.M.L. has had over 50 years experience in fitting shoes, and although, because the magnitude of pedal asymmetry is typically less than half a shoe size, his classification had to be partially subjective, we are confident that no subject classified as asymmetric was symmetric. It is possible, however, that a few subjects classified in the symmetric category may have deserved a rating of ± 1. It should be mentioned, also, that customer cooperation in the project was easily secured, both because J.M.L. has lived in Demopolis all his life and is known by most of the townspeople, and because the town is small and a spirit of cooperation prevails.
5. The exact probability of finding a distribution as unlikely as the one observed is only 3.18×10^{-5}, and we may confidently conclude that in nondextral individuals the distribution of males and females differs with respect to foot-size asymmetry.
6. We express our appreciation to the participants in this study who, though they only sought to be fitted for shoes, graciously gave of their time to make this investigation possible. Perhaps in consequence of our finding that in three-fourths of all people the two feet are of unequal size, it may someday be possible to buy left and right shoes separately, thus securing more comfortable footwear. This work was partially supported by NSF grant BNS 75-23061, a Spencer Foundation grant, and a biomedical grant from the University of Chicago.

19 October 1977; revised 5 April 1978

Substances Moved by Axonal Transport and Released by Nerve Stimulation Have an Innervation-Like Effect on Muscle

Abstract. *Substances which have an innervation-like effect on the cholinesterase activity of organ-cultured rat extensor digitorum longus muscles are moved in nerve by axonal transport, are released from nerve by stimulation, and are present in innervated muscle but apparently absent from denervated muscle. Substances which increase the acetylcholine sensitivity of cultured muscles behave similarly.*

Innervation exerts a profound influence on many of the properties of skeletal muscle (*1–3*). The evidence available suggests that the influence of nerve on muscle is mediated in part by the activity (electrical or mechanical, or both) generated in muscle by nerve (*4*), and in part by mechanisms that are independent of muscle activity (*5–7*). To account for the activity-independent influence of nerve on muscle it has been postulated that muscle properties are influenced by trophic substances (*8*) delivered to muscle by nerve (*1, 3, 5, 9, 10*). Several studies have shown that denervation-like changes occur in muscle when substances that block axonal transport are applied to nerve (*10*). The interpretation of this finding is controversial (*11*), but proponents of the trophic substance hypothesis have interpreted it to mean that substances which normally influence muscle are moved by axonal transport. Musick and Hubbard (*12*) have presented evidence that indicates that protein is released concurrently with acetylcholine (ACh) at the mouse neuromuscular junction and have suggested that this protein might mediate the trophic influence of nerve on muscle.

If the activity-independent influence of nerve on muscle is mediated by trophic substances then one would predict that substances with an innervation-like effect on muscle (i) should be present and moved by axonal transport in nerve, (ii) should be present in innervated muscle (within intramuscular nerve and possibly within muscle itself) but should be absent or present in reduced concentration in denervated muscle, and (iii) should be released from nerve. We have tested each of these predictions examining two muscle properties, cholinesterase (ChE) activity and ACh sensitivity, which are dramatically influenced by innervation. In adult rat muscle, innervation maintains both junctional and extrajunctional ChE activity (*13*) and suppresses extrajunctional ACh sensitivity (*2, 5, 14*). There is evidence that, during development, innervation causes an activity-independent aggregation of ACh receptors from the extrajunctional into the junctional region (*15*). Innervation probably continues to be involved in maintaining high junctional ACh sensitivity in adult muscle, but the extent of this influence is uncertain. Berg and Hall (*16*) have presented data, incidentally and without dis-

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cussion, which may reasonably be interpreted to mean that the number of slowly decaying junctional ACh receptors decreases 37 percent 5 days after denervation. Frank *et al.* (*17*), on the other hand, using a different method in rat soleus found only a slow diminution in the number of junctional ACh receptors after denervation.

