Nerve Trophic Function: In vitro Assay of Effects of Nerve Tissue on Muscle Cholinesterase Activity

Abstract. *The effects of tissue explants and nerve homogenates on cholinesterase activity of muscle of the newt* Triturus *in organ culture were measured. Sensory ganglia, ganglia separated from muscle by a Millipore filter, spinal cord, liver, and nerve homogenates produced greater activity of muscle cholinesterase than occurred in untreated muscle cultured for the same period of time. Boiled ganglia, kidney, oviduct, and spleen were ineffective. This procedure serves as a convenient bioassay for a neurotrophic process and indicates that the trophic effect is mediated by a diffusible chemical substance produced by nerves.*

Trophic effects of neurons can be broadly defined as long-term interactions between nerve cells and the tissues they innervate responsible for the structural, chemical, and functional integrity of the target tissue (*1*). One example of trophic function is the effect of innervation on acetylcholinesterase activity at the motor end plate of skeletal muscle. End plate cholinesterase (ChE) activity appears in developing or regenerating muscle in relation to innervation (*2*) and conversely diminishes when the muscle fiber is denervated (*3, 4*). Denervation, furthermore, produces a more rapid decrease in ChE than in total protein, whereas tenotomy produces a loss of protein without a change in ChE activity of muscle (*4*). Thus, the motor neuron is thought to exert a regulatory effect on the ChE activity at the neuromuscular junction.

It is generally felt that trophic effects are mediated by a chemical or hormonal substance produced by nerve cells which affects other tissues (*5*). Neither the mechanism by which this process is carried out nor the nature of the hypothesized trophic factor have been determined, however. Further progress in this area depends upon the availability of a suitable bioassay system in which the effects of elimination of the nerve supply can be measured and reversed by appropriate replacement therapy (*1*). Recently, it has been shown that nerve extracts infused into denervated limbs of newts stimulate protein synthesis (*6*). I describe here a simple in vitro system in which the effects of nerve tissue and extracts on ChE activity of skeletal muscle can be assayed.

Adult newts, *Triturus viridescens,* were rinsed in sterile distilled water, anesthetized in sterile 2 percent Chloretone, dipped in 70 percent alcohol, and rinsed again in distilled water. Under the dissecting microscope, the skin of the upper forelimb was slit along the limb and freed from the underlying muscle. The dorsal muscle bundle (triceps) was then removed by cutting near the tendinous insertions and origins. Muscle removed in this manner usually weighed about 1 mg depending on the size of the animal; muscle weighing less than 0.5 mg or more than 2.0 mg was not used for assay. The muscle was placed on squares of Dacron cloth supported by a stainless steel grid in plastic organ culture dishes (Fig. 1). The muscle was cultured in an amphibian culture medium (*7*) (Grand Island Biological) at room temperature in a mixture of 95 percent air and 5 percent CO_2. The medium was changed twice weekly. Amphibian muscle can be kept in organ culture for periods of up to 1 month while maintaining structure and capacity to respond to electrical stimulation (*8*). At the end of the culture period, muscle was weighed and homogenized in 0.1*M* phosphate buffer (*p*H 8.0) with a glass homogenizer. The ChE activity was measured with a colorimetric method of Ellman *et al.* (*9*) utilizing acetylthiocholine as substrate on a Beckman DU spectrophotometer. Rates were calculated in micromoles of substrate hydrolyzed per minute per gram of muscle.

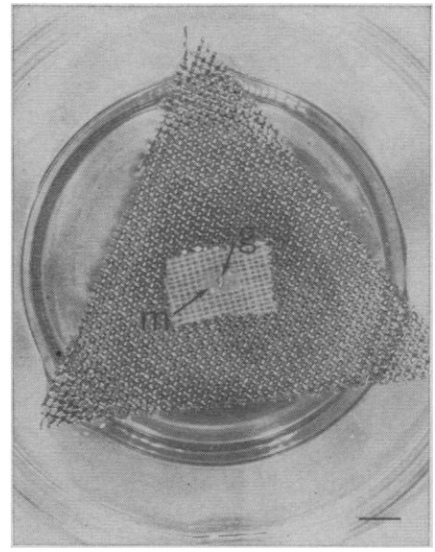

Fig. 1. Center well of an organ culture dish illustrating method of culturing muscle in the presence of a sensory ganglion. The muscle tissue (*m*) lies on dacron cloth supported by a stainless steel grid. The sensory ganglion (*g*) is placed directly on the surface of the muscle bundle. Bar represents 2 mm.

