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Selective Effects of Skeletal Muscle Extract Fractions on Motoneuron Development *in vitro*

R. G. Smith, K. Vaca, J. McManaman, and S. H. Appel

Department of Neurology and Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030

***In vivo* labeling of motoneurons with retrogradely transported wheat germ agglutinin, and *in situ* staining of cholinergic neurons with a monoclonal antibody against CAT, have been used as methods to identify motoneurons in cultures of dissociated 14 d embryonic rat ventral spinal cord. With these two different labeling techniques, we demonstrate that skeletal muscle extracts maintain motoneuron survival, and induce motoneuron morphologic and cholinergic differentiation *in vitro*. Further, we show that several partially purified muscle extract-derived proteins separately augment each of these specific parameters of motoneuron development. A 55,000 Da neutral glycoprotein specifically induces both ACh synthesis and motoneuron-selective process outgrowth and elongation, while a 1200-1500 Da peptide selectively augments both apparent motoneuron survival and ACh synthesis. A third trophic agent, a 33,000-35,000 Da acidic glycoprotein, induces process outgrowth in both motoneurons and nonmotoneurons of the ventral spinal cord, but has no effect on ACh synthesis.**

In earlier reports, we described several muscle-derived protein factors that separately promote the morphologic and cholinergic differentiation of dissociated ventral spinal cord neurons (Smith et al., 1983; Smith et al., 1985; McManaman et al., 1985). Under reducing conditions, neurite outgrowth-stimulating activity was almost quantitatively recovered as a single acidic glycoprotein with an apparent molecular weight of almost 35,000 Da. In contrast, ACh synthesis-stimulating activity was found in multiple species. One high-molecular-weight factor was a glycoprotein of 55,000 Da, whereas several lower molecular weight factors were 17,000, 6000, and 1200–1500 Da. Combinations of the high-molecular-weight glycoprotein with any of the lower molecular weight peptides were additive in stimulating neuron cholinergic activity, while mixtures of the lower molecular weight peptides were not additive in their effects.

Since motoneurons are thought to represent only 5–10% of spinal cord cells (Schaffner et al., 1983), it is not clear whether any of these trophic proteins specifically affect motoneurons. Thus, questions concerning the specificity of the morphological and cholinergic factors for motoneurons and nonmotoneurons need to be addressed in order to clarify the significance of our previous data. Recently, several groups have used injected free-HRP as a probe for motoneurons (Bennett et al., 1980; Slack and Pockett, 1982; Tanaka and Obata, 1983). In such studies, medium conditioned on cultures of chick myotubes increased the apparent retention of chick HRP-labeled motoneurons *in vitro*. However, because this label is sensitive to metabolism and loss from cells (Gonatas et al., 1979; Peyronnard and Charron, 1983; Wan et al., 1982), it is difficult to determine whether conditioned media prevented the loss of HRP reactivity from cells or promoted cell survival.

We have employed two methods for motoneuron labeling *in vitro*. The first technique relies on the *in situ* addition and retrograde motoneuron transport of a labeled wheat germ agglutinin probe (Okun, 1981): Labeled ventral horn cells are identified after cells are dissociated and grown in culture. The second method depends on the recognition and binding of a monoclonal antibody directed against the endogenous cholinergic neuron marker, CAT (Crawford et al., 1982). Using these different motoneuron-labeling techniques, we now show that extracts of skeletal muscle increase motoneuron survival, motoneuron cholinergic parameters, and motoneuron neurite outgrowth in cultures of dissociated ventral spinal cord. Furthermore, we demonstrate that partially purified morphologic and cholinergic factors from skeletal muscle have differential effects on these motoneuron properties.

Materials and Methods

Tissue culture media were obtained from GIBCO Inc. (Grand Island, NY), while horse and goat sera were purchased from Kansas City Biologicals Inc. (Kansas City, MO). Radiochemicals were obtained from IsoTex (^{125}I: Friendswood, TX), or Amersham ([^{3}H]choline chloride; Arlington Heights, IL). All chemicals used were of analytical grade, and were purchased from either Sigma Chemical Company (St. Louis, MO) or Boeringer-Mannheim Biochemicals (Mannheim, Germany).

Cell culture and bioassays

Dissociated 13–14.5 d embryonic Sprague-Dawley rat ventral spinal cord cultures were prepared and grown according to previously described procedures (McManaman et al., 1985; Smith and Appel, 1983; Smith et al., 1985). In this report, cultures were treated with newborn skeletal muscle extract either 6 hr postplating, or 1 d after addition of 5×10^{-6} M cytosine arabinoside (Ara-C). We typically employed muscle supernatants prepared by tissue homogenization and 100,000 × *g* centrifugation. For some experiments, however, muscle supernatant was further purified by ammonium sulfate fractionation, sizing chromatography, preparative isoelectric focusing, and lectin chromatography, as detailed in a previous report (Smith et al., 1985).

Neuron process outgrowth and ACh synthesis assays have been described elsewhere (Smith and Appel, 1983; Smith et al., 1985) and were typically performed 3 and 4 d, respectively, after addition of the muscle fractions. To summarize these methods: Process density (number of processes per cell or process/cell ratio) was obtained by first counting the number of processes with lengths greater than one cell diameter in random microscopic fields, and dividing by the total number of cells in each field. Between 1 and 3% of the total dish surface area was typically

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Correspondence should be addressed to Dr. Stanley H. Appel, Chairman, Dept. of Neurology, Baylor College of Medicine, 1200 Moursund Ave., Houston, TX 77030.

Table 1. Time-dependent uptake of [^{125}I]WGA into spinal cord

Time (hr)	[^{125}I]WGA (dpm/cord)	[^{125}I]WGA + cold WGA (dpm/cord)
1	3180 ± 68	1462 ± 352
2	6462 ± 626	614 ± 225
4	13,418 ± 300	1674 ± 525
6	23,236 ± 627	1150 ± 120

Hindlimbs of 14 d rat embryos were injected with [^{125}I]WGA (200,000 dpm/limb), either in the presence of 100-fold excess unlabeled WGA (5 mg/ml), or without added cold probe. Embryos were then grouped according to the type of injections they received, and incubated for the times indicated at 30°C in aerated Sato's growth medium. Following each incubation period, three spinal cords per group were separated from their embryos, and the [^{125}I]WGA incorporated into each cord was measured on a gamma-emission spectrometer. Results are the mean of three cords per value ± SD.

Note. Total label injected per limb = 200,000 dpm.

Table 2. Specificity of [^{125}I]WGA uptake into spinal cord

Section	[^{125}I]WGA (dpm/cord section)	[^{125}I]WGA + cold WGA (dpm/cord section)
Total cord	10,485	3190
Left ventral	7092	640
Left dorsal	754	706
Right ventral	989	746
Right dorsal	720	700
Left body	590,000	510,000
Right body	17,504	14,256

Rat embryos were unilaterally injected with 500,000–600,000 dpm [^{125}I]WGA into left hindlimbs. Following a 4 hr incubation at 37°C, cords were removed and quadrisected into right, left, dorsal, and ventral aspects. Dissected cords were then assayed for prior uptake of [^{125}I]WGA by gamma-emission spectrometry. Hemisected bodies (without cords) were tested in order to determine the amount of label crossover from the injected to the noninjected side. These results (derived from two embryos) are representative samples of typical cord fractionation studies.

assayed, and cell counts were checked by plate DNA analysis. *De novo* ACh synthesis was measured by a modification of the assay of Johnson and Pilar (1980). Some cultures were also assayed after 4 d for total CAT activity by a modification of the procedure of Fonnum (1969). Specificity of the CAT assay for our cells was verified by addition of napthylvinylpyridine (NVP), a specific inhibitor of CAT (White and Cavallito, 1970). Addition of 1 mM NVP completely inhibited CAT activity relative to untreated sample pairs.