To assay putative trophic substances influencing muscle ChE activity and ACh sensitivity, we denervated in vivo the extensor digitorum longus (EDL) muscles of male Wistar rats (100 to 200 g) and after 2 or 3 days removed the EDL muscles and cultured them for 1 to 3 days in Trowell's T8 medium supplemented in various ways. The ChE activity of cultured muscles was measured according to a modification (*7*) of the method of Ellman *et al.* (*18*), and their ACh sensitivity was measured by ACh iontophoresis (see legends to Figs. 1 to 3).

To determine whether putative trophic substances in nerve are moved by axonal transport we took advantage of the fact that substances moved by axonal transport accumulate in a cut nerve in the region just proximal to the site of section (*19*). The sciatic nerves on both sides of 12 rats were sectioned, segments of control nerve were removed, and the animals were allowed to recover. Four days later, nerve enriched in substances moved by axonal transport was obtained by removing the 2.5-mm segment of nerve just proximal to the site of section from each sciatic nerve. Figure 1 shows that both ChE activity and ACh sensitivity were significantly higher in muscles cultured in enriched as opposed to control nerve extract (*20*).

To determine whether innervated muscle contains putative trophic substances which are absent or present in reduced concentration in denervated muscle, we cultured EDL muscles in the extract of normally innervated muscle or in the extract of muscle which had been denervated for 3 to 7 days (*21*). The ChE activity of muscles cultured in medium supplemented with denervated muscle extract (1.33 ± 0.06) was essentially the same as that of muscles cultured in T8 medium alone (1.33 ± 0.04) but, as shown in Fig. 2, innervated muscle extract significantly increased the ChE ac-