The ChE activity in muscle cultured alone was found to rise initially (Fig. 2). This increase in activity corresponds to the initial denervation hypertrophy observed in other cell constituents (*6, 10*). Thereafter, activity slowly decreases to less than that observed in normal muscle (Fig. 2). This loss of activity is similar to the changes in ChE following denervation of muscle in vivo (*4*).

The effects of tissue explants and extracts on ChE activity were assayed by adding these to muscle from one forelimb and comparing the ChE activity of this muscle with that in the untreated muscle from the opposite limb of the same newt. At least five pairs of muscles were tested in each experiment. Sensory ganglia were used to determine the long-term effects of nerve explants on ChE activity. Sensory ganglia were selected because the sensory supply of the limb by itself is capable of sustaining limb regeneration in the newt (*11*). Furthermore, the effects of normal neuromuscular transmission as might occur with motor neurons are eliminated so that any effect on ChE activity could be attributed to the general trophic action exerted by all types of nerves (*12*). Sensory ganglia (C3 or C4) were removed in a manner already described (*11*) and placed directly on top of the muscle in organ culture (Fig. 1). At the end of the culture period, ganglia were removed from the muscle prior to weighing, homogenization, and ChE measurement. Throughout the 28-day course of this experiment ChE activity was higher in muscle cultured in the presence of sensory ganglia than in control muscle from opposite limbs (Fig. 2). Differences in activity were most pronounced after 7 to 14 days but were less at longer times.

Although ChE activity in muscle cultured in the presence of ganglia remained higher than that in control tissues, it still slowly decreased and fell below normal. Because it seems likely that the ganglia themselves might undergo changes and become less effective in these long-term cultures, ganglia were changed at weekly intervals.

Table 1. Effect of tissues and extracts on cholinesterase (ChE) activity of muscle after 1 week in organ culture. Differences in ChE activity were determined between treated forelimb muscle and untreated muscle from the opposite forelimb of the same animal. At least five pairs of muscles were assayed in each experiment.

Material assayed	Difference in ChE (%)	P
Both muscles untreated	0	
Sensory ganglion	+24	$<.01$
Boiled ganglion	+ 3	$>.05$
Ganglion separated from muscle by Millipore filter	+16	$<.05$
Spinal cord	+11	$<.05$
Kidney	− 1	$>.05$
Oviduct	− 2	$>.05$
Spleen	+ 2	$>.05$
Liver	+12	$<.05$
Homogenate 1	+16	$<.05$
Homogenate 2	+17	$<.01$

In this case, enzyme activity did not decrease as rapidly and was higher at each time than in muscle exposed to the same ganglion (Fig. 2). The ChE activity still diminished when ganglia were changed, indicating a gradual loss of ability of muscle to respond to the nerve tissue.

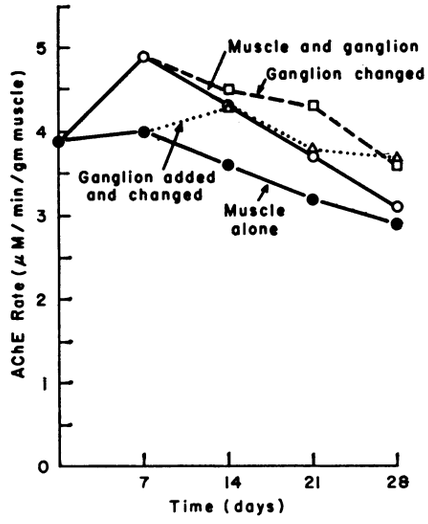

Fig. 2. Effects of sensory ganglia on cholinesterase (ChE) activity of muscle in organ culture. The ChE activity rises slightly and then declines in muscle cultured alone (closed circles). In the presence of a sensory ganglion (open circles), enzyme activity is higher at all times than in muscle cultured alone. When the ganglion is changed at weekly intervals (squares), the difference in ChE activity in comparison to controls is even greater and the rate of decline of activity is less. When the ganglion is added to muscle after 1 week and then changed weekly (triangles), activity rises and is maintained. Except for the controls, each point represents the average of five experiments. The control points are the averages of the ChE activity of muscles from the opposite limbs of all the experimental muscles (15 values).

One effect of the ganglion appears to be to slow the rate of loss of ChE activity. However, if the ganglion is added to the muscle after 1 week in culture, ChE activity increases prior to slowly decreasing again (Fig. 2). Thus, the nerve explants seem to have a direct stimulatory effect on ChE activity.

