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

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ABSTRACT Protein factors derived from skeletal muscle separately promote neurite elongation and acetylcholine synthesis in cultured rat ventral spinal neurons. Morphologic factor activity (neurite-inducing activity) is specifically found in rat skeletal muscle and cord neuron extracts, decreases with the postnatal age of the rats from which muscle extract is prepared, and increases in rat hindlimb muscle after 5 d of denervation. Cholinergic factor activity (acetylcholine synthesis–stimulating activity) is found in extracts of rat cerebral cortex and cardiac muscle in addition to spinal cord and skeletal muscle, increases with animal age, and decreases following 5 d of denervation. Biochemically, the factors responsible for these activities differ in their lability to denaturing conditions, apparent molecular weights, isoelectric points, and lectin-binding specificities. Under reducing conditions, morphologic activity is isolated in a single acidic glycoprotein with an M_r of 35,000, while acetylcholine synthesis–stimulating activity is found in multiple species of different molecular weights. Thus, acetylcholine synthesis–promoting activities and neurite growth–promoting activity appear to reside in different molecules. Significant purification of several of these factors has been achieved.

Skeletal muscle, the target organ for motoneurons, influences the survival, growth, and differentiation of its innervating neurons. In avian and mammalian embryos, procedures which reduce or block muscle function in vivo subsequently prevent the death of many motoneurons (53), delay the reduction of polyneuronal connections (3, 42), and induce motoneuron neurite sprouting (10). Electrical stimulation of such denervated skeletal muscle blocks this terminal sprouting in neurons (7) and increases the rate of loss of polyneuronal innervation (47). These developmental effects have been attributed to skeletal muscle–derived neurotrophic factors acting in a retrograde fashion on motoneurons (24, 54).

The main evidence for the existence of such diffusible neurotrophic factors comes from in vitro experimentation. Media conditioned on cultured myotubes and skeletal muscle homogenates have been shown to augment motoneuron survival (2, 57, 61), general neuron phenotypic appearance including neurite elongation (15, 27, 60), and motoneuron neurotransmitter induction (20, 21, 60). However, since these muscle-derived activities have not been substantially purified, it is not clear whether a single factor possesses both morphologic and cholinergic properties, or whether multiple factors are involved. In vitro experiments with embryonic parasympathetic (45) ganglia suggest that several agents can separately influence different aspects of neuron development. Molecular sieving has been used to separate the ciliary neuron morphologic- and cholinergic-enhancing activities (45).

In vivo experiments with spinal cord also suggest differential regulation of motoneuron development. In rat and chick, the critical periods for motoneuron survival (13, 25, 35), loss of polyneuronal connections (6), and cholinergic differentiation (30, 52) occur at different developmental stages. Process density and hyperinnervation in surviving motoneurons are greatest at the stage of motoneuron death (24), while subsequent postnatal loss of polyneuronal innervation is not accompanied by additional loss of motoneurons (35, 49). Fur-

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ther, in response to partial denervation by either axotomy or pharmacological blockade, motoneuron neurite sprouting from adjacent axons increases (5, 67), while there is a decrease in their capacity to synthesize and transport neurotransmitter (26, 29, 48).

We have previously demonstrated that skeletal muscle extract increases both neurite outgrowth and acetylcholine (ACh)[1] synthesis in cultures of dissociated embryonic rat ventral spinal cord (60). In the present paper, we provide evidence suggesting that the effects of muscle extract on neurite outgrowth and ACh synthesis in vitro are differentially regulated, and that the molecules possessing morphologic- and cholinergic-enhancing properties have distinctly different physical properties which can be readily separated by several biochemical techniques. Furthermore, we report the partial purification of a morphologic-enhancing factor which has no cholinergic-stimulating activity, and several cholinergic-enhancing factors without apparent morphologic-enhancing activity (58, 59).

MATERIALS AND METHODS

Tissue culture media were purchased from Gibco Laboratories (Grand Island, NY). Horse serum was obtained from Kansas City Biologicals Inc. (Kansas City, MO), and was heat inactivated at 50°C for 45 min before addition to media. All chemicals used were of analytical grade. Radiochemicals were purchased from New England Nuclear (Boston, MA), except for [^{3}H]choline chloride, which was obtained from Amersham Corp. (Arlington Heights, IL). All purification steps were performed on ice or in a cold room set to 4–7°C.

Cell Culture: Neuronal cultures were prepared from the dissociated, arachnoid-free ventral spinal cords of 13–14-d embryonic rats (Zivic-Miller, Allison Park, PA). Spinal cords were first separated longitudinally into dorsal and ventral portions. Ventral cord portions were then pooled, exposed to a 0.08% trypsin solution at 37°C for 15 min, and dissociated by gentle trituration through a fire-polished pasteur pipette. The dissociated cells were plated onto polylysine-coated Falcon tissue culture dishes (Falcon Labware, Becton, Dickinson & Co., Oxnard, CA). For morphologic assays, cells were plated at a density of 520 cells/mm^2 on 35-mm culture dishes. For assays of ACh synthesis and accumulation, cells were either treated as above, or plated at a fivefold higher density (2,500 cells/mm^2) onto 24-well multiwell plates. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 2 mM glutamine, 33 mM glucose, and 0.15 mM Garamycin. One half day after plating, cytosine arabinoside (5×10^{-5} M) was added to this medium for 12 to 24 h, to retard the proliferation of non-neuronal cells. After cytosine arabinoside removal, tissue extract fractions were added in constant volumes (usually 50 μl/ml culture medium) of a sterile saline solution. Half of the culture medium was exchanged every other day, and extract fractions were replaced with each feeding.

Tissue Extract Preparation: All tissue extracts were prepared from Sprague-Dawley rats. Washed tissue was homogenized in 3 vol (wt/vol) of 10 mM Dulbecco's phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) with 1 mM EDTA, 0.5 mM EGTA, and 2 mM phenylmethylsulfonyl fluoride, and centrifuged at 32,000 *g* for 1 h. The resulting supernatant (S_1) was re-centrifuged for 2 h at 100,000 *g*. The final supernatant (S_2: crude extract) was dialyzed against PBS for 12–24 h at 4°C, and filter sterilized through 0.22-μm Millex disposable filters (Millipore/Continental Water Systems, Bedford, MA) before use. This supernatant was used for all subsequent purification steps and experiments. Muscle extract, unless otherwise indicated, was prepared from the limb skeletal muscle of newborn rats.

Biological Assays: Where possible, the same cultures were used for testing both cell process outgrowth and ACh synthesis. Alternatively, identical sister cultures were used for different biological assays. The results of multiple experiments were usually pooled and expressed as a percent of the relevant control culture biological activity for each experiment. By this method, differences in control values were minimized, allowing better comparison of changes in biological activities.

[1] *Abbreviations used in this paper:* ACh, acetylcholine; α-BTX, α-bungarotoxin; DTT, dithiothreitol; IEF, isoelectric focusing; MWCO, molecular weight cut-off; NEM, *N*-ethylmaleimide; P/C ratio, the ratio of processes per cell.

To determine the yield of these biological activities from muscle extracts, a biological activity unit was defined. One unit is that amount of sample required to produce a 50% stimulation over control levels, either in cell process density, or in ACh synthesis. Activity levels could be determined from activity/concentration curves for each sample, and quantitated by the best linear or logarithmic curve fit of the resultant concentration analysis data ($r^2 > 0.9$).

To test extract samples for denaturation by pH, protease, nuclease, heat, and reducing agents, concentrations corresponding to initial half-maximal biological activity were used to plot the rate of change in factor activity. Unless otherwise described in the text, only just-saturating concentration data has been presented.

ACh Synthesis: *De novo* ACh synthesis and accumulation was measured after 6 d in culture by a modification of Johnson and Pilar's assay (29). Cultures were depolarized for 10 min in a Tyrode's buffer with 25 mM KCl (to deplete existing stores of ACh), and then incubated in normal Tyrode's solution containing 10 μM [^{3}H]choline chloride (2–4 Ci/mmol, Amersham Corp.) for 30 min. The cultures were washed with PBS three times to remove unused radioactive substrate, and extracted with 15:85 1 N formate/acetone, containing ^{14}C-labeled tetraethylammonium bromide as an internal standard (New England Nuclear). These formic acid/acetone extracts were transferred to glass test tubes and dried by vacuum centrifugation. The contents of each tube were then resuspended in 2 ml of HEPES-buffered Tyrode's solution, pH 7.2, without potassium, and an aliquot was taken for measurement of choline uptake. Samples were centrifuged for 20 min at 2,500 *g*, and the supernatant (containing the choline and ACh) was collected and split into two equal fractions. One fraction from each well was treated with 0.02 U acetylcholinesterase (Boehringer-Mannheim Biochemicals, Mannheim, FRG) at 37°C for 10 min. Free choline was phosphorylated by adding 0.02 U of choline kinase (Sigma Chemical Co., St. Louis, MO), 10 mM ATP, 10 mM $MgCl_2$, and 10 μM eserine sulphate in 10 mM Tris buffer (final pH in solution was 8.2). The ACh was separated from phosphorylcholine by extraction in tetraphenylboron/acetonitrile (19), and carried into a toluene scintillint upper phase, which was removed for counting. The amount of ACh synthesis and accumulation was determined by first correcting for the loss of internal standard, and then taking the difference between the esterase-treated and untreated fractions as the amount of [^{3}H]ACh in each well. [^{3}H]ACh synthesis and accumulation was normalized to cell concentration by analysis of the DNA content in each culture well, and was expressed as the number of picomoles of [^{3}H]ACh/μg DNA per hour.

Morphometric Assay: Cultured cells were examined for process outgrowth by phase-contrast microscopy. As an indicator of average process density, the ratio of processes per cell (p/c ratio) was measured 3–4 d after extract addition. This ratio was obtained by 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. Cell counts were checked by plate DNA analysis, and cell viability at the time of counting was periodically tested by trypan blue exclusion. Twenty to sixty microscopic fields, representing between 1–3% of the total dish surface area, were counted for each plate. Process lengths in these fields were also measured, using a radicule. Periodically, process length and density measurements were photographically verified.