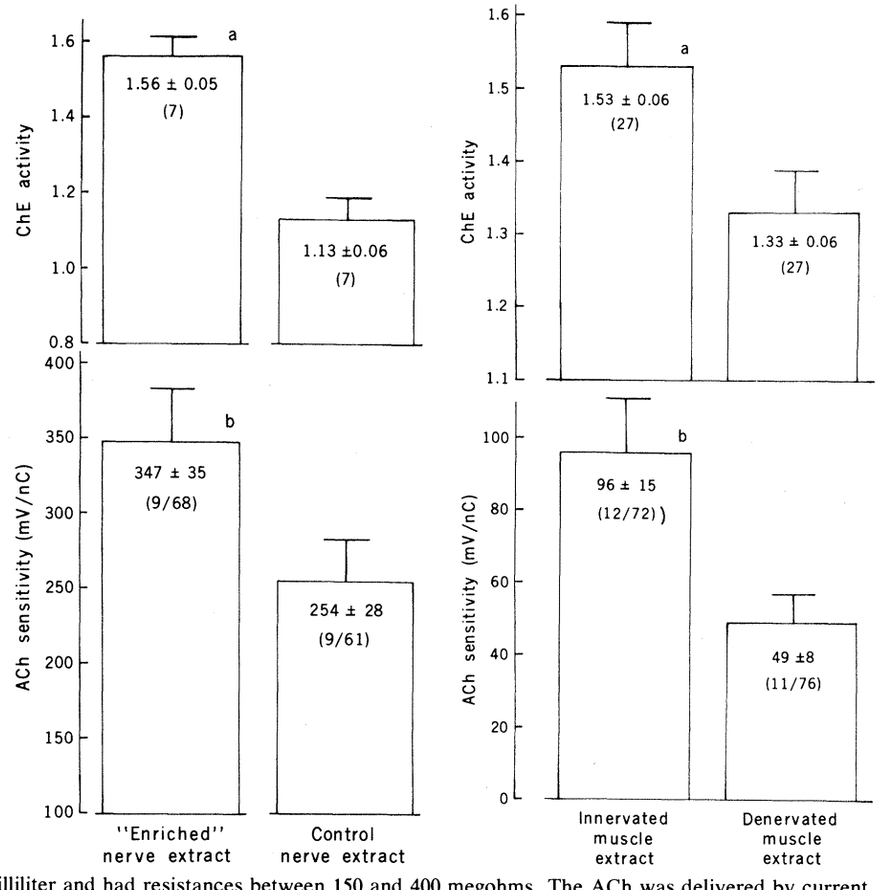

Fig. 1 (left). The influence of substances moved by axonal transport on the ChE activity and ACh sensitivity of cultured muscles. In each experiment both EDL muscles of one rat were denervated in vivo for 3 days; one muscle was then cultured for 2 days in control nerve extract and the other was cultured for 2 days in the extract of nerve enriched in substances moved by axonal transport (see text for details). In all experiments except two, 0.5 mg/ml of control and enriched nerve extract (*20*) was used; in the remaining two experiments, on ACh sensitivity, 0.1 mg/ml was used. Whole muscle ChE activity was measured by a modification (*7*) of the method described by Ellman *et al.* (*18*) and is expressed as micromoles of acetylthiocholine hydrolyzed per minute per gram of muscle. The ACh sensitivity was measured iontophoretically and is expressed in millivolts of depolarization per nanocoulomb of charge delivered (mV/nC) (*5*). To make this measurement, paired muscles cultured in control and enriched nerve extract were mounted side by side in a chamber perfused continuously with oxygenated rat Ringer solution (m*M*: NaCl, 135.0; KCl, 5.0; $MgCl_2$, 1.0; $CaCl_2$, 2.0; $NaHCO_3$, 15.0; Na_2HPO_4, 1.0; glucose, 11.0). To avoid bias in the measurement of ACh sensitivity the measurement was obtained blind with regard to the media in which muscles had been cultured. ACh sensitivity was measured alternately in fibers from the two muscles by using the same iontophoretic pipette and measuring in the same region of each muscle. In these experiments measurements were made on the superficial surface of the distal third of the muscle. The iontophoretic pipettes used contained 0.5 g of acetylcholine chloride per milliliter and had resistances between 150 and 400 megohms. The ACh was delivered by current pulses of 1 to 50 msec and the resulting depolarization was measured intracellularly by means of conventional glass microelectrodes containing 3*M* KCl. Supplements which increase ACh sensitivity tend to have an innervation-like (hyperpolarizing) effect on the resting membrane potential of muscle (*28*). To eliminate any contribution that this might make to increased ACh sensitivity, we corrected the ACh sensitivity (AS) measured in each fiber to the value expected if its resting membrane potential were −78 mV: $AS_{-78\ mV} = (AS_{RMP\ obs})(78 - 15)/(RMP_{obs} - 15)$, where RMP_{obs} was the resting membrane potential observed at the time ACh sensitivity was measured and −15 mV was taken as the reversal potential for ACh (*29*). Values shown are means ± standard error. Numbers in parentheses are the number of muscles and, in the case of ACh sensitivity, the number of fibers examined. (a) $P < .001$ by Student's t-test with respect to muscles cultured in control nerve extract. (b) $P < .05$ by Student's t-test with respect to muscles cultured in control nerve extract. Fig. 2 (right). The influence of innervated and denervated muscle extract on the ChE activity and ACh sensitivity of cultured muscle. Rat EDL muscles were denervated in vivo for 2 days and were then cultured in the extract of normal innervated diaphragm muscle (10 mg/ml) or in the extract of diaphragm muscle (10 mg/ml) which had been denervated for 3 to 7 days prior to extraction (*21*). Muscles in which ChE activity was measured were cultured 2 to 3 days and muscles in which ACh sensitivity was measured were cultured for 3 days. Both ChE activity and ACh sensitivity were measured as described in Fig. 1. Values shown are means ± standard error. Numbers in parentheses are the number of muscles and, in the case of ACh sensitivity, the number of fibers examined. (a) $P < .03$ by Student's t-test with respect to muscles cultured in denervated muscle extract. (b) $P < .01$ by Student's t-test with respect to muscles cultured in denervated muscle extract.

tivity of cultured muscles; ACh sensitivity was also significantly higher in muscles cultured in innervated as opposed to denervated muscle extract (*22*).

