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A sciatic nerve protein has a trophic effect on development and maintenance of skeletal muscle cells in culture

(neurotrophic influence/chicken embryonic muscle culture/muscle maintenance)

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ABSTRACT **Citrate-soluble extracts of chicken sciatic nerve were fractionated biochemically and added to cultures of embryonic chicken skeletal muscle in order to identify the component that exerted trophic influences on the muscle. A protein fraction that expressed trophic activity was obtained by ion-exchange chromatography on DEAE-cellulose followed by gel filtration on Sephadex G-100 superfine. This fraction enhanced the rate and degree of morphological maturation and the level of protein synthesis in embryonic muscle cells. Muscle fibers treated with this fraction after maturation in culture survived for longer periods *in vitro* than did comparable controls. Characterization of the active protein fraction by sodium dodecyl sulfate/polyacrylamide gel electrophoresis revealed the presence of one major protein (molecular weight 84,000) and three minor proteins. Electrophoretic analysis of biologically inactive gel filtration fractions indicated that the three minor proteins were contaminants and that biological activity was associated with the protein of molecular weight 84,000. Analytical isoelectric focusing revealed that the active protein was acidic and focused as four species with isoelectric points (pI) of 5.74, 5.77, 5.92, and 6.15. Maximal incorporation of [^{14}C]-leucine into muscle cell protein was elicited by 20 μg of active protein per culture dish. These data suggest that an acidic protein having trophic influences upon muscle has been identified and partially purified.**

Denervation produces a variety of pathological changes in fetal and adult skeletal muscle (1). In fetal muscle these changes include slowing of maturation and differentiation, while in adult muscle there is a decrease in the resting membrane potential, development of extrajunctional acetylcholine sensitivity, loss of endplate acetylcholinesterase, and muscle atrophy and degeneration. The mechanism by which the motor nerve regulates the metabolic properties of muscle is not known, although three hypotheses have been proposed to account for this phenomenon: (*i*) muscle activity (2–4), (*ii*) acetylcholine transmission (5, 6), and (*iii*) neurotrophic influences (7, 8). Recent evidence has shown that a diffusible, neurohumoral trophic substance, unrelated to nerve-impulse-related agents, is responsible for the regulation of muscle properties and that this neurotrophic influence is modified by alteration or abnormalities in acetylcholine release, muscle activity, or nerve-impulse transmission (see review, ref. 9). However, the biochemical characterization of this putative neurotrophic substance has not been fully accomplished (10–12).

Tissue culture of embryonic and adult skeletal muscle has proved to be a valuable tool for assessing neurotrophic influences (10, 12–17). Using muscle cell culture, Oh (18, 19) demonstrated that extracts of central nervous or peripheral nervous system tissue enhanced the morphological maturation of muscle cells and maintained mature, cross-striated muscle fibers for more than 6 weeks in the absence of innervation. Furthermore, the active agent in peripheral nerve extracts was characterized as a protein with a molecular weight greater than 10,000 (19). It was shown recently that extraction of sciatic nerves with citrate buffer at pH 4.2 stabilized the biological activity of these extracts to the extent that a protein fraction, obtained by gel filtration of citrate-soluble protein on Sephadex G-200, retained its full biological activity and could be partially characterized (20, 21).

In the present study, we report that a biologically active protein has been further purified and characterized from citrate-soluble sciatic nerve protein by ion-exchange chromatography followed by gel filtration on Sephadex G-100 superfine. The active species is an acidic protein with an estimated molecular weight of 84,000. This sciatic nerve protein can influence the maturation and long-term maintenance of cultured muscle fibers in a manner that resembles some components of the enhanced development after innervation (13).

MATERIALS AND METHODS

Preparation of Citrate-Soluble Protein. All steps in the isolation and purification procedure were carried out at 4°C. Sciatic nerves, obtained from adult chickens (Pel-Freez), were homogenized in 10 mM sodium citrate buffer, pH 4.2 (40% homogenate, wt/vol). The homogenate was centrifuged for 2 hr at 105,000 × *g*, and the resulting supernatant was dialyzed for 18 hr against deionized water. The dialyzed protein fraction was centrifuged for 1 hr at 20,000 × *g* to remove precipitated protein and lipid, and the resulting supernatant was lyophilized. The lyophilized powder was stored at −70°C until needed.