Preparation of WGA label

Wheat germ agglutinin (Sigma Chemical Co.) was either directly iodinated, or conjugated to the fluorescent marker Lucifer Yellow vinylsulfone. Iodination of the lectin followed a modification of the method of Mickey et al. (1971), and was performed by incubating 50 μl of WGA (1 mg/ml in 0.1 M $NaHPO_4$, pH 7) to 1 μl of [^{125}I]iodine (10 Ci/ml) for 20 min at room temperature. Free radioactive iodine was then scavenged by a second incubation with excess sodium iodide and tyrosine, and iodinated lectin was separated from other products by Sephadex G-25 sizing chromatography (Pharmacia Fine Chemicals, Piscataway, NJ) and precipitation with 10% trichloroacetic acid. After resuspension, the label was rechromatographed and concentrated for injection.

Alternatively, WGA was linked to Lucifer Yellow vinylsulfone (Aldrich Chemical Co., Milwaukee, WI), using a method described by Okun (1981). Conjugation mixtures, containing 1 mg WGA and 0.3 mg Lucifer Yellow in 200 μl of bicarbonate-buffered Tyrode's solution (pH 9), were incubated for 2 hr at 37°C prior to use. Although iodinated WGA was occasionally stored at 4°C for 1–2 weeks, WGA conjugated to Lucifer Yellow (WGA-LY) was always used within 12 hr of preparation.

Injection and assay of WGA tracer

WGA tracer was injected using a modification of the technique of Lance-Jones (1982). Thirteen to 14 d Sprague-Dawley rat embryos were first beheaded and eviscerated, and spinal cords exposed. Approximately 75 μg of the WGA label was then pressure-microinjected through prefilled glass microelectrodes into two or three distal hindlimb sites, and embryos incubated in aerated Sato's growth medium (Bottenstein and Sato, 1979) at 30°C for 0.5–6 hr. Labeled spinal cords were removed and treated in one of three ways. In initial experiments, iodinated WGA tracer distribution was tested in cords after quadrisection into left and right, dorsal and ventral sections. Hemisected right and left body halves and injected limbs were dissected and separately assayed. Alternatively, the presence of WGA-LY tracer in cryostat-sectioned spinal cord slices was assayed by fluorescence microscopy. Prior to sectioning, cords were prefixed in 2% paraformaldehyde.

Most ventral spinal cords, however, were dissociated and either fractionated through a density gradient, or directly plated at 0.5–2 × 10^6 cells/25 mm well onto polylysine-precoated, 22-mm-diameter glass coverslips. For cell fractionation (Schnaar and Schnaffner, 1981), dissociated tissues were cooled to 4°C, layered onto a sterile 0–50% metrizamide (Sigma) discontinuous gradient, and centrifuged for 15 min at 2500 × *g*. Cells were then collected at interfaces between the 0, 8, 12, 17, 25, and 50% metrizamide gradient steps (corresponding to 1.005, 1.045, 1.065, 1.090, 1.130, and 1.3 density [gm/ml] fractions), and either plated onto coverslips or immediately assayed for [^{125}I]WGA content and CAT activity.

Dissociated WGA-LY-labeled cells were identified using a Zeiss fluorescence microscope equipped for phase microscopy. Cell counts were performed on paraformaldehyde-prefixed cultures. To determine the percentage of neurons containing the WGA tracer, cells were also labeled with tetanus toxin (Smith and Appel, 1983). WGA-LY- and tetanus toxin-labeled cells were typically phase-bright, with raised round, or ellipsoid cell bodies. They could be easily distinguished from the flattened, phase-dark, macrophage-like cells that sometimes became labeled with WGA marker after several days in culture. Toxin-binding and WGA-LY internalization were assayed under epifluorescence. Tetanus toxin visualization required indirect immunofluorescence assay, using a horse anti-tetanus toxin first antibody (Massachusetts Department of Public Health), and a rhodamine-conjugated goat anti-horse second antibody (Cappel; Cochranville, PA).

CAT antibody labeling

A monoclonal antibody directed against CAT was obtained from Drs. Garrett Crawford and Paul Salvaterra. This antibody, named 1E6, has been shown to bind CAT, both by specific *in vitro* immunoprecipitation of the enzyme, and by *in situ* labeling of CNS regions known to contain cholinergic neurons (Barber et al., 1984; Crawford et al., 1982; Houser et al., 1983).

For identification of CAT-labeled neurons, cultures were prepared as previously described and maintained on coverslips until the time of assay. Prior to staining, cells were fixed for 2 hr in buffered 2% paraformaldehyde solution. Cell membranes were next partially solubilized for 15 min in Tris-buffered solution (TBS) (0.1 M Tris buffer, pH 7.4, with 0.85% NaCl) containing 0.8% Triton X-100 detergent, followed by a 15 min rinse in TBS containing 1% goat serum and 0.1% detergent (this solution was used in all subsequent incubations and rinses). Cells were then reacted (1) for 3 hr at room temperature with 2–4 μg 1E6 mouse anti-CAT monoclonal antibody in 40 μl TBS; (2) for 1 hr with a 1:40 dilution of goat anti-mouse antibody (American Qualex; La Mirada, CA); (3) for 1 hr with a 1:60 dilution of mouse peroxidase anti-peroxidase (PAP) complex (Sternberger-Meyer; Jarrettsville, MD). Five minute coverslip rinses were inserted between each cell-staining step. Steps (2) and (3) were each repeated for 30 min in order to intensify staining, and coverslips were then washed a final time for 20 min in three changes of TBS. CAT-labeled cells were visualized in serum-free and detergent-free 0.1 M Tris buffer, pH 7.6, using 0.05% 3,3'-diaminobenzidine·4HCl (DABA) and 0.005% H_2O_2. Remaining DABA was rinsed away, and coverslips were mounted on glass slides in buffered glycerol solution, pH 7.4.

Total cell and cell process counts were made on random 400-power fields with phase optics. Bright-field measurements of CAT antibody-labeled cells and cell processes were counted for the identical fields, as described in Figure 2 (examples of method provided in Figs. 2 and 4). Averaged cell size determinations were made for both CAT antibody-labeled cells and total cells from measurements of cell perimeters, using a Zeiss microscope attached to a Zeiss Videoplan microcomputer.

Table 3. Metrizamide fractionation of [^{125}I]WGA-labeled cells

Density gradient (% metrizamide)	Cell number ($\times 10^5$)		[^{125}I]WGA incorporation (dpm/cell $\times 10^{-3}$)		CAT activity (dpm/cell $\times 10^{-3}$)	
		% Total		% Total		% Total
Pregradient	285.5	100	2.10	100	4.08	100
8	5.9	2	8.69	42	14.09	38
13	11.6	4	0.58	7	2.55	12
17	46.2	16	0.24	7	0.74	15
25	76.1	27	0.14	9	0.24	8
50	37.3	13	0.22	7	0.37	6
Unfractionated cells	50.8	18	0.24	10	0.05	9
Recovery		80		82		88

Four hours after *in situ* labeling of spinal neurons via bilateral hindlimb injection of iodinated WGA, 10 embryonic rat spinal cords were dissected from their bodies, pooled, dissociated, and centrifuged through a discontinuous metrizamide density gradient. Direct hemocytometer counts were performed on aliquots of trypan blue-excluding cells (e.g., intact cells) obtained from both recovered metrizamide gradient fractions and unfractionated dissociated cells. Recovered cells from each gradient fraction were separately counted on a gamma-emission spectrometer, and dissociated cells then homogenized by fraction for assay of CAT activity.