To determine whether nerve stimulation releases putative trophic substances we conditioned (see legend to Fig. 3) T8 medium with sciatic nerve–EDL muscle preparations in which the nerve was stimulated supramaximally at 7 Hz for 30 to 60 minutes. As a control we used T8 medium conditioned with unstimulated EDL muscles. Figure 3 shows that ChE activity was significantly increased by substances released by nerve stimulation. Postsynaptic blockade of neuromuscular transmission with *d*-tubocurarine (0.5 to 1.5 mg/liter) did not reduce the release of the substances which increased ChE activity, but presynaptic blockade of the vesicular release of transmitter with Mg^{2+} (10 m*M*) did significantly reduce the release of these substances (Fig. 3). These observations strongly suggest that substances which increase muscle ChE activity are released from synaptic vesicles along with ACh. Nerve stimulation also released substances which significantly increased the ACh sensitivity of cultured muscles. In these experiments ACh sensitivity was measured not only in the distal region as in previous experiments (Figs. 1 and 2) but also in the proximal region where end plates usually are found. The increase in ACh sensitivity occurred in both regions but was larger and more significant in the proximal region where end plates normally are found.

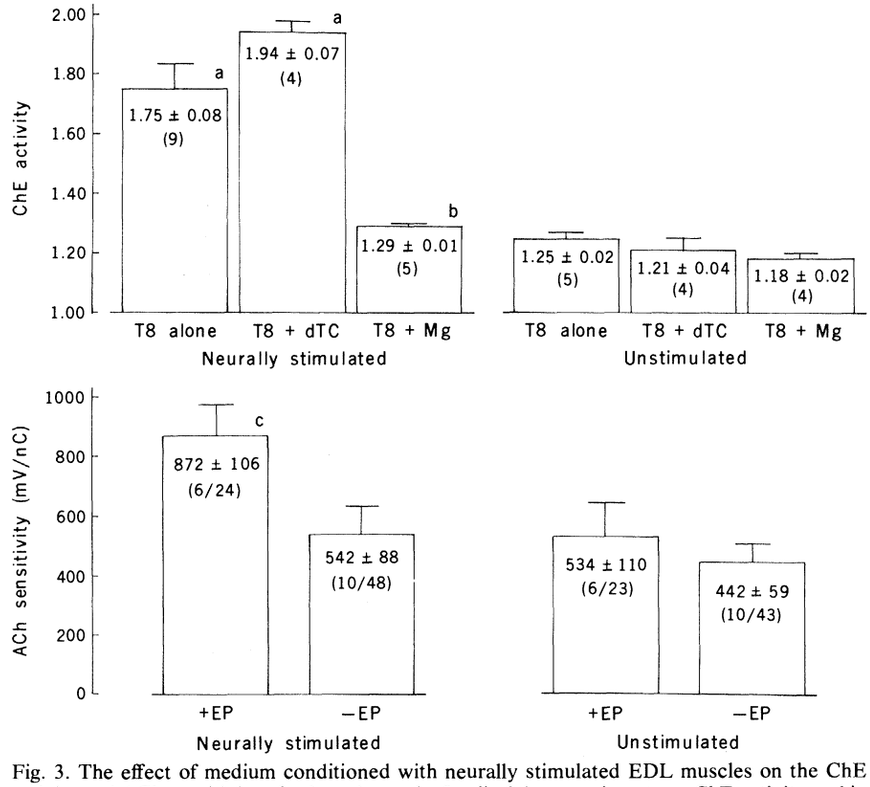

Fig. 3. The effect of medium conditioned with neurally stimulated EDL muscles on the ChE activity and ACh sensitivity of cultured muscle. In all of the experiments on ChE activity and in four of the experiments on ACh sensitivity the following procedure was used to condition media. The sciatic nerve and EDL muscle were dissected, washed with rat Ringer solution, and placed in 5 ml of oxygenated (95 percent O_2 and 5 percent CO_2) T8 medium. The sciatic nerve was stimulated supramaximally through platinum stimulating electrodes at 7 Hz for 15 minutes, and the preparation was then allowed to rest for 5 minutes. The cycle of 15 minutes stimulation and 5 minutes rest was repeated until the preparation had been stimulated for a total of 30 to 60 minutes. The contralateral EDL muscle was used to condition 5 ml of T8 medium in identical fashion except that the muscle was not stimulated. The media conditioned with neurally stimulated and unstimulated EDL muscles were diluted to 20 ml with normal T8 medium in order to be tested. In some experiments 0.5 to 1.5 mg of *d*-tubocurarine (*d*TC) or 10.0 m*M* $MgCl_2$ per liter was added to the T8 bathing both stimulated and unstimulated muscles. In these experiments stimulation at 7 Hz did not begin until contraction in response to nerve stimulation had ceased (usually 30 to 60 minutes), but the procedure was otherwise identical. In six of the experiments on ACh sensitivity a different procedure was used to condition media. The sciatic nerve and EDL muscle were dissected and then perfused for 30 minutes with oxygenated (95 percent O_2 and 5 percent CO_2) rat Ringer solution. The Ringer solution was removed and 5 ml of T8 medium were added for 15 minutes during which the muscle was unstimulated. The "unstimulated" medium was saved, the muscle