Rat Denervation: 6-wk-old Sprague-Dawley rats were denervated under ether anesthesia by cutting and removing a 0.5-cm section of the sciatic nerve at the level of the greater trochantor, or by injecting 2 μg/hindlimb per day of α-bungarotoxin (α-BTX) (Miami Serpentarium Laboratories, Miami, FL) every day. Completeness of denervation was tested each day by assessment of resistance to forced hindlimb flexion and by loss of reflex hindlimb extension. These results were verified at the time of muscle harvest by measurement of denervation-dependent increase in flexor hallucus longus muscle extrajunctional ACh receptors, using an adaptation of the method of Pestronk et al. (50). 6 h before death, iodinated α-BTX (41) was injected into all rat hindlimbs. Flexor hallucus longus junctional regions were identified by staining for acetylcholinesterase after death, and then dissected free from most extrajunctional tissue by cutting the muscles into equal halves. Low affinity ^{125}I α-BTX binding was displaced by a short incubation with excess cold toxin, and specific receptor binding was then measured by gamma counting.

Denaturation: The sensitivities of muscle extract to heat and protease were tested using the S_2 fraction. Thermal lability of the extract was determined by incubatng 0.5-ml aliquots at 60°C for intervals of from 5 to 60 min in a constant temperature shaking water bath. The samples were then cooled on ice and centrifuged to remove any precipitate that formed during the incubation. Procedures for assay of muscle extract trypsin sensitivity are described in Fig. 5*B*. After each incubation interval, extract aliquots were immediately placed on ice and treated with 1 mg/ml soybean trypsin inhibitor for 30 min. All samples were dialyzed in 12,000–14,000 molecular weight cut-off (MWCO) dialysis Spectropor 4 membranes (Spectrum Medical, Los Angeles, CA) for 12–16 h against three changes of PBS, pH 7.4, and filter sterilized (Millex 0.22-μm

filters) prior to culture addition.

Nuclease sensitivity was tested using ribonuclease A and deoxyribonuclease I, purchased from Boehringer-Mannheim Biochemicals (Mannheim, Germany). Enzyme incubations were performed after treatment of the muscle extract with 2 mM phenylmethylsulfonyl fluoride.

The pH stability of the trophic activities was examined by dialyzing aliquots of muscle extract at 4°C in different phosphate or citrate-phosphate buffers ranging from pH 3 to pH 11. The aliquots were then titrated back to pH 7.4, centrifuged for 10 min at 2500 *g*, filter sterilized, and tested for biological activity.

Precipitation by Ammonium Sulfate: Ammonium sulfate fractionation was performed at 0°C by the dropwise addition of 3.9 M $(NH_4)_2SO_4$ (Schwarz/Mann Div., Spring Valley, NY) to crude muscle extract. Ammonium sulfate concentration was increased in steps to complete saturation. After each step addition, extract samples were allowed to equilibrate for 1.5 h, and precipitated proteins were then pelleted by a 30-min centrifugation at 15,000 *g*. Precipitates were resuspended in ⅓ their original volume, and dialyzed for 12–16 h through 6–8,000 MWCO Spectropor 1 membranes against either PBS, or PIPES buffer (Calbiochem-Behring Corp., La Jolla, CA) containing 25 mM NaCl. Dialyzed samples were stored at −80°C for further use, or filter sterilized for assay.

Preparative Isoelectric Focusing (IEF): IEF was performed at 6°C on an LKB Multiphor 2117 apparatus. A Sephadex IEF gel slurry (Pharmacia Fine Chemicals, Uppsala, Sweden), containing muscle 46–60% ammonium sulfate fraction mixed with pH 4–9 ampholines (Serva Fine Biochemicals Inc., Garden City Park, NY), was poured into an electrophoresis tray and focused at 8 W constant power for 16 h. At the end of the run, protein banding was determined by overlaying a strip of Whatman 1 paper (Whatman Inc., Clifton, NJ) on the edge of the gel surface. This paper print was removed, fixed in 10% trichloroacetic acid, and developed with Coomassie Blue. The gel was then separated into 30 fractions, and a small aliquot of each fraction was collected for pH determination. Protein contained in the remainder of each fraction was eluted by the method of Winter et al. (69), using alternating 0.5-ml washes of PBS and 0.5 M NaCl in PBS. Eluted proteins were dialyzed through 1,000 MWCO dialysis membranes and concentrated threefold by lyophilization prior to assay of biological activity.

Gel Filtration Chromatography: Gel filtration steps were performed in LKB and Pharmacia columns. Sizing column flow rates were maintained with an LKB 2132 Microperpex peristaltic pump. All column fractions were collected on Gilson Microfractionator (Gilson Medical Electronics, Inc., Middleton, WI) fraction collectors. UV absorbance was assayed for each fraction at 280 nm on a Gilford 250 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH).

Muscle extract samples were dialyzed in 10 mM phosphate/50 mM NaCl buffer, pH 6.8, and then fractionated on either a 2.5 × 85-cm column of Sephacryl S-200 equilibrated at a flow rate of 40 ml/h, or a 1.0 × 90-cm column containing LKB ACA-202 standardized at a rate of 12 ml/h. Grouped fractions from each column were dialyzed through Spectropor 6 1,000 MWCO membranes against one-quarter normal PBS solution, and concentrated fourfold by lyophilization before addition to cultures.

Determination of Glycoprotein Nature, and Lectin-Affinity Chromatography: Sodium periodate, cyanogen bromide-activated Sepharose 4B, and the lectins Triticum vulgaris, Arachis hypogaea. Lotus tetrogonobolus, Concanavalin A, and Lens culinaris were obtained from Sigma Chemical Co. α-Mannosidase, β-glucosidase, neuraminidase, and Limulin were purchased from Boehringer-Mannheim Biochemicals. Glycosidases and lectins were first conjugated to cyanogen bromide-activated Sepharose 4B (51, 65), and then poured into mini econo-columns from Bio-Rad Laboratories, Richmand, CA. The specific binding activities of these lectin-coupled beads were assayed using radiolabeled sugars. After removal of radiolabeled tracers, muscle fractions were then added to the lectin columns. Choice of running and elution buffers depended upon the specific lectin-sugar pair (51, 65). Columns were washed in 20 vol of lectin-specific buffer, and bound molecules then eluted in 2–5 ml of buffer containing 100 mM (50 mM for sialic acid) specific eluting sugar. Samples were dialyzed against ¼ normal PBS solution through 1,000 MWCO Spectropor 6 membranes, and concentrated fourfold by lyophillization prior to testing biological activity.

Other Procedures: Lactate dehydrogenase activity (45) and acetylcholinesterase activity (18) were both assayed on homogenates of spinal cord cultures either 12 h after initial cell plating, or after 5 d in vitro. Cultures were assayed either in the absence or 4-d presence of 200 μg skeletal muscle extract/ml culture medium. Protein content was assayed by the technique of Lowry et al. (38) or by fluorimetric assay (64). Culture DNA assays were performed according to the method of Hinegardner (28).

RESULTS

Under our culture conditions, 95% of added cells attached to dish substrata within 1 h of plating. When plated at a density of 500 cells/mm^2, 37 ± 7% of the cells remained bound to the culture dish after 6 d in vitro (4 d after removal of cytosine arabinoside). At the higher plating density of 2,500 cells/mm^2, apparent cell survival was 50 ± 8%. 85 ± 10% of these cells were labeled by the neuron-selective marker protein tetanus toxin.

Approximately one-fifth of the plated cells began to extend processes within 12 h of attachment. Although this process extension was influenced by plating density and by culture glial content, the addition of skeletal muscle extract independently augmented both the rate and extent of cell process outgrowth (Fig. 1). When measured 3 d after the addition of saturating concentrations of muscle extract, process density increased from 0.36 ± 0.01 processes per cell in untreated cultures, to 1.51 ± 0.05 processes per cell in extract-treated cultures. The percentage of tetanus toxin–labeled cells extending neurites also increased relative to total tetanus toxin–labeled cells, from 20 ± 5% without treatment, to 60 ± 5% after muscle extract addition. Comparable increases in cell process length were observed, from an average of 50 ± 1 μm in controls, to 163 ± 5 μm after 3 d in extract-supplemented cultures. These effects were not due to the binding of a muscle extract component to the polylysine substratum, since a 16-h extract–substratum incubation before cell addition had no effect on neural process outgrowth, measured 3 d after plating. Further, muscle extract addition neither altered total or tetanus toxin–labeled cell survival, as determined by cell counting and culture DNA content, nor enhanced general cell metabolism. Culture acetylcholinesterase activity increased only 15 ± 5% relative to control values after 4 d in 200 μg extract protein/ml culture medium, while extract similarly produced an insignificant 10 ± 6% increase in total lactate dehydrogenase activity compared to control cultures (even though lactate dehydrogenase activity increased in both extract and control cultures by threefold during the 4-d test period).

Changes in cell morphology occurred more rapidly than did increases in cholinergic activity. Maximal process density was achieved after 3–5 d in culture, while ACh synthesis and accumulation continued to increase in vitro for at least 3 wk. When measured 4 d after the initial addition, the rate of ACh synthesis and accumulation in these cells typically increased threefold, from 2.52 ± 0.24 pmol [^{3}H]ACh synthesized/μg DNA per hour in control cultures, to 7.98 ± 0.34 pmol [^{3}H]-ACh/μg DNA per hour in muscle extract–treated neurons. Factor activities, however, varied somewhat between individual extract preparations. The concentration of muscle extract required to give a half-maximal increase in ACh synthesis in spinal neuron cultures (50 μg protein/ml culture medium) was only one-fourth of that necessary for the same change in either process density (200 μg protein/ml culture medium) or process length. These findings suggest that factors regulating process density and ACh synthesis in ventral spinal cord may be different. Three biological criteria are described below providing further evidence for this hypothesis.