Other procedures

Culture lactate dehydrogenase activity was measured by the method of Nishi and Berg (1981), while AChE activity in cultured spinal cord homogenates was analyzed using the procedure of Ellman et al. (1961). Protein content was determined by the method of Lowry et al. (1951) or by fluorescence assay (Udenfriend et al., 1972). Assays of culture DNA were performed using the technique of Hindgardner (1971).

Results

Selectivity of WGA for labeling motoneurons in situ

Iodinated WGA was transported in a retrograde fashion to the ventral spinal cord. Uptake of label was assayed by quantitation of gamma emissions from injected limbs, quadrisected spinal cords, and hemisected embryo bodies. [^{125}I]WGA was first observed in the ventral spinal cord 60–90 min after hindlimb injection, while maximal rates of incorporation by cord were found within 2–3 hr. Four hours after injection of 75 μg WGA into hindlimbs, 1.5–5% of the total injected radioactivity was typically localized in the spinal cord. This uptake was specific and saturable, since only 0.3–0.8% of the iodinated lectin was transported after premixing with 100-fold excess of unlabeled WGA (Table 1). With unilateral limb injection, greater than 90% of this specifically incorporated WGA was found in the ipsalateral ventral portion of the spinal cord (Table 2). Less than 5% of the total transported label was observed on the contralateral side of the cord, corresponding to an equal crossover of total body counts.

Uptake of labeled WGA was further localized to cholinergic neurons of the ventral cord. WGA comigrated with CAT activity in ventral spinal cord cells fractionated through a metrizamide density gradient (Table 3). Approximately 40% of both total CAT activity and total transported WGA was found in a gradient fraction previously shown to be enriched in motoneurons (Schnaar and Schnaffner, 1981).

Although uptake of iodinated WGA was readily demonstrated within the ventral cord, the label could not be visualized in individual cells. For this reason, WGA-LY was employed to visualize motoneurons. This marker, like its iodinated counterpart, was selectively transported into ventral spinal cord ipsilateral to the site of injection, and could be visually demonstrated in frozen lumbar cord slices (Fig. 1*a*). Further, WGA-LY was retained in neurons after their dissociation and plating. Six hours after plating, cord cells from WGA-LY-injected rat embryos were assayed for the binding of tetanus toxin, and for the presence of internalized WGA-LY. Of the tetanus toxin-labeled cells, 3.5% contained a yellow fluorescence, distributed evenly throughout the cytoplasm. These WGA-LY-labeled cells had neuron-like morphologies, and were larger than average cells, with ovoid somata of 15–25 μm × 10–15 μm diameter (Fig. 1, *b* and *c*).

Muscle extract and retention of WGA-labeled cells

In culture, the apparent survival of many WGA-LY-labeled neurons required the presence of muscle extract. In untreated cultures, after 4 d 1.3% of the tetanus toxin-labeled cells and 0.9% of the total surviving cells contained the WGA-LY marker. When maintained with 200 μg muscle extract protein/ml culture medium, however, the number of surviving WGA-labeled cells was increased to 3.3% of the neurons and 2.5% of the total cells (Table 4). Neither neuron number nor total cell survival underwent extract-dependent changes. These results were not due to motoneuron-selective Ara-C toxicity, since similar differences were observed in the absence of prior antimitotic agent addition.

However, one limitation to the use of WGA as a stable cell marker was that cell fluorescence became less distinct over a several day period *in vitro*. Further, the label may have become toxic, since in many WGA-LY-labeled cells there was a roughened or "foamy" appearance to surface membranes. These cells extended few, shortened processes. After 5 or 6 d in culture, some of these labeled neurons may have lysed, since WGA-LY-containing inclusion granules were now found in a significant number of macrophage-like cells.

ACh synthesis in WGA-labeled cultures

Measurements of cholinergic activity also suggested an effect on motoneuron survival. If cultures identical to those tested in the previous experiment (Table 4) were treated for 4 d with 200 μg muscle extract protein/ml culture medium, cell ACh synthetic activity was nearly 500% of 4 d control values, and was 2-fold higher than initial values (Table 5A). However, cholinergic activity in untreated cultures decreased between 6 and 36 hr *in vitro*, even though subsequent extract addition still significantly increased ACh synthesis and accumulation (Table 5B). These results suggested that observed changes in ACh synthesis represented a combination of effects, increasing apparent survival

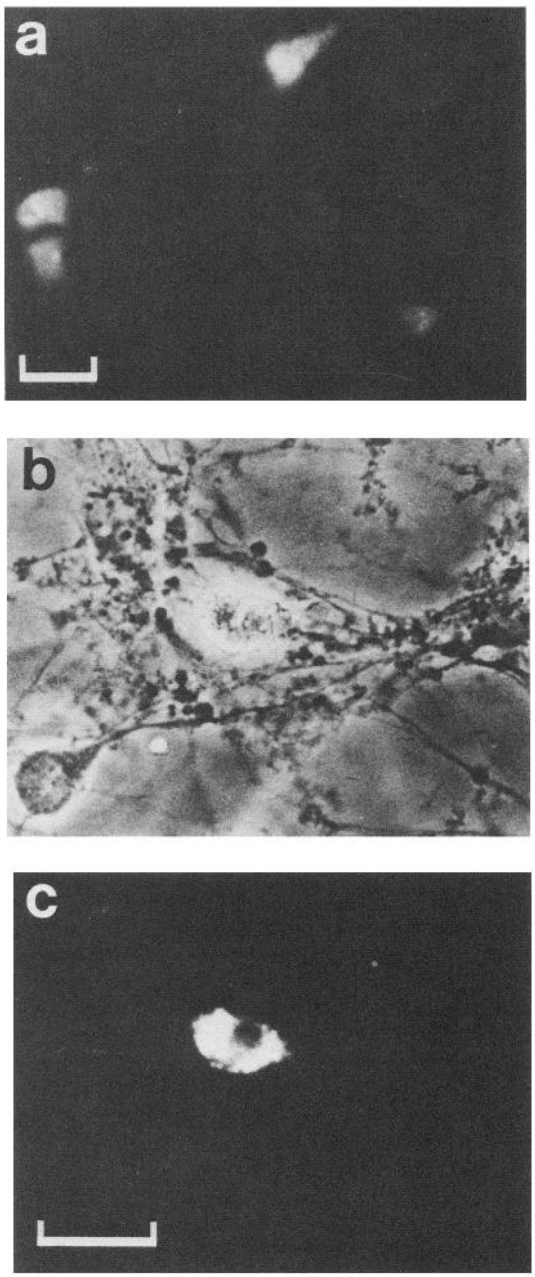

Figure 1. Use of WGA-LY in motoneuron visualization. 75–100 μg of labeled WGA was injected *in situ* into rat 14 d embryo hindlimbs. After a 4 hr incubation at 30°C, spinal cords were removed from the embryos, and prepared as described in the text. *a,* Portion of anterior horn slice taken from thoracolumbar region of embryonic cord. Individual cells in the slice are labeled with the probe. No staining was observed in the dorsal portion of the spinal cord (×500). *b,* Phase-contrast and *c,* fluorescence micrographs of WGA-LY-labeled cells, tested in culture for 4 d in the presence of 200 μg muscle extract/ml culture medium (×800). Bars, 20 μm.

of some cholinergic neurons and inducing cholinergic activity in surviving neurons.

These effects appeared to be selective for cholinergic cells in the ventral cord. Four day addition of equivalent concentrations of muscle extract to identically prepared cells increased AChE activity by 15 ± 5% relative to control, while culture lactate dehydrogenase (LDH) activity increased by only 10 ± 6% over control values. During the same 4 d time period, however, total LDH levels in both control and extract-treated cultures increased approximately 3-fold. Extract addition also produced no significant increase in cellular uptake of gamma-aminobutyric acid, when measured in a similar cell population (J. L. McManaman, personal communication).