was perfused rapidly with rat Ringer solution for 5 minutes, the Ringer solution was removed, and 5 ml of T8 medium were added for 15 minutes during which the nerve was stimulated supramaximally at 7 Hz. The "stimulated" medium was then saved and the muscle again perfused rapidly for 5 minutes with rat Ringer solution. The entire cycle (rest-perfusion-stimulation-perfusion) was performed four times generating 20 ml of T8 medium conditioned with unstimulated EDL muscle and 20 ml of medium conditioned with neurally stimulated EDL muscle. No dramatic difference was detected in media collected by the two procedures so the data obtained were pooled to give the ACh sensitivity data shown in Fig. 3. To test these media we denervated EDL muscles for 3 days in vivo and then cultured the muscles in 20 ml of oxygenated (95 percent O_2 and 5 percent CO_2) T8 medium conditioned with either neurally stimulated EDL muscles or with unstimulated EDL muscles (media were filtered prior to culturing to remove bacteria). Muscles in which ChE activity was measured were cultured for 1 day, and muscles in which ACh sensitivity was measured were cultured for 3 days. Both ChE activity and ACh sensitivity were measured as described in Fig. 1. Numbers in parentheses are the number of muscles and, in the case of ACh sensitivity, the number of fibers examined. (+EP and −EP indicate the presence or absence of end plates.) (a) $P < .001$ by Student's *t*-test with respect to muscles cultured in identical medium conditioned with unstimulated EDL muscle. (b) $P < .001$ by Student's *t*-test with respect to muscles cultured in T8 medium conditioned with neurally stimulated EDL muscle. (c) $P < .04$ by Student's *t*-test with respect to fibers in the +EP region cultured in T8 medium conditioned with unstimulated EDL muscle.

In these experiments we have supplemented T8 medium in several ways in an effort to demonstrate trophic substances. To avoid being misled by nonspecific influences of the supplements on the parameters being measured we prepared and tested control media that were, as much as possible, identical to the experimental media except for the absence of putative trophic substances. We also examined the ChE activity and the ACh sensitivity of muscles cultured in T8 medium alone. The ChE activity of muscles that had been denervated for 3 days and then cultured in T8 medium alone was the same or slightly lower than that of muscles cultured in control media, so the significant innervation-like effect of supplemented media on the ChE activity of cultured muscles was also evident when comparison was made with muscles cultured in T8 alone (*23*). The ACh sensitivity of muscles cultured in T8 medium alone was, in each case (Figs. 1 to 3), higher than that of muscles cultured in control media and, as a result, the increase in ACh sensitivity caused by supplemented media ceased to be significant when comparison was made with muscles cultured in T8 medium alone (*24*). The use of appropriate control media was, therefore, unimportant in demonstrating the effect of putative trophic substances on ChE activity but was critical in demonstrating their effect on ACh sensitivity.