Tissue Specificity

Fig. 2 provides evidence that extracts of different newborn rat tissues differentially augment neurite outgrowth and cho-

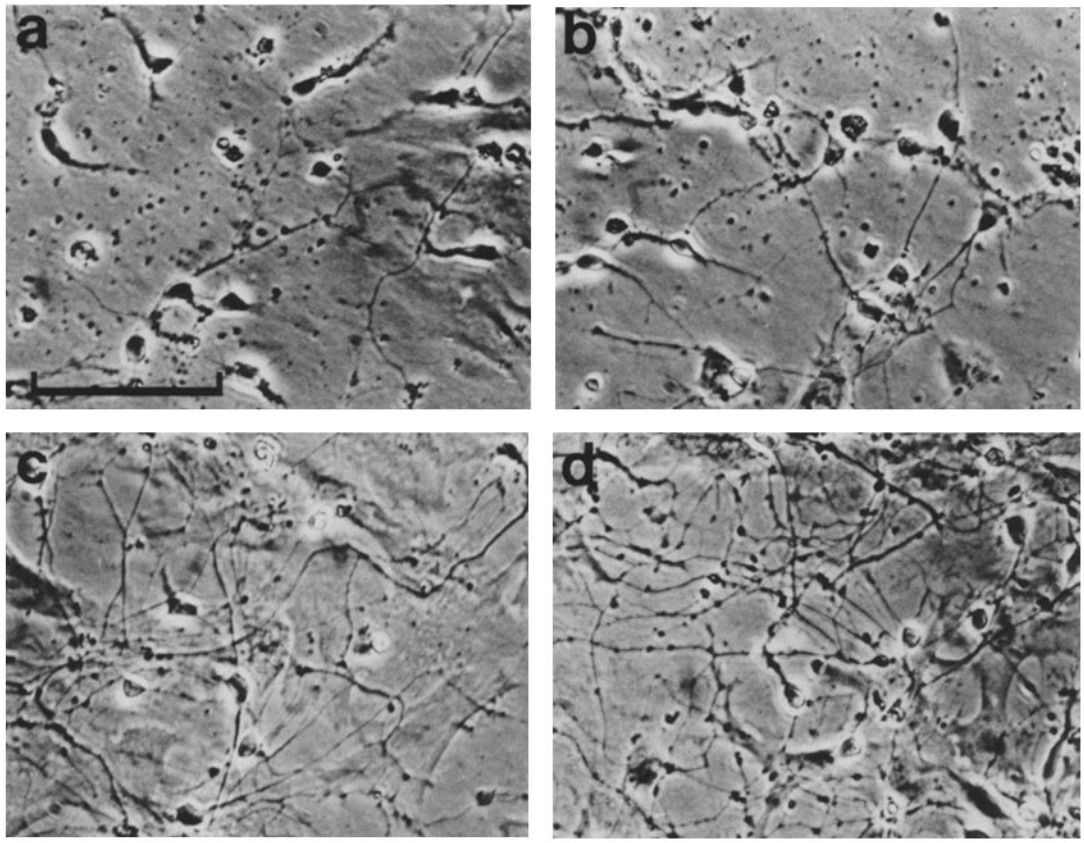

FIGURE 1 Effect of addition (three consecutive days) of muscle extract on neurite extension in cultured ventral spinal cord cells. Control cultures (*a*) were maintained in normal growth medium, while experimental cultures were further treated with 25 (*b*), 100 (*c*), or 400 (*d*) μg muscle extract protein/ml of culture medium. Bar, 100 μm. × 250.

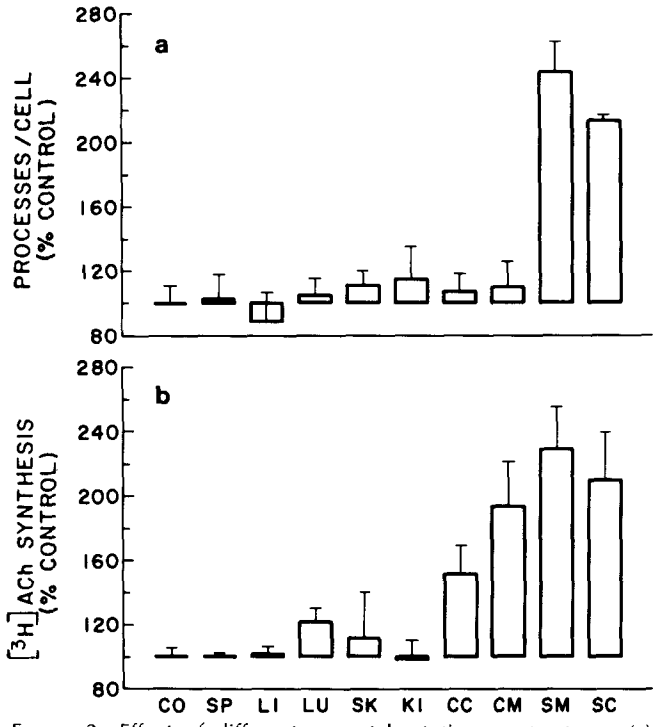

FIGURE 2 Effect of different neonatal rat tissue extracts on (*a*) neurite density and (*b*) [^{3}H]ACh synthesis and accumulation in cultured ventral cord neurons. Extracts of spleen (*SP*), liver (*LI*), lung (*LU*), skin (*SK*), kidney (*KI*), cerebral cortex (*CC*), cardiac muscle (*CM*), skeletal muscle (*SM*), or spinal cord (*SC*) were individually added in equal 200 μg extract protein/ml culture medium aliquots to triplicate cultures. In each experiment, cultures were assayed 3 d later for neurite outgrowth. Additionally, triplicate sister cultures were tested after 4 d for the amount of [^{3}H]ACh synthesized/μg DNA per hour, relative to control (*CO*) values. These results are the means of four experiments ± SDs. The value for mean control morphologic activity was 0.47 processes per cell with an interexperiment SD of 0.31, and an intraexperiment SD of 0.14. The ACh synthesis mean value was 2.45 pmol [^{3}H]ACh/μg DNA per hour with an SD between experiments of 0.89, and an SD of 0.07 within an experiment.

linergic activity. 3 d after the addition of 200 μg protein/ml extract aliquots to ventral cord cultures, only skeletal muscle and spinal cord homogenates significantly stimulated cell process outgrowth. In sister cultures, cholinergic activity was not only increased by skeletal muscle and spinal cord extracts, but also by homogenates of other tissues with dense cholinergic innervation, such as cardiac muscle and cerebral cortex. Even at higher concentrations, these latter two extracts had little effect on process outgrowth, suggesting that an increase in cholinergic activity is apparent with two tissue extracts that do not promote morphologic differentiation. Neurite outgrowth in heart and brain extracts was not actively prevented by the production of a specific inhibitory factor, because mixing just-saturating activity levels of skeletal muscle extract with activity-saturating levels of cardiac muscle extract prior to cell addition did not block process outgrowth.

Age Dependence

The cholinergic and morphologic activities of muscle ex-

tract also display different age dependencies. The levels of morphologically active factor obtained from muscle extracts of different aged rats decreased with the increasing age of those rats (Fig. 3). When expressed per milligram of muscle protein (Fig. 3*A*), there was an 80% loss in this activity during the first 3 wk after birth. Morphologic activity levels obtained from the combination of newborn and adult muscle extracts suggested that this loss was the result of a decrease in activating factor concentration rather than an increased amount of an inhibitory substance. In contrast, cholineric-stimulating activity in older animal muscle was at least slightly greater than that found in newborn tissues. When expressed as total activity per limb, a 400% increase in cholinergic activity was observed with maturation (Fig. 3*B*).

Response to Denervation

To determine whether the age-dependent changes in morphologic and cholinergic factor activities could be reversed by axotomy, we examined the results of physical (sciatic nerve ligation) or chemical (repeated hindlimb injection with α-BTX) denervation of adult rat hindlimb muscle on in vitro tests of these two indices of neuron maturation. Both of these methods produced a loss of distal hindlimb function within 1 d of treatment, and a 35-fold increase in muscle extrajunctional ACh receptor levels (measured by ^{125}I-α-BTX binding) at the time of muscle homogenization.

After denervation, the low morphologic-stimulating activity of adult muscle extract increased almost to newborn activity levels (Fig. 4). With combined physical and chemical denervation, there was no further increase in morphologic activity. In contrast, the high adult cholinergic-stimulating activity decreased after either ligation of α-BTX-induced denervation. None of these responses were a result of the brief exposure of all gastrocnemius muscles to ^{125}I-α-BTX. There was no difference in either parameter of development when muscle extract prepared from control animals 6 h after ^{125}I-α-BTX-injection was compared to muscle extract prepared from non-injected, age-matched controls.

Differential Effects of Denaturation

The morphologic- and cholinergic-enhancing activities of muscle extract could be separated by their biochemical as well as biologic properties. For example, they differed with respect to their rates of heat inactivation. Although both factor activities were destroyed by heating to 60°C for 1 h, the morphologic factor lost activity more rapidly than did the cholinergic factor (Fig. 5*A*). At 60°C, this rate of morphologic activity loss was nonlinear, and appeared to follow an exponential curve with half-maximal activity decay at 8.8 min. At just-saturating activity concentrations, the rate of loss in cholinergic activity with heating was linear, with half of its activity being lost in 29 min.