Table 4. Counts of WGA-LY- and tetanus toxin-labeled cells

	WGA-LY-labeled cells (cells/field)	Tetanus toxin-labeled cells (cells/field)	Total cells (cells/field)
Day 0 (6 hr)	23.3 ± 0.6	665 ± 40	1025 ± 74
Day 4			
Control + Ara-C	4.3 ± 0.3	339 ± 15	456 ± 26
Extract + Ara-C	11.7 ± 0.7	350 ± 8	459 ± 20
Control − Ara-C	5.9 ± 0.4	500 ± 20	—
Control − Ara-C	18.6 ± 0.7	542 ± 32	—

Cultures of WGA-LY-labeled cells from caudoventral spinal cord were plated at a concentration of 2 × 10^6 cells/22 mm well on glass coverslips and treated with 5 × 10^{-6} M Ara-C for 12 hr. At the time of Ara-C treatment, cells also received either 200 μg muscle extract/ml culture medium (extract), or an equal volume of carrier buffer (control). Four days after the initial sample addition, cells were fixed and tested for tetanus toxin-binding. Counts of total cells, tetanus toxin-binding neurons, and WGA-LY-labeled neurons were made for each of twenty 250-power microscope fields/dish. Differences in WGA-LY-labeled cell numbers are compared both for cultures treated for 4 d with 200 μg muscle extract/ml culture medium and for untreated control cultures. Results are the mean of four cultures per group ± SD.

CAT antibody cell-labeling

In order to obtain a less toxic method for visualizing cholinergic cells *in vitro,* we modified an indirect immunoperoxidase staining technique for 1E6 CAT-directed monoclonal antibody (Fig. 2). Specificity of this antibody for CAT has been previously defined, both by biochemical assays and by *in vivo* cell-labeling tests (Crawford et al., 1982; Houser et al., 1983). Further, under our assay conditions, cell-staining was only observed in the presence of the CAT antibody. No staining of cells was found if nonimmune mouse sera replaced the antibody, or if any of the subsequent antisera were removed. Labeled cells had neuron-like morphologies; each possessed central round or ovoid somata and one or more well-defined processes (Fig. 2, *a, b*). Moreover, the observed percentage of CAT-stained cells/total

Table 5. ACh synthesis in WGA-LY-labeled cells

Time	ACh synthesis (pmol [^{3}H]ACh/μg DNA/hr) Control	Extract
	A	
Day 0 (6 hr)	6.1 ± 1.1	
Day 4 (104 hr)	2.5 ± 0.7	12.2 ± 2.0
	B	
Day 0 (36 hr)	2.5 ± 1.0	
Day 4 (132 hr)	2.3 ± 0.4	7.5 ± 1.1

Sister cultures to those described in Table 4 were assayed for ACh synthesis and accumulation. In part A, 6 hr after plating, 200 μg muscle extract/ml culture medium was added simultaneously with a pulse of Ara-C to these cultures. In part B, cells were treated with 5 × 10^{-6} M Ara-C prior to addition of the extract. In both cases, ACh synthesis was assayed 4 d after initial extract addition. Values are the mean of quadruplicate cultures per treatment group ± SD, in this representative experiment.

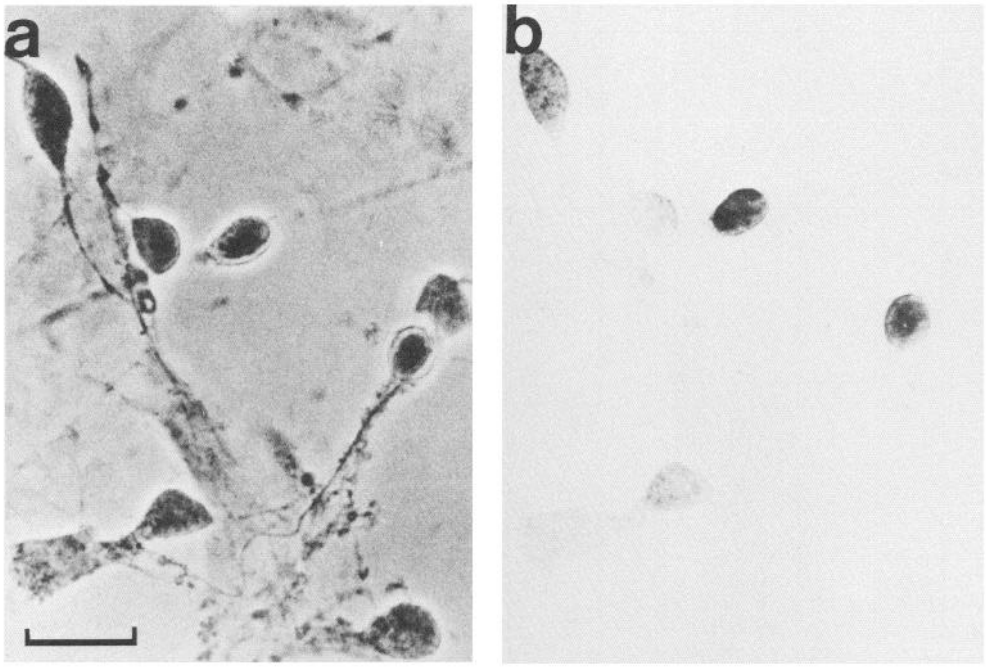

Figure 2. Phase-contrast (*a*) and bright-field (*b*) photomicrographs depicting CAT antibody-labeling cells. Cells are visually assayed for diaminobenzidene reaction product by subjectively assigning each cell in random microscopic fields to one of four categories: (1) heavy or dense labeling, (2) moderate labeling, (3) light or indeterminate labeling, and (4) no labeling. *Heavily stained and moderately stained cells* are grouped together as "positively labeled," while *unstained and indeterminately stained cells* are defined as "not labeled." By such subjective analysis, two heavily labeled and one moderately labeled CAT-staining cells are extending processes in this image, made after 5 d in the presence of 400 μg crude muscle extract/ml culture medium. Note the presence of two lightly stained and two nonreactive neuron-like cells, together with several unstained nonneuronal cells (×1000). Bar, 20 μm.

cells in whole cord cultures was only 40% of that found in dishes containing equal numbers of identically plated and treated dissociated ventral cord cells, suggesting that the ventral cord provided most of the CAT antibody-labeled neurons (Fig. 3). In dissociated ventral spinal cord, the degree to which neurons were labeled was variable, with both heavily and moderately stained cells observed. 1E6 antibody also stained cultured cholinergic neurons from chick ciliary ganglion. These neurons, however, were all uniformly stained. Passaged nonneuronal cells derived from rat spinal cord did not react either with anti-CAT antibody or with tetanus toxin.

Muscle extract and retention of CAT antibody-labeled cells

The apparent survival of many CAT antibody-labeled cells required muscle extract addition (Fig. 4). Typically, in the absence of extract, 7.8 ± 2.7% of cells labeled with anti-CAT antibodies after 4.5 d in culture. The addition of 200 μg muscle extract shortly after plating increased the number of CAT-stained cells to 17.5 ± 4.2% of the ventral cord cell population, measured after 4.5 d. This effect of the extract was concentration-dependent and saturable (Fig. 3). It was also specific for labeled neurons, since general neuron survival was not significantly altered by its addition (Fig. 5*a*). In the absence of extract, 50% of the CAT-containing neuron population was lost within 36 hr of plating (Fig. 5*b*). However, twice daily addition of muscle extract in saturating concentrations helped support the apparent survival of CAT-labeled cells for 2 weeks in culture.