DEAE-Cellulose Ion-Exchange Chromatography. DEAE-cellulose (Cellex-D, Bio-Rad), washed as described by Himmelhoch (22), was packed into a 0.9 × 60 cm column and equilibrated with 10 mM imidazole·HCl buffer (pH 7.5) at a flow rate of 12.5 ml/hr. Citrate-soluble protein (60–80 mg) was fractionated in a 300-ml linear gradient of 0–200 mM NaCl in 10 mM imidazole·HCl buffer (pH 7.5). Fractions of 3.0 ml were collected, and eluted protein was detected by monitoring the absorbance at 280 nm. Fractions were pooled and biological activity was assessed in culture (refs. 19 and 20; see below). The biologically active protein peak eluted by 60–75 mM NaCl was dialyzed against deionized water for 18 hr and lyophilized.

Sephadex G-100 Superfine Chromatography. Active nerve protein, obtained by ion-exchange chromatography, was chromatographed on a column of Sephadex G-100 superfine (1.6 × 100 cm) with 10 mM sodium phosphate, pH 7.2/150 mM NaCl serving as elution buffer. Flow rate was maintained at 4.8 ml/hr, and fractions of 2.0 ml were collected. Eluted protein was detected by monitoring the absorbance at 280 nm. Fractions were pooled and biological activity was assessed in tissue culture (refs. 19 and 20; see below).

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Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; IEF, isoelectric focusing.

Disc Polyacrylamide Gel Electrophoresis. The electrophoresis was carried out in 7.5% running gels (5 × 75 mm) in Tris glycine buffer (pH 8.3) as described by Davis (23). Gels were electrophoresed at 2.5 mA/tube until the tracking dye, bromophenol blue, had migrated to within 1 cm from the tube bottom. Proteins were visualized with Coomassie brilliant blue as described by Fairbanks *et al.* (24).

Sodium Dodecyl Sulfate ($NaDodSO_4$) Flat-Bed Polyacrylamide Gel Electrophoresis. Protein samples were denatured at 100°C for 10 min in a buffer consisting of 10 mM Tris·HCl (pH 8.0), 1 mM EDTA, 2% $NaDodSO_4$, 5% 2-mercaptoethanol, 4 M urea, 25% surcose, and 0.001% bromophenol blue. $NaDodSO_4$/polyacrylamide gel electrophoresis in flat-bed polyacrylamide gels (15 × 17 × 0.2 cm) was carried out by a modification* of the conditions described by King and Laemmli (25). Molecular weights were estimated by comparing the relative migration distance with those of known standards (high and low molecular weight standards; Pharmacia) run with each gel (26).

Analytical Isoelectric Focusing (IEF). Analytical IEF in flat-bed polyacrylamide gels containing 2% pH 4–6 Ampholines (Brinkmann) and 2% pH 5–8.5 Ampholines (LKB) was carried out in an LKB Multiphor as described by Righetti and Drysdale (27). Temperature of the gels during IEF was maintained at 5°C by means of a refrigerated water circulator. Proteins were focused for 2.5 hr at 20 W constant power. The pH of the Ampholine gradient was determined on gel slices at 25°C with a Metrohm pH meter. Gels were fixed and stained in a solution of 0.05% Coomassie brilliant blue and 0.1% cupric sulfate in acetic acid/ethanol/deionized water, 19:25:65 (vol/vol) for 4 hr (27).

Cell Culture. Myogenic cells were obtained (28) from dissociated breast muscle of 11-day-old chicken embryos (18, 29). The myogenic cells (5×10^5) were plated on Linbro plastic dishes (35 × 10 mm; FB-6-TC) that had been coated with collagen (acid-soluble collagen, 50 μg/dish; Calbiochem). The cultures were maintained in 1 ml of culture medium at 36°C in a humidified atmosphere of 95% air/5% CO_2. Standard culture medium consisted of 87.5% Dulbecco's modified Eagle's medium (GIBCO), 10% horse serum (heat-inactivated and Millipore-filtered; North American Biological, Miami, FL) and 2.5% chicken embryo extract (EE_{50}). Chicken embryo extract was prepared from 10-day-old chicken embryos as described by Paul (30), except that the brains and spinal cords were excluded. The medium was replaced every 3 days and no antibiotics were used.