Morphologic- and cholinergic-stimulating activities also differed in their rates of inactivation by trypsin. In the presence of 0.1 mg/ml trypsin at 37°C, loss of morphologic activity followed a linear time course, with a $t_{1/2}$ of inactivation observed at 48 min (Fig. 5*B*). Loss of cholinergic activity was nonlinear with time, however, and could be modeled by two

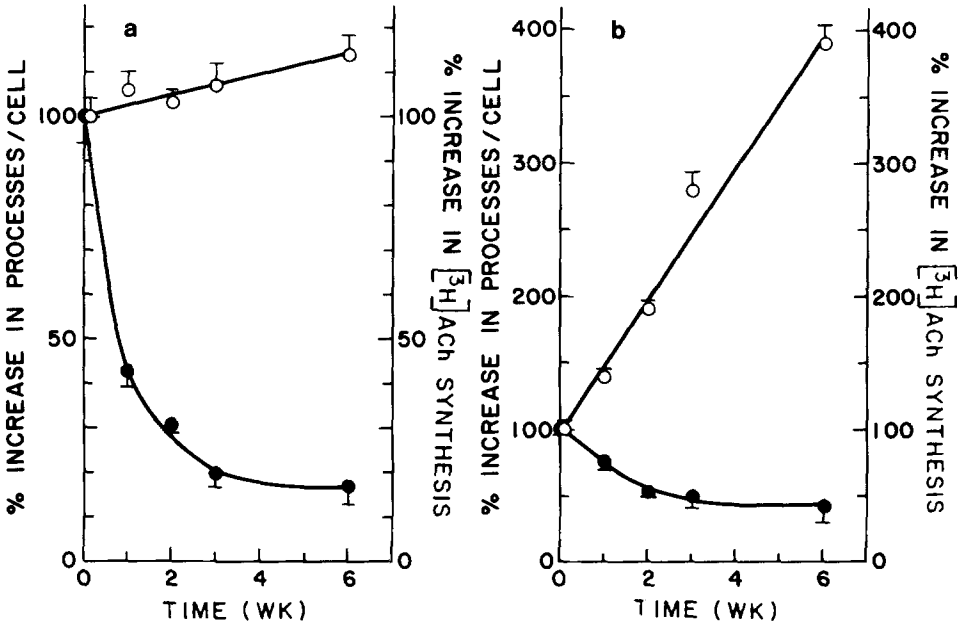

FIGURE 3 Comparison of rat limb muscle development to muscle extract-induced changes in neurite extension and cholinergic development in vitro. Extracts of rat hindlimb muscle, obtained at ages ranging from birth to 6 wk, were added to ventral spinal cord cultures. Although multiple extract protein concentrations were tested, this figure depicts the results (for each rat age) of addition of single subsaturating 100 μg muscle extract protein/ml culture medium concentration, on process density (●), measured after 3 d, or on [^{3}H]ACh synthesis (○), assayed 4 d after initial extract addition. For each experiment, data from triplicate cultures are expressed as a percent of newborn extract activity (100%), relative to control culture values (0%). The results are normalized per mg muscle extract protein in *a*, or per limb (*b*), and are the mean of four experiments (12 cultures per point) ± SEM. Actual mean values for process outgrowth are 0.41 ± 0.19/0.09 for control and 1.63 ± 0.30/0.17 for newborn extract-treated cultures, where interexperiment SD is expressed above intraexperiment SD. Comparable mean values for cholinergic activity were 2.59 ± 0.66/0.09 pmol [^{3}H]ACh/μg DNA per hour, and 5.62 ± 0.74/0.16 pmol [^{3}H]ACh/μg DNA per hour for control and newborn extract-treated cultures, respectively.

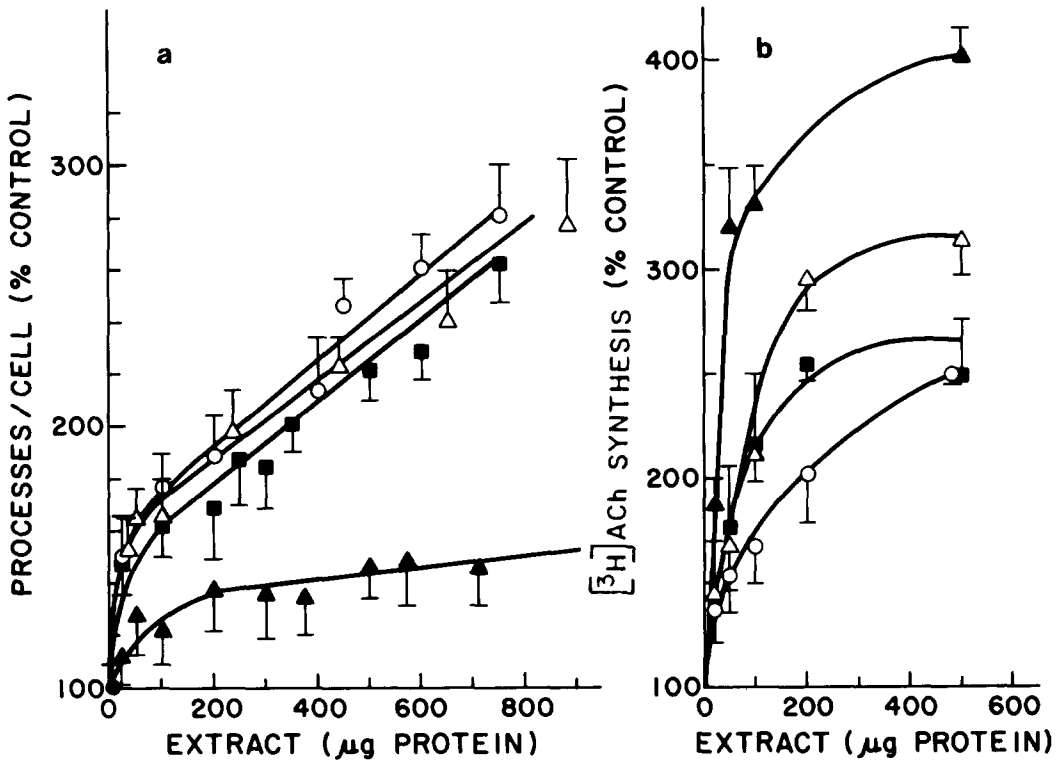

FIGURE 4 Denervation-induced changes in process density and cholinergic activity. Muscle extracts were prepared 5 d after distal hindlimb denervation, either by cutting the sciatic nerve (■), by daily injections of 2 μg α-BTX per limb per day (△), by a combination of these two methods (○), or after sham operation and daily saline injections (▲). These extracts were added to dissociated ventral spinal cord neurons. Concentration-dependent effects of the extracts were assayed in triplicate cultures 3 d after initial addition for differences in (*a*) the P/C ratio or in (*b*) [^{3}H]ACh synthesis, measured in triplicate sister cultures after 4 d. Results are expressed as a percent of untreated culture values, and are the mean of three experiments (9 cultures per point) ± SEM. Control mean values for morphologic and cholinergic activities were 0.63 ± 0.27/0.10 processes per cell, and 2.21 ± 0.74/0.14 pmol [^{3}H]ACh/μg DNA per hour, respectively (mean control value + interexperiment SD/intraexperiment SD).

lines that provided an activity loss $t_{1/2}1$ at 16 min and a $t_{1/2}2$ at 23 min. This effect was blocked by pretreatment of the enzyme with 1 mg/ml soybean trypsin inhibitor. Neither extract incubation at 37°C, nor addition of soybean trypsin inhibitor to extract, had any effect on the activities induced by muscle extract.

The cholinergic and morphologic activities exhibited different sensitivities to pH (Fig. 5*C*). The morphologic activity was stable at acid pH, but was destroyed above pH 7.5. Conversely, cholinergic-stimulating activity was stable between pH 6 and 9, with only partial activity being lost below pH 6. Total cholinergic activity was destroyed, however, at pH greater than 9.

Both activities were stable to a 6-h incubation with either DNAase I or RNAase A. Further, neither activity was lost when incubated with disulfide reducing agents like 1–5 mM dithiothreitol (DTT) or 2-mercaptoethanol. In fact, when tested at subsaturating activity concentrations, a 12-h incubation with 2 mM DTT increased total cholinergic activity in muscle extract by 2.2-fold. This increased activity was preserved after subsequent incubation with 2–5 mM *N*-ethylmaleimide (NEM). Because DTT and NEM were dialyzed from the extract before addition to cultures, these agents did not directly effect the survival or differentiation of the cells.

Precipitation by Salts

Both morphologic- and cholinergic-stimulating activities of muscle extract could be reversibly precipitated by the step-gradient addition of ammonium sulphate. Most morphologic factor activity and cholinergic factor activity was precipitated and recovered in the 47–62% saturated ammonium sulfate fraction of muscle extract. When morphologic- and cholinergic-stimulating activities were also tested for susceptibility to denaturation at high salt concentrations, both were stable to 2 M NaCl, but were labile to treatment with 6 M guanidine hydrochloride.

Separation by Size

As a direct test for the presence of multiple neurotrophic species, the active 46–62% ammonium sulfate sample was fractionated by Sephacryl S-200 gel filtration chromatography (Fig. 6). In the presence of the reducing agents DTT and NEM, a single morphologically active species was found with an M_r of 33,000–37,000 (Fig. 6*C*). In contrast, two peaks of cholinergic activity were found, with M_r's of 55,000–65,000 and 14,000–17,000 (Fig. 6*D*). If DTT and NEM were not added to the extract before S-200 sizing chromatography, morphologic activity eluted as several activity peaks with M_r's averaging greater than 200,000, 125,000, 100,000, 67,000, and 33,000 (Fig. 6*A*). Omission of DTT and NEM also increased the number of cholinergic activity peaks. These species eluted with M_r's of greater than 200,000, 75,000–85,000, 55,000–65,000, 40,000–45,000, and 14,000–17,000 (Fig. 6*B*).