Before detachment from the culture substratum, many CAT antibody-labeled neurons became small and crenated. In control cultures, such crenated cells never extended processes, and few survived for more than 1 d. The addition of muscle extract did not prevent the appearance of small CAT antibody-labeled cells, but did sustain their survival for several days *in vitro*. With extract, small cells constituted up to 25% of the CAT antibody-labeled neuron population.

Changes in specific CAT activity in culture paralleled changes observed for CAT-antibody binding (Fig. 6). In untreated cultures, total dish CAT activity decreased approximately 60% over a 36 hr period. Muscle extract prevented this decrease in total culture CAT activity.

Muscle extract actions on morphologic differentiation in CAT antibody-labeled cells

Unfractionated muscle extracts also induced process outgrowth in cholinergic neurons (Fig. 7). Neurite extension was dependent upon the concentration of extract, with similar amounts of muscle homogenate inducing process elongation in both the total neuron and cholinergic neuron populations. Neurite outgrowth from CAT-labeled cells proceeded at the same rate as observed for other neurons, beginning after 6–12 hr in culture.

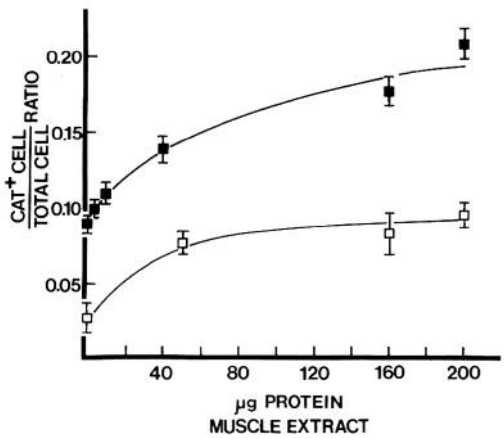

Figure 3. Concentration-dependent effects of muscle extract on CAT antibody-labeled cell survival. Survival of CAT-stained cells is expressed as the ratio of CAT+ cells to total cell number, under conditions where total cell number is not significantly changing. The effect of increasing muscle extract concentrations on this ratio is depicted, both for dissociated ventral spinal cord (■), and dissociated total cord (□). Each *data point* represents the mean of quadruplicate cultures ± SD. Total averaged percentage of CAT-labeled cells in untreated cultures (relative to total ventral cord cells) is 7.8 ± 2.7%, measured after 5 d in culture (culture $n = 30 \pm$ SD, in seven experiments). The mean percentage of CAT-labeled cells in extract-treated ventral spinal cord cultures after the same *in vitro* interval is 17.5 ± 4.2% (culture $n = 30 \pm$ SD, in seven experiments).

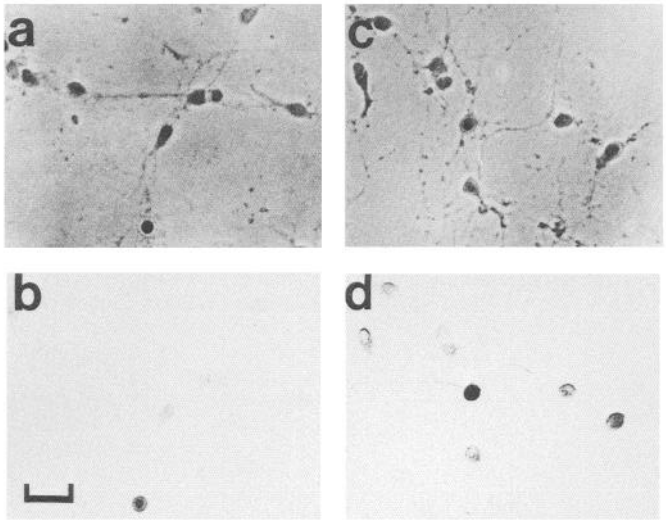

Figure 4. Effect of 400 μg muscle extract/ml culture medium on the number of CAT antibody-labeled cells. Cultures were plated at 500,000 cells per coverslip, and grown for 5 d either in the absence (*a, b*) or presence (*c, d*) of added muscle extract. Note the larger number of *heavy* (2) and *moderate* (5) *staining cells* in the culture treated with 200 μg muscle extract/ml culture medium than in the control culture (1 heavily labeled cell and 1 moderately labeled cell: ×150). Bar, 50 μm.

Effect of partially purified muscle extract fractions on CAT antibody-labeled cells

Partially purified muscle-derived extract fractions increased apparent cholinergic neuron survival, process outgrowth, and ACh synthesis (Table 6). While 4 d addition of crude muscle homogenate affected all these parameters of motoneuron development, purified extract components were more selective in their actions (Fig. 8). The 35,000 Da morphologic factor (MF; Fig. 8, *e, f*), previously shown to possess only morphologic activity on ventral spinal cord cells (Smith et al., 1985), nonselectively altered process outgrowth in both the general and cholinergic neuron populations. Two of the four previously described muscle-derived cholinergic factors were also tested on CAT-labeled cells. When assayed with the 1E6 antibody, 4 d addition of a 55,000 Da "high-molecular-weight" factor (HMWF) had no effect on cholinergic neuron survival, but did induce the outgrowth of neuronal processes in CAT antibody-labeled cells (Fig. 8, *c, d*). Conversely, the 4 d addition of the 1500 Da "low-molecular-weight" cholinergic factor (LMWF) increased the number of cholinergic neurons retained on the culture dish, while having little effect on labeled cell morphologic appearance or differentiation (Fig. 8, *a, b*). Thirty to forty percent of these retained cells were identical in appearance to the small, round cells described earlier. Recombination of the fractionated species (at activity-saturating concentrations) reconstituted the total morphologic and cholinergic activity levels observed with crude extracts.

Discussion

A combination of exogenous and endogenous labeling techniques have been employed to assess the effects of muscle extracts on embryonic rat motoneuron survival and development *in vitro*. Because sensitivity and stability are important characteristics of the probe, we have used wheat germ agglutinin as one motoneuron label. This protein, when conjugated to [^{125}I]iodine, Lucifer Yellow, or HRP, is many times more sensitive than free-HRP for marking rat and chick neurons. WGA was also more stable than other probes with respect to diffusion from the site of injection and to degradation in the cell (Okun,