The supplemented media used in these experiments had ChE activity, so the in-

crease in ChE activity observed in cultured muscles could conceivably have been due to ChE molecules in the media which became bound to the cultured muscles (*25*). If this were the case one would expect the increase in the ChE activity of cultured muscles to be accompanied by a decrease in the ChE activity of the culture media. In 12 experiments we measured the ChE activity of media conditioned with neurally stimulated EDL muscles before and after muscles were cultured. In all of these experiments muscle ChE activity increased; but in nine of these experiments the ChE activity of the culture medium also increased, so the binding of ChE molecules in the supplemented media to cultured muscles probably does not account for the increase in muscle ChE activity observed.

In the present experiments substances moved by axonal transport and released by nerve stimulation increased ChE activity and ACh sensitivity in a remarkably parallel manner (Figs. 1 to 3). The substances found to influence ChE activity are almost certainly to be involved in the normal maintenance of muscle ChE activity by innervation. The physiological function of the substances found to increase extrajunctional ACh sensitivity is less obvious because innervation normally suppresses extrajunctional ACh sensitivity. Our working hypothesis is that the substances which, in these experiments, increased ACh sensitivity when applied globally to denervated cultured muscle are substances which, in vivo, are involved in the maintenance of junctional ACh sensitivity. In vivo, these substances are presumably delivered in highest concentration to the junctional region, and this, coupled with the generalized suppression of ACh receptor synthesis by muscle activity (*26*), could contribute to the pattern of high ACh sensitivity restricted to the junctional region which is characteristic of innervated adult muscle.

Previous studies have shown that ChE activity, ACh sensitivity, and several other muscle properties can be influenced in cultured muscle by a variety of nerve-related substances (*27*). These studies are of great interest because they suggest that cultured muscle can be used to assay trophic substances which are normally delivered to muscle by nerve. The difficulty in interpreting these studies is that the changes observed in them could have been caused by nonspecific factors and not by physiologically relevant trophic substances. The emphasis in our experimentation has been to establish that the innervation-like change which we have induced in cultured muscles is due to substances which are normally delivered to muscle by nerve. Our data indicate that substances with a substantial and highly significant innervation-like influence on ChE activity are (i) moved in nerve by axonal transport, (ii) released from nerve by stimulation, and (iii) present in innervated muscle but apparently absent from denervated muscle. These results provide very strong evidence that muscle ChE activity is normally maintained, in part, by substances delivered to muscle by nerve. The results of parallel experiments on ACh sensitivity suggest that substances delivered to muscle by nerve may also be involved in the maintenance of junctional ACh sensitivity.