As in the case of denervated muscle in vivo (*4*), a substantial amount of activity remains in the cultured muscle. This activity appears to represent background ChE activity in the muscle that is not affected by innervation (*4*). Because the ChE content of the entire muscle is measured, changes in specific areas such as the motor end plate are not detected with the biochemical procedure. To determine the changes occurring in the neuromuscular junction, the thiolacetic acid–lead nitrate method for the histochemical demonstration of ChE activity was employed (*13*). In normal muscle, enzyme activity was intensely localized to the motor end plate which appeared as an elongated plaque-like area on the surface of the muscle fiber (Fig. 3a). The junctional folds of the end plate were apparent as light bands alternating with dark regions produced by accumulation of reaction product in the synaptic cleft. After 1 week in culture, enzyme activity was markedly reduced in intensity (Fig. 3c). Activity occurred as small, isolated deposits of reaction product along the end plate region, but junctional folds were absent or indistinct. In contrast, in muscle cultured in the presence of sensory ganglia, localization of activity maintained the normal pattern (Fig. 3b). The end plate was reduced in extent and activity was not as intense, but the basic organization of junctional folds and site of activity was preserved. These results are indicative of an effect of the nerve explants on motor end plate ChE.

For routine assay of materials, 1 week in culture was selected as a time period. At this time, the difference in ChE activity in muscle in the presence of sensory ganglia and control muscle was large and use of longer intervals became cumbersome. Assays were performed on five pairs of muscles by treating one forelimb experimentally and using the muscle from the opposite forelimb as a control. Whereas individual newts may vary considerably in ChE activity, values from untreated opposite limbs of the same newt were the same (Table 1). Because the pairs of muscles are nonindependent samples, differences between the muscles of each pair can be analyzed by treating each change as a single observation. The changes in cholinesterase activity were tested by the t distribution at the .05 and .01 significance levels. With the use of pairs of muscles in this manner, a given mean change has a greater significance than the same difference in two independently selected groups of samples.

Sensory ganglia produced the greatest difference in ChE activity (Table 1). Boiling the ganglia for 1 minute prior to placing it on the muscle abolished the effect. The ganglion did not have to be in direct contact with the muscle to have an effect. When the ganglion was placed in a separate chamber separated from the muscle by a filter (*14*) (TH filter, pore size 0.45 μm, Millipore Corporation), ChE activity was higher than it was in con-

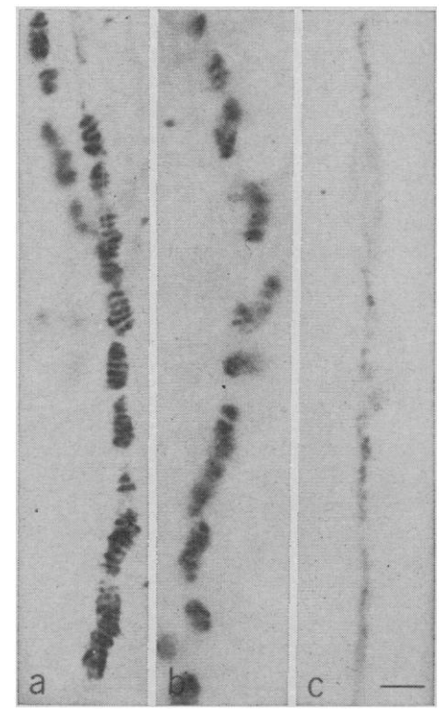

Fig. 3. Histochemical demonstration of cholinesterase (ChE) activity in motor end plates. (a) Normal motor end plate showing intense localization of enzyme activity. The light bands alternating with dense reactive areas represent junctional folds. (b) Motor end plate of muscle cultured in the presence of a sensory ganglion for 1 week. Activity is still intense and junctional folds are apparent although not as distinct as normally. (c) Muscle cultured alone for 1 week. Activity is reduced in intensity and localized to small droplets. The width of the reactive zone is considerably smaller and junctional folds cannot be distinguished. Bar represents 2 μm.

trols. Spinal cord explants also produced a difference, but not as great as did sensory ganglia. Other tissues were assayed and kidney, oviduct, and spleen produced no significant differences. Liver, however, which itself is capable of regeneration, resulted in higher ChE activity.