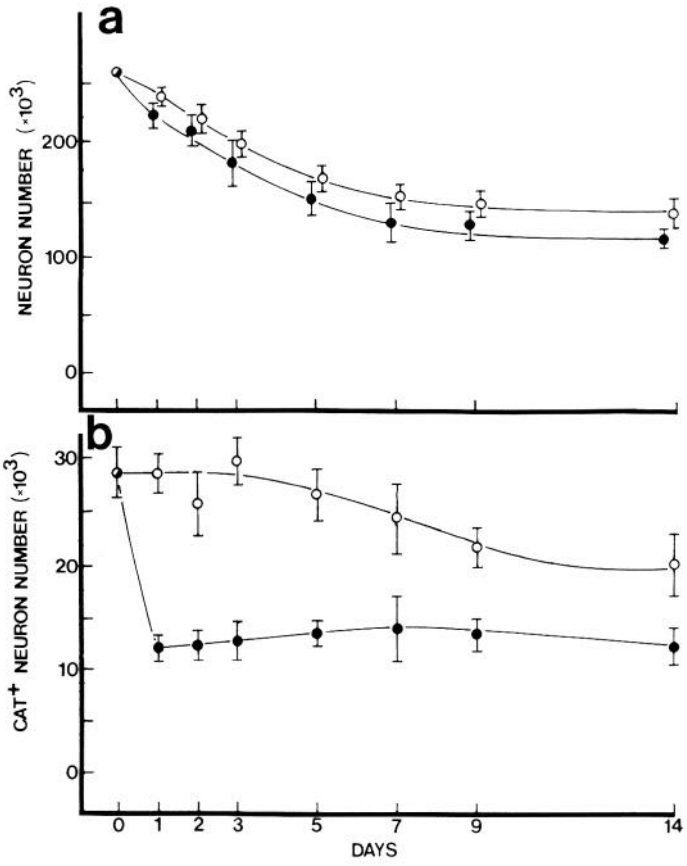

Figure 5. Time course of muscle extract effects on (*a*) total cell survival and (*b*) CAT-antibody-labeled cell survival. Cells were treated by addition of 200 μg muscle extract/ml culture medium to coverslips 6 hr after cell-plating (500,000 cells per dish). Cells were maintained with extract for up to 14 d (○). Untreated cultures were likewise assayed at each timepoint (●). Each value represents the average number of cells per coverslip for that time and treatment, as determined by counting between 20 and 30 125-power microscopic fields/coverslip. Results are the mean of eight values/timepoint, collected in two experiments, ± SD.

1981; Schwab et al., 1978; Wan et al., 1982). We have shown that both [^{125}I]WGA and WGA-LY were specifically taken up by cholinergic neurons of the ventral cord of the embryonic rat. Although superior to many other exogenously added markers, WGA was neither a quantitative nor a permanent label for motoneurons, in part because it may have had toxic long-term effects.

A monoclonal antibody directed against the endogenous marker, CAT, was employed as a second method for identifying motoneurons *in vitro*. Since all motoneurons are thought to be cholinergic, this label should be quantitative. Cell-labeling was also permanent insofar as cells continued to express cholinergic properties. Observed variability in the density of cell-labeling was probably not an artifact of culture (Barber et al., 1984), and may reflect actual variation in individual motoneuron CAT content (Kato and Murashima, 1985). The introduction of toxic substances into the cultures was avoided. One drawback of this technique may be the existence of cholinergic cells other than motoneurons in the ventral cord (i.e., autonomic preganglionic neurons), but these are rare at the stage of rat development from which the cultures were prepared.

While attempts at co-labeling cord neuron populations with endogenous and exogenous probes have met with technical difficulties (Smith, unpublished results), the relative selectivity and

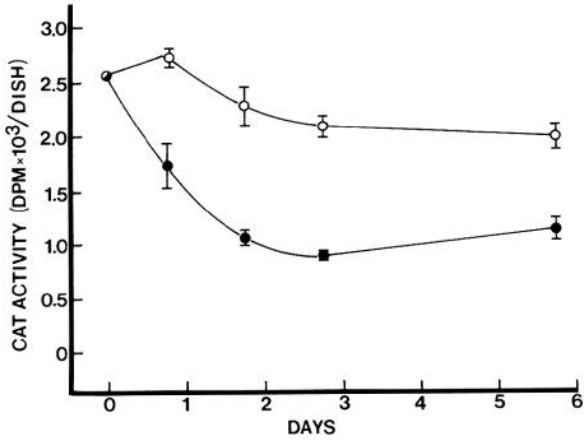

Figure 6. Loss of CAT activity in cultures of dissociated ventral spinal cord as a function of time *in vitro*. The effect of addition of 200 μg muscle extract/ml culture medium (○) is compared to no treatment of culture wells (●), at various times after the initial addition of muscle extract (4 hr after plating 500,000 cells per dish). Each *time point* represents the mean of triplicate cultures ± SD.

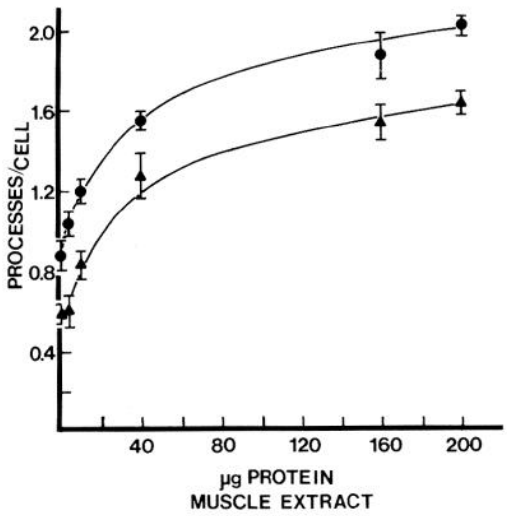

Figure 7. Process outgrowth measured both for total cells (●) and for CAT-labeled neurons (▲), as a function of muscle extract concentration. Results are expressed as mean values obtained from quadruplicate coverslips (counting 20–30 low-power microscopic fields/coverslip) ± SD.

efficiency of these two methods may be indirectly compared. Because both WGA-LY- and CAT antibody-staining techniques label ventral horn neurons, they each can be used to provide an estimate of spinal cord motoneuron number. Values derived from WGA-LY uptake suggest that motoneurons comprise 3.3% of caudoventral spinal cord cells (1.5% total cells). This is probably an underestimate of the "true" motoneuron number, since retrograde transport of a probe into motoneurons is a relatively inefficient, albeit highly selective method of staining. In contrast, the postplating percentage of CAT-labeled cells is almost 11% of the ventral cord population (approximately 5% of total cord cells). This value corresponds closely to another recently reported estimate of motoneuron number (Schaffner et al., 1983). Unfortunately, since the percentage of CAT-antibody-labeled cells increases relative to total neuron number with time in culture (possibly due to the expression—and subsequent staining—of CAT in maturing nonmotoneuron cholinergic cells), this assay begins to overestimate the number of spinal cord motoneurons after several days in culture. Thus, after 4–5 d *in vitro*, control ventral cord populations typically contain almost 8%

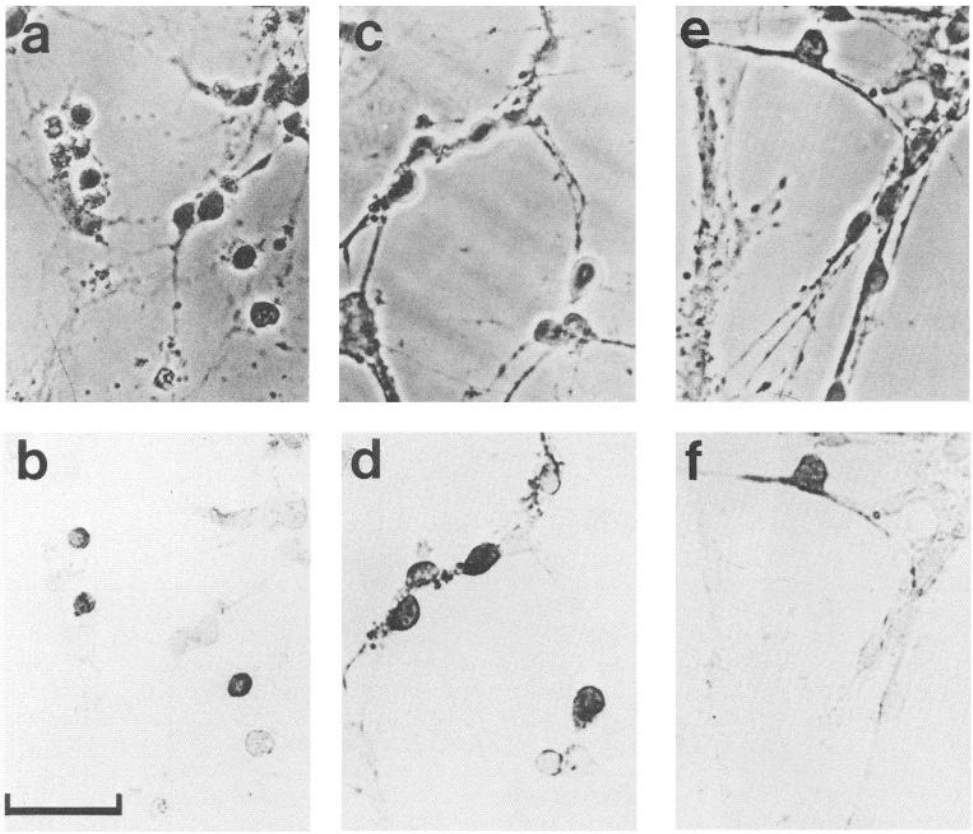

Figure 8. Actions of partially purified fractions of muscle extract on ventral spinal cord cultures. Phase-contrast and bright-field images of labeled cells 4 d after the addition of the LMWF (*a, b,* 0.5 μg/ml), HMWF (*c, d,* 5 μg/ml), and MF (*e, f,* 0.05 μg/ml) (×325). Bar, 50 μm.