Biochemical Assays. The incorporation of [^{14}C]leucine (320 Ci/mol, 1 Ci = 3.7×10^{10} becquerels; New England Nuclear) into cell protein after a pulse-label of 1 μCi/culture dish per 18 hr was determined as described (19). Protein was determined by the Lowry procedure (31), with lipid-free bovine serum albumin (fraction V; Sigma) as standard.

RESULTS

Biological activity of individual, pooled column fractions was assessed by the ability of the individual fractions to enhance the morphological maturation of cultured muscle cells or to stimulate the incorporation of [^{14}C]leucine into muscle cell protein (Table 1) (19, 20). Biological activity was not simply a reflection of an increase in plating efficiency since fractions were added to cell cultures no sooner than 18–24 hr after plating, when all viable myogenic cells had attached to the collagen substrate (20).

A single peak of biologically active protein was eluted from DEAE-cellulose by ion-exchange chromatography (Fig. 1). Under the chromatographic conditions used, the protein fraction was eluted from DEAE-cellulose by 60–75 mM NaCl at pH 7.5 in 10 mM imidazole·HCl buffer. Disc and flat-bed $NaDodSO_4$/polyacrylamide gel electrophoresis revealed the presence of seven and nine protein bands, respectively. The level of protein recovered in this active fraction represented 5–8% of the citrate-soluble sciatic nerve protein applied to the column.

The active protein fraction recovered by ion-exchange chromatography was pooled and fractionated by gel filtration on Sephadex G-100 superfine (Fig. 2). Biological activity was associated with the major protein peak. The active fraction obtained by gel filtration represented a 31-fold purification of

Table 1. Effect of column fractions on protein synthesis and morphological development of skeletal muscle cells *in vitro*

Fraction added	[^{14}C]Leucine incorporation,* dpm/dish × 10^{-3}	Morphological development,† stage
Control	36.7 ± 0.6‡	2
DEAE fractions		
Active	47.6 ± 0.3‡§	4
Inactive	36.1 ± 0.2	2
Sephadex fractions		
Active	44.7 ± 0.6‡§	4
Inactive	36.6 ± 0.9	2

* Sample tubes collected during ion-exchange chromatography (Fig. 1) or gel filtration (Fig. 2) were pooled into arbitrary fractions. A portion of each fraction (100 μl) was added to each of four myotube cultures (4-day-old). Subsequently, a pulse-label of 1 μCi of [^{14}C]-leucine (320 Ci/mol) was added to each culture dish, and incorporation of [^{14}C]leucine into cell protein was determined after 20 hr (19). The biologically active fractions, corresponding to the respective shaded areas in Figs. 1 and 2, were the only fractions that significantly increased the incorporation of [^{14}C]leucine into protein as compared to the control. As such, the value for the respective "inactive fractions" as shown here represents their mean [^{14}C]leucine incorporation ±SD of these fractions (DEAE: five fractions, $n = 20$; Sephadex G-100 superfine: three fractions, $n = 12$).

† Fractions were added to cultures at 24 and 96 hr after myogenic cells were plated. The stage of morphological development was assessed at 6 days *in vitro*. Stages of morphological development were arbitrarily determined as described (19): 1, single mononucleated myoblasts; 2, thin polynucleated myotubes; 3, wide multinucleated myotubes; 4, mature muscle fibers with cross striations.

‡ Each value is the mean ±SD of four dishes.

§ Statistically significant increase ($P < 0.001$) over the control value (Student's t test).

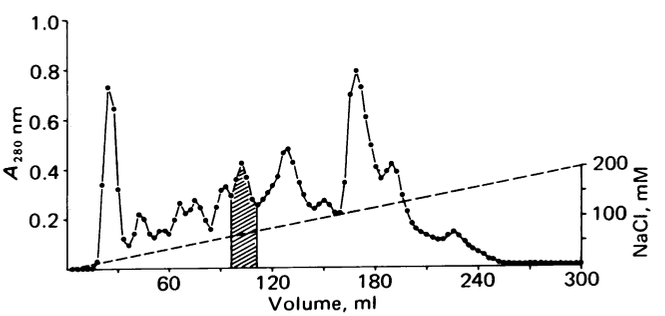

FIG. 1. DEAE-cellulose ion-exchange chromatography. Approximately 75 mg of citrate-soluble sciatic nerve protein was applied to a 0.9 × 60 cm column of DEAE-cellulose. Protein was eluted by means of a linear gradient of 0–200 mM NaCl in 10 mM imidazole·HCl (pH 7.5). The protein peak eluted by 60–75 mM NaCl (shaded) had biological activity.