Homogenates of nerve tissue were also assayed. Sensory ganglia, brachial nerves, and cervical spinal cord were removed and homogenized with a glass homogenizer in 1 ml of phosphate buffer (*p*H 7.4), centrifuged for 15 minutes at 600 rev/min, filtered through a Millipore filter (Type HA, pore size 0.45 μm), and added to the culture medium. In one experiment, 15 ganglia and nerves and five cords were used (homogenate 1) and in the second, 50 ganglia and nerves and 13 cords were homogenized (homogenate 2). With the latter, approximately 0.1 ml of filtered homogenate containing 0.1 mg of protein was added to each milliliter of culture medium. The nerve homogenates had an effect on ChE activity. The more concentrated extract was only slightly more effective than the extract prepared from less tissue.

These results show that nerve explants prevent, slow, or reverse the decrease in ChE activity occurring as a result of denervation in cultured skeletal muscle. Measurements can be performed easily and relatively quickly, making this system potentially suitable as a bioassay for a neurotrophic process. The observations that sensory ganglia, ganglia separated from muscle by a filter, and filtered homogenates are effective in maintaining ChE activity provides additional evidence that the trophic effect is mediated by a chemical substance or nerve trophic factor. Use of the assay system should allow further investigation into the nature of the factor and its mechanism of action on muscle.

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Glucocorticoid Receptors in Lymphoma Cells in Culture: Relationship to Glucocorticoid Killing Activity

Abstract. *Mouse lymphoma cells in culture which are killed by adrenal steroids contain specific cortisol receptors that may be involved in the initial events of hormone action. The similarity of these receptors to those in hepatoma tissue culture cells, where adrenal steroids induce tyrosine aminotransferase, suggests that certain aspects of steroid action are similar in the two systems. In three steroid-resistant lymphoma cell populations specific binding was less than in the parent lines, suggesting that conversion to steroid resistance may be associated with changes in specific steroid binding.*

Glucocorticoid hormones have diverse actions on different responsive tissues. For example, these steroids induce the synthesis of tyrosine aminotransferase in rat hepatoma cells (HTC cells) (*1*), and cause cell death in mouse lymphoid tumor cells (*2–5*). The activities of different steroids in these two systems are similar and steroids that block cortisol induction of tyrosine aminotransferase synthesis in HTC cells also block the cortisol lymphoma cell killing activity (*4–7*). These features, and the existence of "specific" steroid receptors that appear to be involved in the hormonal responses in HTC cells and many target tissues (*8–12*), led us to examine steroid binding in lymphoma cells.

Characteristics of the cell lines used (*3–6*) and general techniques used for measurement of steroid uptake (*10*) have been described. The steroid-sensitive cells (lines S49.1, S1AT.4 and S1A.4.8) are killed by cortisol concentrations as low as $3 \times 10^{-8} M$ (*4–6*). The growth of cells of the "transitory sensitive" line S1AT.8 is transiently inhibited in the presence of cortisol, but the cells are not killed (*6*). The resistant variants (lines S49.1H.1, S49.1TB.-2H from S49.1; and S1AT.4TB.2H from S1AT.4) are unaffected by cortisol concentrations as high as $10^{-5} M$

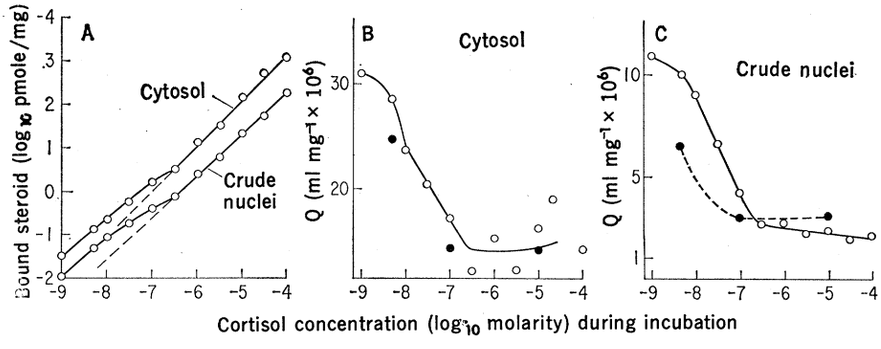

Fig. 1. Cortisol associated with lymphoma cell fractions. (A) Uptake of [^{3}H]cortisol (44 c/mmole) by S49.1 (steroid-sensitive) lymphoma cells (ordinate in picomoles per milligram of protein in the fraction) is plotted (logarithmically) against the concentration of cortisol during incubation. (B and C) The data shown in (A) plotted as Q, the ratio of relative concentrations of bound to free steroid, as a function of the cortisol concentration (○). (B) Cytosol; (C) nuclei. (B) and (C) also show the cortisol associated with the steroid-resistant cell line S49.1H.1 measured in the same experiment (●).