Under both nonreducing and reducing conditions, morpho-

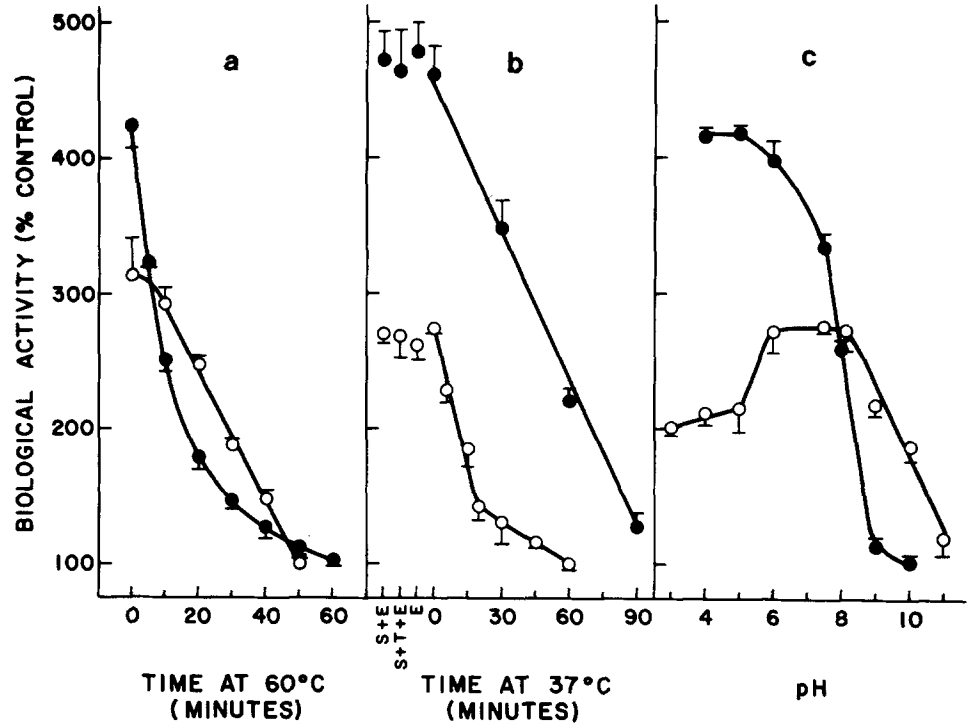

FIGURE 5 The effects of heat, trypsin, and extremes of pH on the loss of muscle extract morphologic factor activity and cholinergic factor activity. Muscle extracts were (*a*) heated to 60°C for 5–60 min, (*b*) incubated for 90 min at 37°C (*E*) with either soybean trypsin inhibitor (*E+S*) or trypsin inactivated by 1 mg/ml soybean trypsin inhibitor (*S+T+E*), treated with active 0.1 mg/ml trypsin for 5–90 min, or (c) dialyzed for 6 h against different buffered solutions of pH 3–11 prior to addition to cultures. Prior to use, trypsin and soybean trypsin inhibitor (Sigma Chemical Co.), were both assayed for proteolytic activity with the protease substrates toluene-sulphonyl-L-arginine methyl ester and benzoyl-L-arginine ethyl ester. In each experiment, samples were assayed in triplicate ventral spinal cord cultures for either neurite outgrowth (●), or ACh synthesis (○). Reported results of heat and trypsin denaturation of muscle extract are the mean of three experiments (9 cultures per point) ± SEM, whereas assays of extract pH stability are the mean of two experiments (6 cultures per point) ± SEM. Control values for morphologic activity were 0.34 ± 0.02 processes/cell for *a*, 0.58 ± 0.23/0.09 processes for *b*, and 0.35 ± 0.06/0.04 for *c*. Control values for cholinergic activity were 2.42 ± 0.57/0.20 pmol [^{3}H]ACh/μg DNA per hour for *a*, 2.13 ± 0.88/0.14 pmol [^{3}H]ACh/μg DNA per hour for *b*, and 2.55 ± 1.22/0.17 pmol [^{3}H]ACh/μg DNA per hour for *c*. Results refer to the mean control values ± interexperiment SD or range/intraexperiment SD. This method for normalizing biological activity provides consistent quantitative comparisons, both between preparations of muscle extract, and between experiments.

logic activity was quantitatively recovered from the gel filtration column (Table I). In contrast, most of the cholinergic stimulating activity added to the S-200 column was not recovered (Table II). Prior to reduction, total recovered activity accounted for 30% of that originally added to the column. Addition of DTT and NEM, even though increasing the recovery of activity in the 55,000–65,000 and 14,000–17,000-mol-wt peaks, decreased total recovered cholinergic-stimulating activity to only 20% of that added to the column.

Since much ACh synthesis–inducing activity was lost by passage through the S-200 column, a similar ammonium sulfate fraction was added to an LKB ACA-202 sizing column. On this column (which we empirically found to resolve protein molecular weight standards in the range of 25,000–2,000), morphologic-stimulating activity eluted in a single peak at the column void volume, while four cholinergic factor activity peaks were identified. The first peak of activity eluted at the void volume (presumably corresponding to the 55,000-mol-wt factor), while other cholinergic peaks eluted with M_r's of 16,000–18,000, 5,000–7,000, and 2,000. Total cholinergic activity recovery was ~77% (Table III). The lowest molecular weight cholinergically active peak has since been resolved by Bio-Rad P-6 and P-2 gel chromatography to an approximate molecular weight of 1,200–1,500 (40).

Separation by Preparative IEF

Morphologic and cholinergic activities also had different apparent isoelectric points (Fig. 7). After electrofocusing of the 46–62% extract ammonium sulfate fraction, morphologic activity was usually found to focus as a single band at pH 4.8, while the cholinergic activity focused as multiple bands at pH 9.2, 7.7, 5.2, and 4.1. On occasion, a very small amount of morphologic activity was also found at a pH range corresponding to a pI of 7.5–8.

The M_r's of the four cholinergically active IEF fractions were determined by gel filtration on S-200 or ACA-202 gels. Samples were pooled by pI activity peak and concentrated by lyophilization prior to addition to a sizing column. Cholinergic activity initially recovered from the basic fraction (pI = 9.2), eluted from an ACA-202 column at an M_r of 14,000–18,000 (Fig. 8*A*). The fraction corresponding to a pI of 7.7 contained an activity peak with an M_r of 55,000–65,000, and when chromatographed on ACA-202, also produced a second activity peak with an M_r of 5,000–8,000 (Fig. 8*B*). The pH 5.2 and 4.1 fractions contained only low molecular weight (<5,000) cholinergically active species. After S-200 sizing chromatography, the morphologically active isoelectric frac-

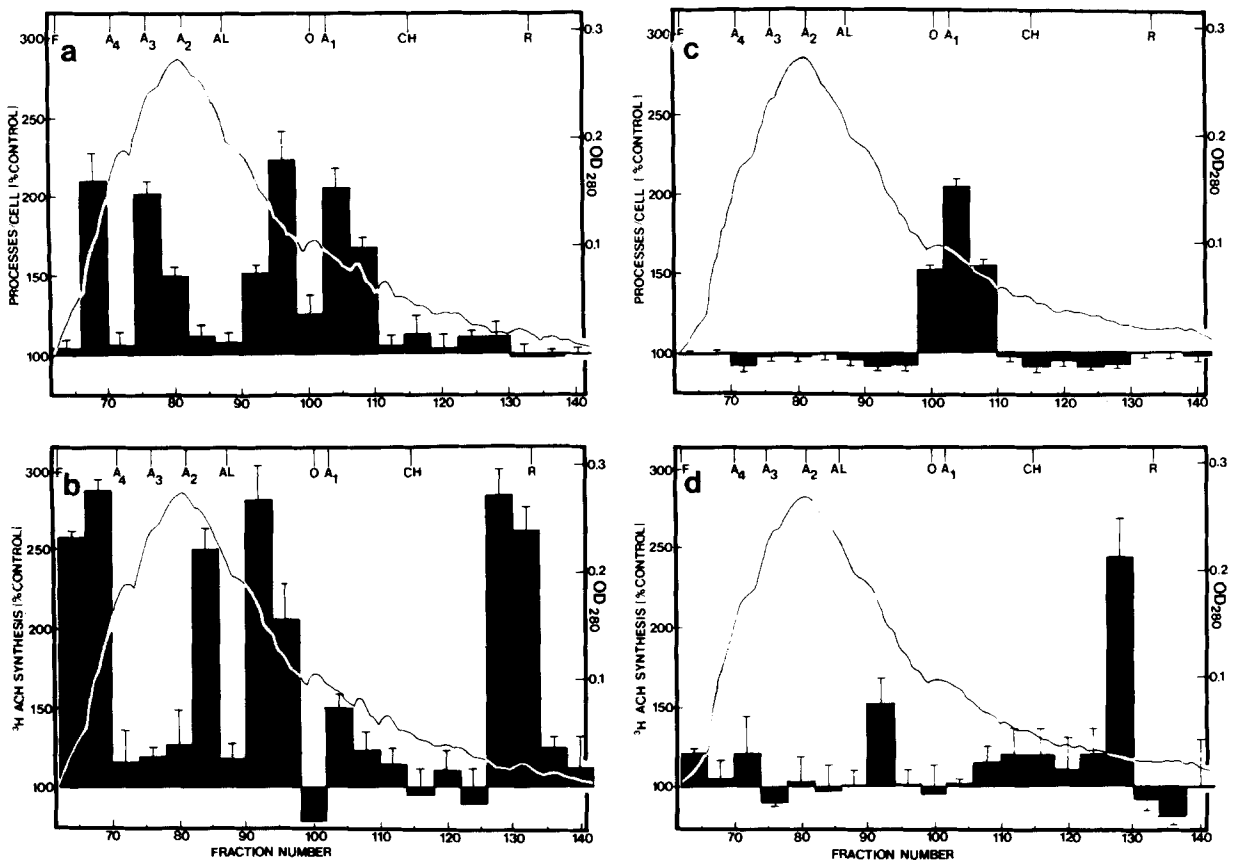

FIGURE 6 Separation of morphologic and cholinergic factor activities by Sephacryl S-200 molecular sieving chromatography, with or without prior reduction and alkylation. A 2.0-ml aliquot of a 46–62% ammonium sulfate-precipitated fraction of muscle extract was added to a Sephacryl S-200 sizing column, and each group of four consecutively eluted 2.5-ml fractions was pooled and added to triplicate cultures for bioassay of neuron process outgrowth (*a*), and ACh synthesis (*b*). After reduction and alkylation, an identical extract sample was fractionated on the same column, and assayed in triplicate cultures for extract fraction-induced changes in neuron process density (c) or ACh synthetic activity (*d*). Comparison of the absorbance at 280 nm of each fraction (—) with its corresponding pooled fraction biological activity is depicted for both column elutions. Each activity bar represents the mean of two different assays for these fractions (six cultures total) ± SD. Control values for morphologic activity were 0.78 ± 0.21/0.05 processes per cell for unreduced fraction experiments, and 0.88 ± 0.28/0.06 for reduced fraction tests, where values refer to the mean + interexperiment range/ mean intraexperiment SD. Control values for ACh synthesis were 1.64 ± 0.64/0.13 pmol [^{3}H]ACh/μg DNA per hour for assays with unreduced fractions, and 2.87 ± 1.19/0.31 pmol [^{3}H]ACh/μg DNA per hour. Molecular weight standards shown are ferritin (*F*), subunits of aldolase (*A1–4*), bovine serum albumin (*AL*), ovalbumin (*O*), chymotrypsinogen (*CH*), and Ribonuclease A (*R*).