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References and Notes

1. L. Guth, *Physiol. Rev.* **48**, 645 (1968).
2. A. J. Harris, *Annu. Rev. Physiol.* **36**, 251 (1974).
3. E. Gutmann, *ibid.* **38**, 177 (1976).
4. T. Lømo and J. Rosenthal, *J. Physiol. (London)* **221**, 493 (1972); L. Guth, *Exp. Neurol.* **24**, 508 (1969); D. B. Drachman and F. Witzke, *Science* **176**, 514 (1972); T. Lømo and R. H. Westgaard, *J. Physiol. (London)* **252**, 603 (1975); D. K. Berg and Z. W. Hall, *ibid.* **244**, 659 (1975).
5. R. Miledi, *J. Physiol. (London)* **151**, 1 (1960); *ibid.* **154**, 190 (1960).
6. J. V. Luco and C. Eyzaguirre, *J. Neurophysiol.* **18**, 65 (1955); N. Emmelin and L. Malm, *Q. J. Exp. Physiol.* **50**, 142 (1965); P. A. Lavoie, B. Collier, A. Tenenhouse, *Nature (London)* **260**, 349 (1976); S. S. Deshpande, E. X. Albuquerque, L. Guth, *Exp. Neurol.* **53**, 151 (1976); A. Pestronk, D. B. Drachman, J. W. Griffin, *Nature (London)* **260**, 352 (1976).
7. B. Davey and S. G. Younkin, *Exp. Neurol.* **59**, 168 (1978).
8. Since the chemical nature of the postulated trophic material is unknown, we have arbitrarily elected to use the plural, trophic substances, in referring to it.
9. S. S. Tower, *Physiol. Rev.* **19**, 1 (1939).
10. W. W. Hofmann and S. Thesleff, *Eur. J. Pharmacol.* **20**, 256 (1972); H. L. Fernandez and B. U. Ramirez, *Brain Res.* **79**, 385 (1974); H. L. Fernandez and N. C. Inestrosa, *Nature (London)* **262**, 55 (1976); T. Tiedt, P. L. Wisler, S. G. Younkin, *Exp. Neurol.* **57**, 766 (1977); E. X. Albuquerque, J. E. Warnick, J. R. Tasse, F. M. Sansone, *Exp. Neurol.* **37**, 607 (1972).
11. A. Cangiano, *Brain Res.* **58**, 255 (1973); T. Lømo, *Nature (London)* **249**, 473 (1974); ——— and R. H. Westgaard, *Cold Spring Harbor Symp. Quant. Biol.* **40**, 263 (1976); A. Cangiano and J. A. Fried, *J. Physiol. (London)* **265**, 63 (1977).
12. J. Musick and J. I. Hubbard, *Nature (London)* **237**, 279 (1972).
13. L. Guth, R. W. Albers, W. C. Brown, *Exp. Neurol.* **10**, 236 (1964); Z. W. Hall, *J. Neurobiol.* **4**, 343 (1973).
14. J. Axelsson and S. Thesleff, *J. Physiol. (London)* **149**, 178 (1959).
15. M. J. Anderson, M. W. Cohen, E. Zorychta, *J. Physiol. (London)* **268**, 731 (1977); M. J. Anderson and M. W. Cohen, *ibid.*, p. 757.
16. D. K. Berg and Z. W. Hall, *ibid.* **252**, 771 (1975).
17. E. Frank, K. Gautvik, H. Sommerschild, *Cold Spring Harbor Symp. Quant. Biol.* **40**, 275 (1976).
18. G. L. Ellman, K. D. Courtney, V. Andres, Jr., R. M. Featherstone, *Biochem. Pharmacol.* **7**, 88 (1961).
19. L. Lubinska, S. Niemierko, B. Oderfeld, L. Szwarc, *J. Neurochem.* **11**, 493 (1964); P. Banks, D. Mangnall, D. Mayor, *J. Physiol. (London)* **200**, 745 (1969); S. Ochs and N. Ranish, *Science* **167**, 878 (1970); L. Lubinska and S. Niemierko, *Brain Res.* **27**, 329 (19 → L. M. Partlow, C. D. Ross, R. Motwani, D. B. McDougal, Jr., *J. Gen. Physiol.* **60**, 388 (1972).
20. Nerve extract was prepared by homogenizing nerve segments in 4 ml of Trowell's T8 medium at 4°C in a Potter-Elvehjem homogenizer. After addition of 4 ml of T8 medium the extract was centrifuged in the cold (4°C) at 2700*g* for 10 minutes. Additional T8 medium was then added to the supernatant medium so that each milliliter of the final supplemented medium contained the extract of 0.1 or 0.5 mg of sciatic nerve (wet weight).