* Pharmacia Product Bulletin no. 17-0445-01.

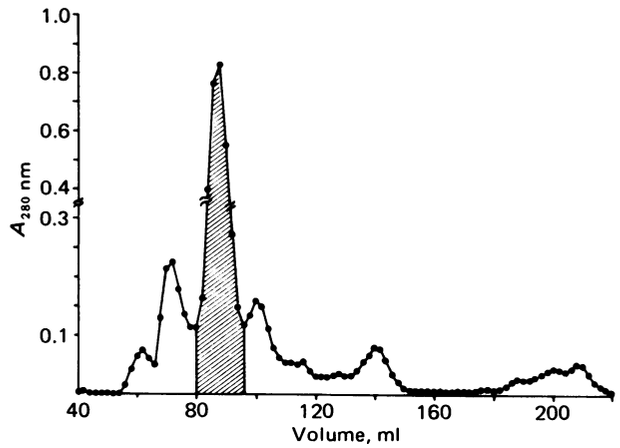

FIG. 2. Sephadex G-100 superfine column chromatography. Approximately 17 mg of active protein recovered from DEAE-cellulose was applied to a 1.6 × 100 cm column of Sephadex G-100 superfine. Protein was eluted with phosphate-buffered saline (pH 7.2) at a flow rate of 4.8 ml/hr. The major protein peak (shaded) had biological activity.

citrate-soluble protein. Disc gel electrophoresis of the active fraction revealed a single protein band (Fig. 3) which migrated as a doublet when low levels (4–6 μg) of protein were electrophoresed. By contrast, $NaDodSO_4$ gel electrophoresis on flat-bed gels (Fig. 4) revealed a major protein band (M_r 84,000), a diffuse minor "doublet" band (M_r 68,000–70,000), and two minor single bands (M_r 65,000 and 45,000). The major protein band accounted for 92% of the electrophoresed protein as determined by densitometric analysis.

Densitometry of $NaDodSO_4$/polyacrylamide gels (Fig. 5) revealed that the protein of M_r 84,000 was almost absent in biologically inactive gel filtration peaks. By contrast, proteins with molecular weights of 45,000–74,000 were enriched in these inactive gel filtration peaks as compared to the low levels of these proteins found in the active gel filtration peak. Thus, it seems apparent that the minor proteins (M_r 45,000–70,000) present in the active fraction represent contaminants and that the protein of M_r 84,000 represents the biologically active species.

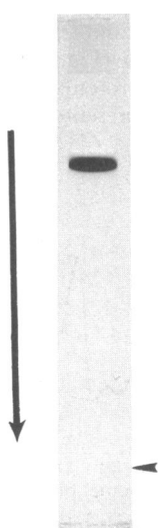

FIG. 3. Disc gel electrophoresis of active gel filtration fraction. Ten micrograms of active protein was applied to each gel and electrophoresed. Arrowhead marks the position of the tracking dye, bromophenol blue.