TABLE I. *Morphologic Activity: 1,140 Units Added to Column*

	Before reduction		After reduction and alkylation	
Size	Specific activity	Total activity	Specific activity	Total activity
mol wt	*U/mg protein*	*U*	*U/mg protein*	*U*
250,000	203 ± 16	105 ± 6	0	0
115,000–125,000	32 ± 5	70 ± 5	0	0
63,000–67,000	1,415 ± 150	455 ± 25	0	0
33,000–36,000	1,360 ± 125	313 ± 13	4,457 ± 305	1,425 ± 72

Recovery and fractionation of factor activities by sizing chromatography. Results are primarily derived from the column fractionation experiment depicted in Fig. 3. Morphologic-specific activity from Sephacryl S-200 columns were assessed in peak activity fractions by regression analysis of activity/concentration curves. Total activities were determined from all fractions contributing to a peak. The data are accompanied by the range for each value, as delineated from the regression curves. Average total recovery (92% before reduction and 124% after reduction and alkylation) was obtained from five different S-200 column fractionation experiments.

tion yielded a single activity peak with an M_r of 34,000–38,000 (Fig. 8*C*).

Additivity of Cholinergic Activities

When tested together on cultured cells, the cholinergically active ammonium sulfate fractions produced additive increases in cholinergic activity. To test whether the four cholinergic gel filtration fractions might also provide additive stimulations of ACh synthesis, active species were first partially purified by ammonium sulfate fractionation, ACA-202 gel chromatography, and preparative IEF. As previously observed (Figs. 6*D* and 8*B*), the 55,000-mol-wt factor provided a smaller maximal stimulation at saturating activity than did

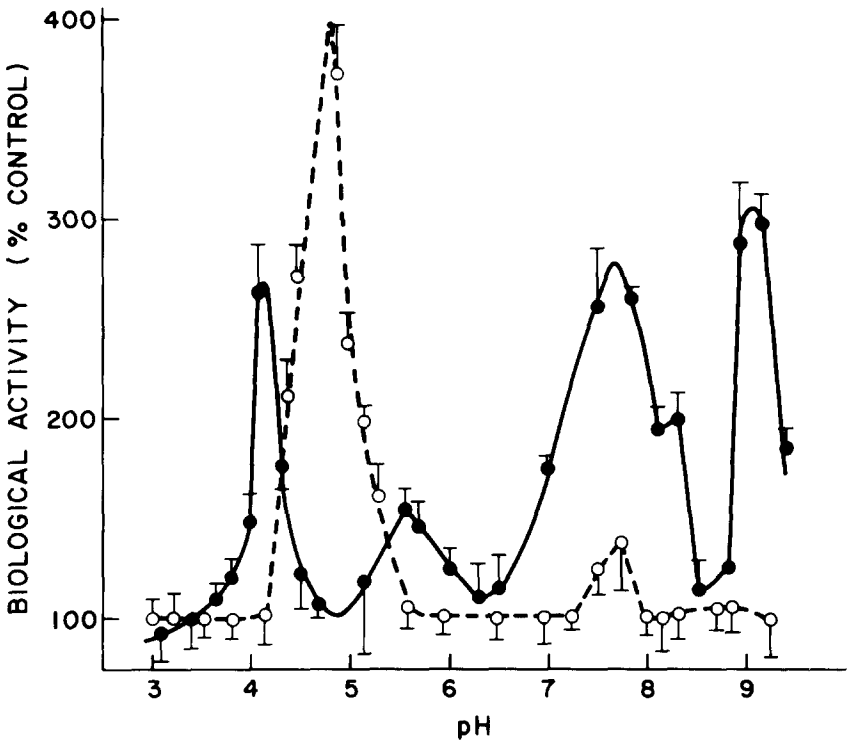

FIGURE 7 Biological activity profile from preparative IEF of a 46–62% ammonium sulfate fraction of muscle extract. Morphologic factor activity (---) and cholinergic factor activity (—) obtained from triplicate culture assays of IEF fractions, depicted as a function of the pH of each fraction. Results are the mean of three experiments (9 cultures per point) ± SEM. Mean control values for morphologic and cholinergic activity were 0.65 ± 0.18/0.03 processes/cell and 2.70 ± 1.06/0.07 pmol [^{3}H]ACh/μg DNA per hour, where interexperiment SD is expressed above intraexperiment SD.

TABLE II. *Cholinergic Activity: 1,880 Units Added to Column*

	Before reduction		After reduction and alkylation	
Size	Specific activity	Total activity	Specific activity	Total activity
mol wt	*U/mg protein*	*U*	*U/mg protein*	*U*
250,000	651 ± 63	214 ± 20	0	0
75,000–85,000	86 ± 42	117 ± 57	0	0
55,000–65,000	96 ± 10	67 ± 7	100 ± 11	62 ± 7
14,000–17,000	4,250 ± 618	136 ± 26	7,143 ± 1,587	343 ± 76

Recovery and fractionation of factor activities by sizing chromatography. Results are primarily derived from the column fractionation experiment depicted in Fig. 3. Cholinergic-specific activity from Sephacryl S-200 columns were assessed in peak activity fractions by regression analysis of activity/concentration curves. Total activities were determined from all fractions contributing to a peak. The data are accompanied by the range for each value, as delineated from the regression curves. Average total recovery (32% before reduction and 21% after reduction and alkylation) was obtained from five different S-200 column fractionation experiments.

TABLE III. *Cholingic Activity: 4,400 Units Added to Column*

	After reduction and alkylation	
Size	Specific activity	Total activity
mol wt	*U/mg protein*	*U*
25,000	145 ± 21	200 ± 21
14,000–17,000	1,000 ± 46	1,580 ± 88
5,000–7,000	400 ± 15	194 ± 31
2,000	30,000	1,394 ± 13

Recovery and fractionation of factor activities by sizing chromatography. Results are primarily derived from the column fractionation experiment depicted in Fig. 3. Cholinergic-specific activity from LKB ACA-202 columns were assessed in peak activity fractions by regression analysis of activity/concentration curves. Total activities were determined from all fractions contributing to a peak. The data are accompanied by the range for each value, as delineated from the regression curves. Average total recovery (77%) was obtained from three different ACA-202 column fractionation experiments.

any of the other three smaller species (Fig. 9*A*). Moreover, even though these three lower molecular weight fractions had comparable saturating activity levels, their maximal activities were significantly less than that of the crude high speed supernatant.

When the 55,000-mol-wt factor was added to any of the lower molecular weight species, the combined cholinergic activity was equal to that found in the crude homogenate (Fig. 9*B*). However, when the lower molecular weight factors were mixed with one another and added to cultures, no such additivity of cholinergic factor stimulation was observed.

Determination of Glycoprotein Nature

Both the morphologic- and cholinergic-stimulating activities of skeletal muscle extract appeared to be produced by glycoproteins. Each activity was completely destroyed by a 20-min, 37°C incubation with 5 mM sodium periodate. This effect was not the result of direct damage to cells, since periodate was removed by dialysis prior to addition to cultures. However, because periodate may have denatured muscle extract activities by some other action than the cis-diol cleavage of sugar groups, morphologic and cholinergic factor binding to lectin columns was also assessed (Table IV). Muscle extract fractions, each partially purified by ammonium sulfate fractionation, sizing chromatography, and preparative IEF, were added to lectin columns. Columns were then washed with 10–20 ml of a PBS carrier solution containing calcium, magnesium, and/or manganese ions, as needed, to stabilize

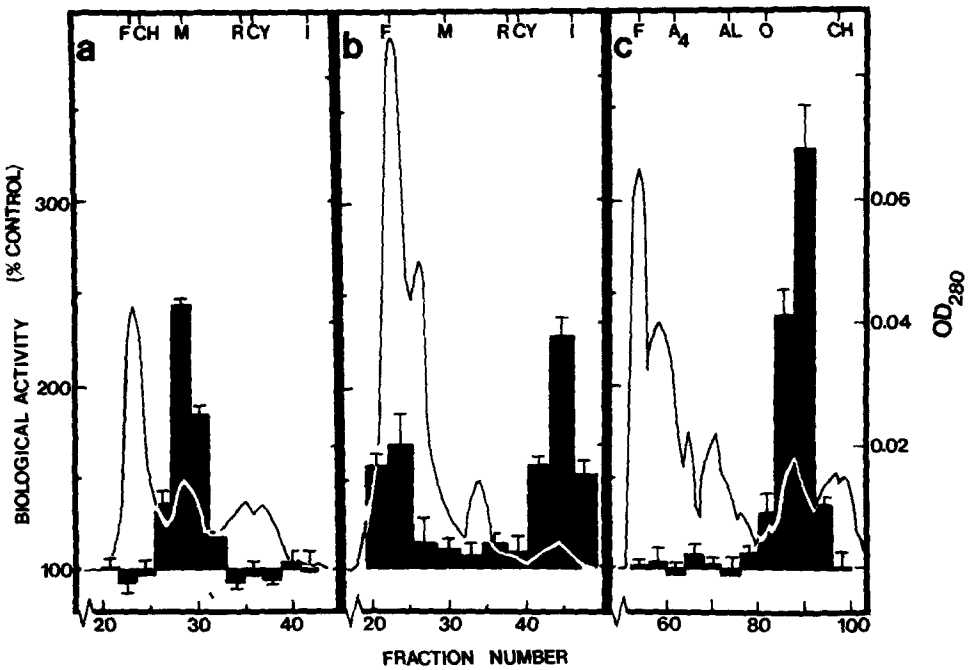

FIGURE 8 Sizing chromatography of biologically active IEF fractions. ACA-202 molecular sieving elution profile of the cholinergic factor activities associated with pIs of (*a*) 9.0–9.5, and (*b*) 7.0–8.4. (*c*) Sephacryl S-200 chromatography fractionation of morphologic factor activity with pI of 4.5–5.1. Optical density at 280 nm is depicted, by elution fraction number, with the relevant biological activity obtained in each experiment by bioassay of quadruplicate cultures. Activity is depicted by bars, representing the increase above control values for a mean of two experiments (8 cultures per group) ± SD. Control values for process outgrowth and ACh synthesis were 0.67 ± 0.20/0.05 processes/cell, and 2.69 ± 0.41/0.14 pmol [^{3}H]ACh/μg DNA per hour, where numbers refer to mean control activity levels ± range of values between experiments/mean SD of values within individual experiments. Sample volumes, fraction processing, and molecular weight standards shown are as depicted in Fig. 6.

lectin–sugar binding. Glycoproteins were eluted with buffer containing an excess of the relevant specific lectin-binding sugars.