21. Muscle extract was prepared exactly like nerve extract [see (*20*)] except that each milliliter of the final supplemented medium contained the extract of 10.0 mg of muscle. Extracts were made from diaphragm because this muscle can be dissected into end plate–containing (+EP) and end plate–free (−EP) segments. Experiments were performed on extracts of +EP and −EP segments and both ChE and ACh sensitivity were found to be higher in muscles cultured in the extract of +EP as opposed to −EP segments, but the differences were not significant so the data on +EP and −EP segments were pooled to give Fig. 2.
22. The experiments on innervated muscle extracts do not definitively establish that trophic substances can be extracted from pure muscle, because innervated muscle contains intramuscular axons and nerve terminals which degenerate after denervation and which could account for the innervation-like effects seen.
23. The ChE activity of muscles denervated 5 days in vivo was 0.99 ± 0.02, the ChE activity of muscles denervated 3 days in vivo and cultured 2 days in T8 alone was 1.02 ± 0.03, and the ChE activity of muscles denervated 3 days in vivo and cultured 2 days in control nerve extract (0.5 mg/ml) was 1.13 ± 0.06; whereas the ChE activity of muscles denervated 3 days in vivo and cultured 2 days in enriched nerve extract (0.5 mg/ml; Fig. 1) was 1.56 ± 0.05. The significant innervation-like influence of supplemented medium on ChE activity was apparent, therefore, regardless of the muscles used for baseline measurement.
24. Comparison of muscles cultured in supplemented media with muscles cultured in T8 medium alone showed that ACh sensitivity was 8 percent higher in enriched nerve extract, 30 percent lower in innervated muscle extract, and 28 percent higher in the +EP region of muscles cultured in medium conditioned with indirectly stimulated muscle, but none of these differences was significant. The significant increase in ACh sensitivity caused by supplements was apparent only when muscles cultured in supplemented media were compared with muscles cultured in appropriate control media (Figs. 1 to 3). This occurred because muscles cultured in control media in each case had an ACh sensitivity which was lower than that of muscles cultured in T8 medium alone. The reason for the apparent suppression of ACh sensitivity by control media (Figs. 1 to 3) is not clear, but the substances responsible for it must certainly have been present in enriched nerve extract and in medium conditioned with neurally stimulated muscle, and probably were also present in innervated muscle extract, so the comparisons shown in Figs. 1 to 3 are the appropriate ones for demonstrating the effect of putative trophic substances on ACh sensitivity.
25. T. H. Oh, J. Y. Chyu, S. R. Max, *J. Neurobiol.* **8**, 469 (1977).
26. C. G. Reiness and Z. W. Hall, *Nature (London)* **268**, 655 (1977).
27. T. Lentz, *Science* **171**, 187 (1971); *J. Cell Biol.* **55**, 93 (1972); *Exp. Neurol.* **45**, 520 (1974); H. Kuromi and S. Hasegawa, *Brain Res.* **100**, 178 (1975); T. H. Oh, *Exp. Neurol.* **46**, 432 (1975); *ibid.* **50**, 376 (1976); J. K. Engelhardt, K. Ishikawa, S. J. Lisbin, J. Mori, *Brain Res.* **110**, 170 (1976); S. Hasegawa and H. Kuromi, *ibid.* **119**, 133 (1977); C. N. Christian, M. Daniels, H. Sugiyama, Z. Vogel, P. G. Nelson, *Soc. Neurosci. Abstr.* **3**, 533 (1977).
28. S. G. Younkin, L. H. Younkin, R. S. Brett, B. Davey, *Soc. Neurosci. Abstr.* **3**, 537 (1977).
29. J. del Castillo and B. Katz, *J. Physiol. (London)* **125**, 546 (1954); A. Takeuchi and N. Takeuchi, *ibid.* **154**, 52 (1960).
30. We thank M. E. Fellows for excellent technical assistance. This work was supported by NIH grant NS 13027.

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