Table 6. Effects of separated muscle-derived factors on motoneuron survival, process outgrowth, and ACh synthesis

Treatment	Total cell P/C ratio (±SE)	n	CAT Ab-labeled neuron P/C ratio (±SE)	n	CAT+ cell: total neuron ratio (±SE)	n	ACh synthesis (% control) (±SE)	n
PBS	1.11 ± 0.05	4	0.32 ± 0.02	4	0.15 ± 0.01	4	1.00 ± 0.06	6
Crude NRMX	1.85 ± 0.05	4	0.70 ± 0.05	4	0.23 ± 0.01	4	2.38 ± 0.15	6
LMWF	1.00 ± 0.05	8	0.26 ± 0.03	8	0.24 ± 0.02	8	1.98 ± 0.01	6
HMWF	1.19 ± 0.05	6	1.01 ± 0.07	6	0.14 ± 0.01	6	1.43 ± 0.06	6
MF	1.88 ± 0.07	4	0.80 ± 0.08	4	0.12 ± 0.01	4	n.d.	
HMWF + MF + LMWF	1.80 ± 0.05	4	0.95 ± 0.06	4	0.24 ± 0.01	4	2.45 ± 0.13	3

Dissociated ventral spinal cord cultures, plated on coverslips at a density of 5×10^5 cells/22 mm well, were treated both with Ara-C and extract fractions 6 hr postplating. Twelve hours later, cells were refed both growth medium and saturating concentrations of extract fractions. Tested factors included unfractionated newborn rat muscle extract (crude NRMX: 400 μg/ml), low-molecular-weight cholinergic factor (1500 Da LMWF: 0.5 μg/ml), high-molecular-weight cholinergic factor (55,000 Da HMWF: 5 μg/ml), and morphologic factor (35,000 Da MF: 50 ng/ml). These substances had previously been partially purified by a combination of ammonium sulfate fractionation, sizing chromatography through Sephacryl S-200, LKB ACA-202, or Bio-Rad P-2 gels, preparative isoelectric focusing, and lectin chromatography (Smith et al., 1985). Four days after the first addition of either extract or extract-derived factor, cells were labeled with CAT antibody. Cell and process counts were made on twenty to thirty 125-power fields per coverslip in order to assay changes in general cell and CAT+ cell-specific process density (process/cell or P/C ratio) for effects on CAT+ cell number (relative to total neuron number), and for increases in ACh synthesis. Both total cell number and total neuron number remained fairly constant in all cultures. Results are the mean values for between three and eight wells per group (n) ± SEM, as tested in two experiments.

labeled cells (11% of ventral cord neurons; 3.5% of total cord cells), while extract-treated cultures have, on average, 17.5% of ventral cord cells staining with CAT antibody (23.3% of ventral cord neurons; 7.9% of total cord cells). As a result, the CAT antibody-labeling technique provides a very efficient, but possibly less selective, motoneuron-marking technique.

Another major result of the WGA-LY- and CAT antibody-labeling studies was the suggestion of muscle extract-dependent, motoneuron-specific survival. The entire decrease in cholinergic cell number, measured by the loss of probe-containing cells, occurred within the first 30 hr of cell culture. Although this simultaneous loss of CAT- and WGA-LY-staining might have been a result of intracellular protein leakage from damaged, dissociated cells rather than actual cell death, such loss was totally reversed by the initial addition of muscle extract at the time of plating. An effect on motoneuron survival was also suggested by measurement of cholinergic activity. Both total CAT activity and ACh synthesis in control cultures decreased with time, with major losses occurring between day 0 and day 1 *in vitro*. The magnitude of this loss was comparable to decreases found in sister culture WGA-LY-labeled neuron number. Addition of muscle extract prevented both the apparent loss of labeled motoneurons and the decrease in cholinergic synthetic capacity. Since these changes temporally and quantitatively paralleled previous results of CAT antibody cell-labeling, the data suggest that most presumed loss of label occurred during this early period of culture.

The data confirm and extend the work of others using free-HRP to test the effects of muscle-conditioned medium on chick motoneuron survival *in vitro* (Bennett et al., 1980; Nurcombe et al., 1984; Slack and Pockett, 1982; Tanaka and Obata, 1983). In our experiments, however, the degree to which stained motoneurons disappeared from untreated cultures was much less than previously described. Since we have recently replicated the results of others using free-HRP as a rat motoneuron label, this difference was probably due to greater sensitivity and better retention of the WGA and CAT markers in motoneurons.

The effects of muscle extract were not limited to enhancing apparent motoneuron survival. The extract also produced an enhancement of ACh synthesis that was larger than could be explained solely by motoneuron loss. After 4 d in extract, these differences amounted to net increases of 2–3-fold. They were comparable to the total increase normally observed if extract addition was delayed until 24–36 hr postplating. Since most cholinergic cell loss occurred during the first 1.5 d *in vitro*, the increase in ACh synthesis probably resulted from direct induction, either of choline uptake, CAT, or ACh storage capacity. These data were not a measure of general neuron development, however, since neither culture lactate dehydrogenase activity, AChE activity, nor GABA uptake similarly increased after extract addition.

We have also been able to use CAT antibody-labeling to assess some of the specific actions of partially purified active muscle proteins on ventral cord neurons. As previously described, the 17,000, 6000, and 1500 Da proteins each had cholinergic activity; however, the high-molecular-weight glycoprotein was additive to each of the lower molecular weight peptides (Smith et al., 1985). As a representative of these low-molecular-weight species, the 1500 Da LMWF was shown not only to increase ACh synthesis capacity (McManaman et al., 1985), but also to promote the retention of CAT-antibody-labeled neurons in culture. However, many of these cells were small crenated cells and may not have been functionally active. It did not noticeably affect the morphology of the surviving CAT-positive cells. We have previously suggested that these cholinergic species may act similarly to a factor recently described by Kaufman et al. (1985). In contrast, the 55,000 Da HMWF specifically promoted neurite outgrowth in CAT-positive cells, but not other ventral cord neurons. It did not prevent early cell loss of CAT-positive cells but enhanced the capacity for ACh synthesis in those that remained. The actions of this species are thus similar to those of a factor recently reported by Gurney (1984). The 35,000 Da morphologic factor had a general effect in promoting neurite outgrowth in ventral cord neurons, including CAT-positive cells. This result thus extends our own previous data, as well as the work of others (Dribin and Barrett, 1982; Henderson et al., 1982; Smith and Appel, 1981, 1983). These three classes of proteins were additive in their effects on CAT-containing neurons, together reproducing the effects of the crude muscle extract.