The 34,000–37,000-mol-wt morphologically active factor bound selectively to Lens culinaris, concanavalin A, and Lotus tetrogonobolus lectins, and was eluted by α-methyl-mannoside (Table IV). Although these lectins all have specificity for α-mannosyl (4, 34) residues, there was no loss of morphologic factor activity when muscle extract fractions were incubated for 1 h with sepharose-linked β-glucosidase or α-mannosidase.

The cholinergically active 14,000–17,000- and 55,000–60,000-mol-wt factors did not bind to concanavalin A or Lens lectin columns, but did bind to a sialic acid–specific lectin, eluting with *N*-acetylneuraminic acid (Table IV). In addition, incubation of dialyzed muscle extract at 37°C with 0.1 U of sepharose-linked neuraminidase greatly reduced its ability to stimulate ACh synthesis ($t_{1/2}$ = 7.5 min), but had no effect on morphologic factor activity. The lowest molecular weight cholinergically active species did not bind to columns specific for either of these sugar types, and was not destroyed by neuraminidase.

Factor Purification

We have partially purified several of the biologically active muscle extract–derived species. Beginning with ~100 ml of crude rat muscle homogenate, ammonium sulfate fractionation, sizing gel chromatography, preparative IEF, and lectin column chromatography were sequentially performed to increase the purity of some of the biologically active factors. By these methods, the morphologically active protein has been estimated to be purified ~75,000-fold with more than a 35% yield (Table V).

The analysis of purification for the 14,000–17,000- and 55,000–60,000-mol-wt cholinergic factors, however, has posed problems. This difficulty was due to both the presence of multiple cholinergically active species that contribute to the total biological activity, and to the continual problem of loss of the low molecular weight factors from less purified fractions. At present, the 55,000–60,000-mol-wt factor is more than 100-fold more active, per mg protein, than is the crude muscle homogenate. There was a 3% recovery of cholinergically active material in this molecular weight range relative to total activity (Table V). The 14,000–17,000-mol-wt factor has, by the same scheme, been purified almost 3,700-fold with a final yield of 18%.

DISCUSSION

Our in vitro data indicate that skeletal muscle–derived trophic activities separately enhance spinal cord neurite outgrowth and ACh synthesis. The biological evidence for this conclusion is fourfold: (*a*) although both morphologic- and cholinergic-enhancing activities are present in extracts of skeletal muscle and spinal cord, cholinergic-stimulating activity is also selectively found in two tissues without morphologic activity (e.g., cardiac muscle and cerebral cortex); (*b*) morphologic and cholinergic factor activities from muscle differ with respect to time course of action and concentration dependence; (*c*) morphologic-enhancing activity declines significantly as a function of age, with skeletal muscle extracts from 3-wk-old rats possessing only 20% of the activity of newborn rat skeletal muscle, whereas cholinergic-enhancing activity in muscle in-

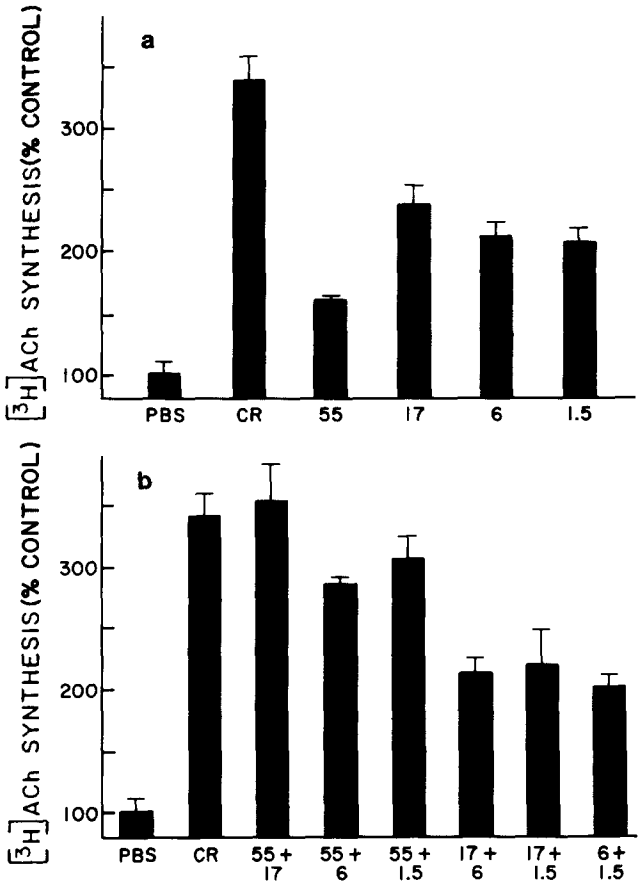

FIGURE 9 Additivity of cholinergic factor activities. (a) Maximal levels of factor-induced ACh synthesis (depicted here by solid bars) were determined by preparation of activity/concentration curves for each of the size-separable cholinergically active factors. (*b*) Comparison of the maximal level of cholinergic-stimulating activity from crude muscle extract with levels obtained by adding together, at saturating concentrations, the partially purified cholinergic factor activities (indicated in the legend). Each bar represents the bioassay of triplicate cultures for each of two experiments ± SD. Control values for cholinergic activity was 2.56 ± 0.39/0.24 pmol [^{3}H]ACh/ μg DNA per hour, with the range of interexperiment values expressed above the mean SD for individual experiments. Phosphate-buffered saline carrier (PBS), high speed supernatant of muscle extract (*CR*), and 55,000- (*55*), 17,000- (*17*), 6,000- (*6*), and 1,500- (*1.5*) mol-wt cholinergically active factors.

creases over the same time period; (*d*) 5 d after denervation, extracts from adult rat skeletal muscle demonstrate a fourfold increase in morphologic activity (per milligram of muscle tissue), and a twofold decrease in cholinergic-enhancing activity.

These changes in morphologic activity with age and denervation parallel the in vivo loss of rodent polyneuronal innervation (6) and enhancement of neurite sprouting (5, 7), suggesting decreases and increases respectively in the availability of muscle-derived trophic agent (46, 55). Likewise, the age-dependent increase in cholinergic-enhancing activity tested in vitro independently follows in vivo motoneuron neurotransmitter differentiation (8, 30), while the loss in cholinergic-stimulating activity after denervation correlates with reversible decreases in neurotransmitter synthesis and transport in vivo after nerve crush or ligation (26, 48). The separate developmental time courses for these effects in vitro bear similarities to in vivo data on the time course of retinal neuron embryonic development (56) and post-axotomy neuron differentiation (26, 47, 67). Such results suggest the existence of two or more trophic activities, although they alone do not rule out the separate actions of a single trophic substance (63).

The morphologic- and cholinergic-enhancing activities in crude muscle extract have different sensitivities to heat, protease digestion, and changes of pH (Table VI). While these biochemical tests also do not define the existence of separate factors, direct evidence for factor separation comes from the chromatographic properties of these activities. Under reducing conditions, gel filtration provided a single peak of morphologic activity, and several peaks of cholinergic activity (each devoid of endogenous ACh synthetic activity). Similar results have been obtained when muscle extract was fractionated by IEF and ion exchange chromatography, providing several fractions with cholinergic activity and a single major fraction with morphologic activity. Finally, lectin-affinity chromatography separated the morphologic protein, which appears to be an α-mannosyl residue–containing glycopeptide (4, 34), from several cholinergic species. These cholinergic species could themselves be subfractionated into those which contained essential sialic acid residues and those without apparent sugar moieties. Using the protocol defined in this paper, we have achieved a 75,000-fold purification of the 35,000-mol-wt morphologic factor, which migrates as a single

TABLE IV. *Lectin Binding Specificity of Factor Activities*

	Lectin	Eluting sugar	Activity in wash	Activity in eluate
			% *total added* ± *SD*	% *total added* ± *SD*
A.	Triticum vulgaris	*n*-Acetylglucosamine	103 ± 12	8 ± 16
	Arachis hypogaea	Galactose	104 ± 20	3 ± 18
	Lotus tetragonobolus	L-Fucose	99 ± 33	53 ± 32
	Concanavalin A	*a*-Methylmannopyranoside	24 ± 15	90 ± 22
	Lens culinaris	*a*-Methylmannopyranoside	27 ± 21	105 ± 20
	Limulin	*N*-Acetylneuraminic acid	86 ± 15	15 ± 20
B.	Lens culinaris	*a*-Methylmannopyranoside	— (*a*)	—
	Concanavalin A	*a*-Methylmannopyranoside	105 ± 17 (*b*)	4 ± 20
	Limulin	*N*-Acetylneuraminic acid	90 ± 15* (*a*)	12 ± 17
			86 ± 30* (*b*)	5 ± 20
			4 ± 6 (*a*)	98 ± 15
			34 ± 12 (*b*)	54 ± 16

Binding and recovery of morphologic factor activity (*A*) and cholinergic factor activity (*B*) from various immobilized lectins. Values presented are the percentage of total activity applied to each lectin column and recovered in the buffer wash, or in the displacement sugar eluate.
* Data for (a) 55,000- and (b) 17,000-mol-wt factors.

TABLE V. *Purification of Factor Activities*

Treatment	Specific activity	Purification	Total activity	Yield
	U/mg protein		*U*	%
Morphologic (35,000 mol wt) factor activity				
Tissue homogenate	<1	—	—	—
100,000 *g* supernatant*	10	1	5,100	100
46–60% $(NH_4)_2SO_4$ fraction	122	12	4,670	92
S-200 sizing chromatography	4,456	446	3,910	77
Preparative IEF	384,600	38,500	2,295	45
Lectin chromatography	747,000	75,000	1,940	38
Cholinergic factor activity				
Tissue homogenate	<2	—	—	—
100,000 *g* supernatant*	17	1	5,500	100
46–60% $(NH_4)_2SO_4$ fraction	190	11	5,450	99
S-200 sizing chromatography	105‡ (*a*)	6 (*a*)	200 (*a*)	4 (*a*)
	1,000‡ (*b*)	59 (*b*)	1,600 (*b*)	29 (*b*)
Preparative IEF	935 (*a*)	55 (*a*)	170 (*a*)	3 (*a*)
	22,730 (*b*)	1,337 (*b*)	1,150 (*b*)	21 (*b*)
Lectin chromatography	2,055 (*a*)	123 (*a*)	170 (*a*)	3 (*a*)
	66,250 (*b*)	3,680 (*b*)	970 (*b*)	18 (*b*)

* Also referred to as crude muscle extract.
‡ Data for (*a*) 55,000- and (*b*) 17,000-mol-wt factors.

TABLE VI. *Summary of Physical Properties for the Morphologic and Cholinergic Factor Activities of Muscle Extract*

Treatment	Morphologic activity	Cholinergic activity
Heat	Labile $t_{½1}$ 60° = 9 min Labile $t_{½2}$ 60° = 31 min	Labile $t_{½}$ = 29 min
Trypsin	Labile $t_{½}$ 37° = 48 min	Labile $t_{½1}$ = 16 min Labile $t_{½2}$ = 23 min
pH	Stable at acidic pH Labile at basic pH	Maximal activity at pH 5–8.5
DTT	Stable	Stable
β-Mercaptoethanol	Stable	Stable
NEM	Stable	Stable
Periodate	Labile 20 min at 37°C	Labile 20 min at 37°C
Neuraminidase	Stable	Partial loss $t_{½}$ = 7.5 min
α-Mannosidase	Stable	Stable
β-Glucosidase	Stable	Stable
Lectin binding	Con A, Lens, Lotus	Limulin
Ammonium sulfate	35–60%	47–62%
DEAE-cellulose	0.1–0.3 M NaCl	0.70–0.95 M NaCl
CM-cellulose	—	0.04–0.08 M NaCl 0.25–0.40 M NaCl 0.55–0.62 M NaCl 0.9–1.0 M NaCl
pI	4.8	≥ 9.2, 7.7, 5.2, 4.1
Sizing (post-reduction)	33,000–35,000-mol-wt	50,000–60,000-mol-wt 14,000–17,000-mol-wt 5,000–7,000-mol-wt 1,200–1,500-mol-wt

band on one-dimensional SDS gel electrophoresis, and lesser degrees of purification for the various cholinergic factors.

Our results are consistent with in vivo experimental data, and extend previous work using co-cultured muscle (20), conditioned medium from cultured myotubes (15, 21, 27), and extracts of muscle tissue (58, 60) to stimulate spinal cord development. Further, our data complement recent work by other groups. For example, several attempts have been made to purify motoneurotrophic factors using assays that measure only total neuron survival and cell process outgrowth. Since motoneurons comprise a small percentage of ventral spinal cord cells, though, it is possible that their survival and development are regulated differently than other cord neurons (46, 61). Thus, even though the age and denervation-dependent changes in our morphologic activity paralleled specific effects on in vivo motoneuron neurite sprouting and retraction, >60% of the neurons in the cultures increased neurite outgrowth in response to factor addition. It may not be surprising, then, that other reported molecular weights for soluble factors effecting general spinal cord (16, 27, 62), cortical neuron (33), and parasympathetic motoneuron (17) survival and process sprouting are roughly equivalent to our reported values for nonreduced morphologic factor aggregates. Under reducing conditions, our total morphologic activity was quantitatively recovered as a 35,000-mol-wt acidic glycoprotein factor. Similarly, the bovine brain factor that directs neurite outgrowth in dissociated bovine cortical neurons is also a single acidic 37,000-mol-wt glycoprotein (33).

These morphologically active, soluble proteins augment neurite growth rate (16) in a different manner than do substratum-binding proteoglycans (37), which are mechanically essential for cell substratum attachment and subsequent neurite outgrowth (11). Although a few reports (9, 36) have suggested that a substrate-binding proteoglycan complex containing laminin is the only neurite-promoting factor present in muscle-derived fractions, our morphologic factor neither bound to dish substratum nor altered serum components. It thus behaves more like a previously reported soluble substance which augments neurite outgrowth in cultures already containing the substratum-bound factor (12).

Tests of cholinergic activity are more selective of motoneuron function than are general in vitro morphologic assays. Thus, the identification of cholinergic-enhancing factors by several groups (20, 21, 32, 45, 57, 60, 68) may provide more specific insights into motoneuron development than can observation of general growth or survival agents. Yet, our separation of spinal cord neuron morphologic and cholinergic

factors does not exclude the possible existence of a motoneurotrophic hormone which specifically enhances both motoneuron process outgrowth and neurotransmitter synthesis. In fact, the detection of such a factor has been suggested by muscle extract IEF. From several gels, we isolated a small amount of mophologic activity at a pI corresponding to that of the 55,000-mol-wt cholinergic-enhancing factor. This heat-labile, neutral 55,000-mol-wt glycoprotein further appears to have selective effects on in vitro motoneuron development. When added to ventral cord neuron cultures at saturating 1–5 μg/ml concentrations, it promoted both neurite outgrowth and cholinergic development in choline acetyltransferase-antibody–labeled neurons, without altering motoneuron survival, the survival and morphologic development of other cord neurons or non-neuronal cells, or culture protein content (61). Equivalently specific in its actions is the 50,000-mol-wt protein from iris muscle–containing eye extracts that induces cholinergic activity in ciliary ganglion motoneurons without affecting general neuron growth or non-neuronal cell division (45). Further, monoclonal antibodies directed against a similar 56,000-mol-wt neurotrophic species (or a co-purifying antigen) have selectively prevented denervation-induced sprouting in motoneurons (23).

Several other reports have identified acid-extractable, heat-stable 50,000–60,000-mol-wt proteins derived from heterologous sera or whole rat embryos, which generally act to promote "neuronotypic" cell survival (31) and mesodermal cell mitogenesis (39). However, the effects of our high molecular weight cholinergic factor were normally observed in the presence of serum-containing media, and were additive to the actions of horse and calf serum on choline acetyltransferase antibody–labeled cell growth and cholinergic activity. Therefore, this protein is probably not such a serum-derived factor.

Our 55,000-mol-wt species was additive to each of the three lower molecular weight species in reconstituting the maximal cholinergic-enhancing activity of the unfractionated extract. Further, the 14,000–17,000-mol-wt glycoprotein (which has a pI of ~9.2), had a very different effect on general cell growth than did the high molecular weight factor. At factor concentrations equal to its cholinergic-stimulating levels, the 14,000–17,000-mol-wt species increased culture protein synthesis, and induced a 2–3-fold increase in cell division when added to rat L_6 myoblasts, 3T3 fibroblasts, and primary rat astrocytes (Smith, R. G., unpublished results). This species may be analogous to a recently purified 20,400-mol-wt protein from eye extract (1), which augments the survival and growth of ciliary ganglionneurons, and complements the function of a 50,000-mol-wt cholinergic factor (45). It is also similar in its actions to basic (pI = 9.0) 40,000–45,000-mol-wt cholinergically active proteins isolated from skeletal and cardiac muscle–conditioned media, that increase choline acetyltransferase activity in cultured sympathetic neurons (68), and both cholinergic activity and general cell growth in spinal cord neurons (32). During size fractionation of the skeletal muscle–derived factor, active 13,000- and 7,000-mol-wt species were sometimes isolated, associated with a decrease in activity of the 40,000-mol-wt parent compound. We have similarly identified a minor 40,000–45,000-mol-wt cholinergic-stimulating factor component in nonreduced, size-fractionated muscle extract (Fig. 6*b*), which is lost on reduction, commensurate with increasing 14,000–17,000- and 5,000–7,000-mol-wt factor activity.

Although our low molecular weight species may be proteolytic fragments of larger species, several other 25,000–38,000-mol-wt cationic trophic glycoproteins have been shown to readily dissociate into 13,000–17,000-mol-wt subunits in the presence of reducing agents. The members of this genetically related family of substances (44), including platelet-derived growth factor, $p28^{sis}$, and fibroblast-derived growth factor, all contain functionally important disulfide linkages, have pIs in the range of 9–10, and (though not tested for neurotrophic activity) have similar mitogenic actions on fibroblast and glioblast cell populations (14, 66).

The smallest (1,200-mol-wt) factor accounts for most of the observed ACh synthesis–stimulating activity. Since muscle extracts were usually dialyzed before reduction and sieving chromatography, this peptide was also probably derived from larger molecular weight species, either by reduction of disulfide bonds or by proteolysis of precursor proteins (22). Yet, several lines of evidence suggest that the low molecular weight species is not a product of the 55,000-mol-wt protein. When tested together, the activities of the low molecular weight factor and high molecular weight factor were additive, thereby implying different mechanisms of action. Additionally, both the 17,000 and the 55,000-mol-wt factors selectively attached to an *n*-acetylneuraminic acid–specific lectin column and lost most activity after treatment with neuraminidase, while the low (1,200) molecular weight factor neither bound to sialic acid–specific columns nor was inactivated by neuraminidase. Because another recent report describes the neurotrophic effects of a low molecular neurotrophic peptide (43), peptides may also serve as trophic agents, helping to support the survival and development of neurons.

The later stages of motoneuron development, including terminal axon elongation to muscle cells, competition for survival, and induction of neurotransmitter synthesis, all appear to require interactions with the skeletal muscle target tissue. In a previous paper, we showed that soluble extracts of skeletal muscle could reproduce in vitro the effects of muscle cells on spinal cord neuron morphologic and cholinergic differentiation (60). In the present report, we have demonstrated that two trophic activities present in muscle extract can be differentiated by their respective functional dosages, tissue distribution, age of animals from which the extract was prepared, and state of innervation. Additionally, the proteins responsible for these separate activities are biochemically distinct. Some of the cholinergically trophic species indentified in this paper may act through different mechanisms to increase ACh synthetic levels, either by inducing neurotransmitter synthesis, or by stimulating cholinergic neuron survival (61). Further, the physical properties of some of these proteins bear similarities to other reported trophic agents.

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