Motoneurons go through several characteristic stages in their development, including axon growth, competition for survival, and full expression of their transmitter-specific function. In an earlier paper, we showed differential expression of some trophic activities in muscle extract as a function of muscle activity and development (Smith et al., 1983, 1985). We then biochemically separated and partially purified proteins responsible for distinctive activities on dissociated ventral spinal cord cultures. In the present paper, we have demonstrated that these proteins act on motoneurons, in some cases with relative specificity. Our results further suggest the possibility that trophic control might occur as the concerted action of a number of factors, each differently influencing spinal cord development.

References

Barber, R. P., P. E. Phelps, C. R. Houser, G. D. Crawford, P. M. Salvaterra, and J. E. Vaugn (1984) The morphology and distribution of neurons containing choline acetyltransferase in the adult rat spinal cord: An immunocytochemical study. J. Comp. Neurol. *229:* 329–346.

Bennett, M. R., K. Lai, and V. Nurcombe (1980) Identification of embryonic motoneurons in vitro: Their survival is dependent on skeletal muscle. Brain Res. *190:* 537–542.

Bottenstein, J. E., and G. H. Sato (1979) Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc. Natl. Acad. Sci. USA *76:* 514–517.

Crawford, G. D., L. Correa, and P. M. Salvaterra (1982) Interaction of monoclonal antibodies with mammalian choline acetyltransferase. Proc. Natl. Acad. Sci. USA *79:* 7031–7035.

Dribin, L. B., and J. Barrett (1982) Characterization of neurite outgrowth-promoting activity of conditioned medium on spinal cord explants. Dev. Brain Res. *4:* 435–441.

Ellman, G., K. Courtney, V. Andres, and R. M. Featherstone (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. *7:* 88–95.

Fonnum, F. (1969) Radiochemical micro-assays for the determination of choline acetyltransferase and acetylcholinesterase activities. Biochem. J. *115:* 465–472.

Gonatas, N. K., C. Harper, T. Mizutani, and J. O. Gonatas (1979) Superior sensitivity of conjugates of horseradish peroxidase with wheat germ agglutinin for studies of retrograde axonal transport. J. Histochem. Cytochem. *27:* 728–734.

Gurney, M. E. (1984) Suppression of sprouting at the neuromuscular junction by immune sera. Nature *307:* 546–548.

Henderson, C. E., M. Huchet, and J.-P. Changeaux (1981) Neurite outgrowth from embryonic chicken spinal neurons is promoted by media conditioned by muscle cells. Proc. Natl. Acad. Sci. USA *78:* 2625–2629.

Hinegardner, R. T. (1971) An improved fluorimetric assay for DNA. Anal. Biochem. *39:* 197–201.

Houser, C. R., G. D. Crawford, R. P. Barber, P. M. Salvaterra, and J. E. Vaugn (1983) Organization and morphological characteristics of cholinergic neurons: An immunocytochemical study with a monoclonal antibody to choline acetyltransferase. Brain Res. *266:* 97–119.

Johnson, D. A., and G. Pilar (1980) The release of acetylcholine from post-ganglionic cell bodies in response to depolarization. J. Physiol. (Lond.) *299:* 605–619.

Kato, T., and Y. L. Murashima (1985) Choline acetyltransferase activities in single motor neurons from vertebrate spinal cords. J. Neurochem. *44:* 675–679.

Kaufman, L. M., S. R. Barry, and J. N. Barrett (1985) Characterization of tissue-derived macromolecules affecting neurotransmitter synthesis in rat spinal cord neurons. J. Neurosci. *5:* 160–166.

Lance-Jones, C. (1982) Motoneuron cell death in the developing lumbar spinal cord of the mouse. Dev. Brain Res. *4:* 473–479.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall (1951) Protein measurement with folin phenol reagent. J. Biol. Chem. *193:* 265–275.

McManaman, J. L., R. G. Smith, and S. H. Appel (1985) Low molecular weight peptide stimulates cholinergic development in ventral spinal cord cultures. Dev. Biol. *112:* 248–252.

Mickey, D. D., P. N. McMillan, S. H. Appel, and E. D. Day (1971) The specificity and cross-reactivity of antisynaptosome antibodies as determined by sequential adsorption analysis. J. Immunol. *107:* 1599–1610.

Nishi, R., and D. K. Berg (1981) Two components from eye tissue that differentially stimulate the growth and development of ciliary ganglion neurons in culture. J. Neurosci. *1:* 505–513.

Nurcombe, V., M. A. Hill, K. L. Eagleson, and M. R. Bennett (1984) Motor neuron survival and neuritic extension from spinal cord explants induced by factors released from denervated muscle. Brain Res. *291:* 19–28.

Okun, L. M. (1981) Identification and isolation in vitro of neurons marked in situ by retrograde transport. In *New Approaches in Developmental Biology—1981 Short Course Syllabus,* D. I. Gottlieb, organizer, pp. 109–121, Society for Neuroscience, Bethesda, MD.

Peyronnard, J.-M., and L. Charron (1983) Decreased horseradish peroxidase labeling in deafferented spinal motoneurons of the rat. Brain Res. *275:* 203–214.

Schaffner, A. E., P. A. St. John, and J. L. Barker (1983) Purification of embryonic mouse motoneurons by flow cytometry. Soc. Neurosci. Abstr. *9:* 7.

Schnaar, R. L., and A. E. Schaffner (1981) Separation of cell types from embryonic chicken and rat spinal cord: Characterization of motoneuron-enriched fractions. J. Neurosci. *1:* 204–217.

Schwab, M. E., F. Javoy-Agid, and Y. Agid (1978) Labeled wheat germ agglutinin (WGA) as new, highly sensitive retrograde tracer in the rat brain hippocampal system. Brain Res. *152:* 145–150.

Slack, J. R., and S. Pockett (1982) Motor neurotrophic factor in denervated adult skeletal muscle. Brain Res. *247:* 138–140.

Smith, R. G., and S. H. Appel (1981) Evidence for a skeletal muscle protein that enhances neuron survival, neurite extension, and acetylcholine (ACh) synthesis. Soc. Neurosci. Abstr. *7:* 144.

Smith, R. G., and S. H. Appel (1983) Extracts of skeletal muscle increase neurite outgrowth and cholinergic activity of fetal rat spinal motor neurons. Science *219:* 1079–1081.

Smith, R. G., J. McManaman, and S. H. Appel (1983) Evidence for biochemical separation of morphologic and cholinergic-active trophic factors from extracts of newborn rat skeletal muscle. Soc. Neurosci. Abstr. *9:* 616.

Smith, R. G., J. McManaman, and S. H. Appel (1985) Trophic effects of skeletal muscle extracts on ventral spinal cord neurons in vitro: Separation of a protein with morphologic activity from proteins with cholinergic activity. J. Cell Biol. *101:* 1608–1621.

Tanaka, H., and K. Obata (1983) Survival of HRP-labeled spinal motoneurons of chick embryo in tissue and cell cultures. Dev. Brain Res. *9:* 390–395.

Udenfriend, S., S. Stein, P. Bohlen, W. Dairman, W. Leimgruber, and M. Weigele (1972) Fluorescamine: A reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. Science *178:* 871–872.

Wan, X.-C. S., J. Q. Trojanowski, and J. O. Gonatas (1982) Cholerotoxin and wheat germ agglutinin conjugates as neuroanatomical probes: Their uptake and clearance, transganglionic and retrograde transport and sensitivity. Brain Res. *243:* 215–224.

White, H. L., and C. J. Cavallito (1970) Inhibition of bacterial and mammalian choline acetyltransferases by styrylpyridine analogues. J. Neurochem. *17:* 1579–1589.