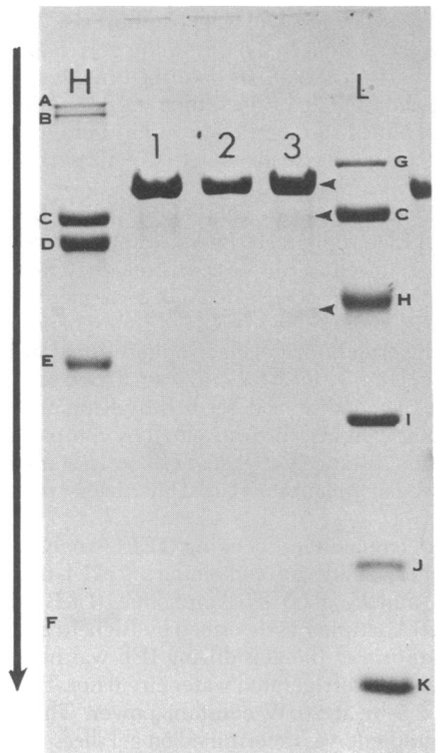

FIG. 4. $NaDodSO_4$ gel electrophoresis of active gel filtration fraction. Sixteen micrograms of protein was applied to lanes 1 and 3; 10 μg was applied to lane 2. Electrophoresis was carried out as described in *Materials and Methods*. Arrowheads (lane 3) mark the position of protein bands (top, M_r 84,000; middle, three closely allied bands of M_r 70,000, 68,000, and 65,000; bottom, M_r 45,000). Lane H, high molecular weight standards: A, thyroglobulin (330,000); B, ferritin half-unit (220,000); C, albumin (67,000); D, catalase (60,000); E, lactate dehydrogenase (36,000); F, ferritin (18,500). Lane L, low molecular weight standards: G, phosphorylase B (94,000); H, ovalbumin (43,000); I, carbonic anhydrase (30,000); J, trypsin inhibitor (20,100); K, α-lactalbumin (14,400).

Analytical IEF in the pH range 4–8.5 revealed that the active protein was heterogeneous. Protein bands were observed at approximate pI values of 5.74, 5.77, 5.92, and 6.15, with a variation of ±0.07. This same heterogeneity was observed whether IEF was carried out in the pH range 4–8.5, 5–8.5, or 3–10, with Ampholines obtained from LKB or Brinkmann.

Muscle cultures treated with the active (M_r 84,000) protein differentiated into mature, cross-striated muscle fibers by 6 days *in vitro* (Fig. 6*b*). By contrast, control cultures treated with inactive sciatic nerve protein or phosphate-buffered saline were composed of immature thin myotubes at 6 days *in vitro* (Fig. 6*a*) and did not develop cross striations until 10 days in culture. For several days thereafter, the control cultures maintained their structural integrity, but then began to undergo muscle fiber atrophy and degeneration; spontaneous contractions disappeared and the fibers became thinner and shorter and appeared to lose their cross striations. Addition of the active protein to these degenerating, control cultures prevented or reversed the degenerative process. In the presence of the active protein, well-differentiated muscle fibers were maintained for more than 5 weeks, a time when control cultures had completely degenerated (Fig. 6 *c* and *d*). If active protein was withdrawn from these treated cultures, muscle fibers began to degenerate within 3–5 days, indicating that the continuous presence of the active protein was essential for the long-term maintenance of muscle fibers in culture.

Fig. 7 demonstrates that maximum incorporation of [^{14}C]-

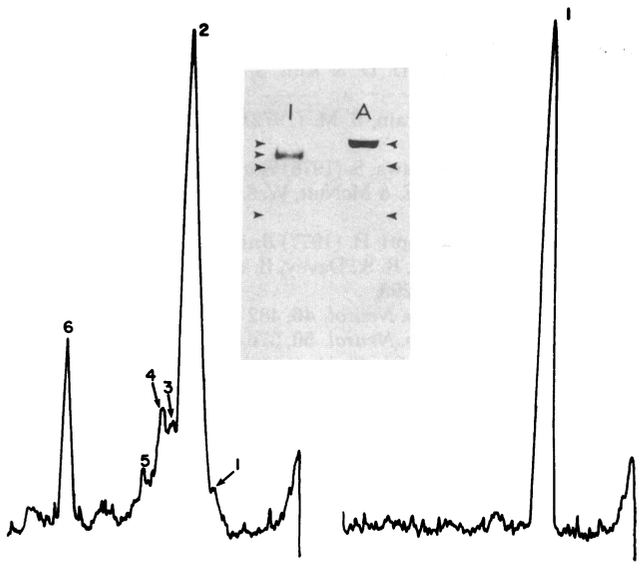

FIG. 5. Densitometry of inactive (*Left*) and active (*Right*) gel filtration fractions. (*Inset*) $NaDodSO_4$ gel electrophoresis was carried out on 4 μg of inactive or active gel filtration protein. Densitometry was performed at 570 nm on gel slices with a Gilford spectrophotometer-densitometer set at 0.2 OD unit full-scale deflection. Molecular weights of peaks as estimated from standards: 1, 84,000; 2, 74,000; 3, 68,000; 4, 65,000; 5, 60,000; 6, 45,000.

leucine into muscle cell protein was elicited by 20 μg of active protein per culture dish (20 μg/ml). This level of active protein would correspond to 0.24 μM, assuming that the molecular weight of the active protein is 84,000. Similarly, the effective dose eliciting 50% of maximum leucine incorporation into muscle protein (ED_{50}) corresponded to 9 μg of active protein per ml (0.11 μM). As demonstrated previously (20), the observed stimulation of cell protein synthesis is not caused by a

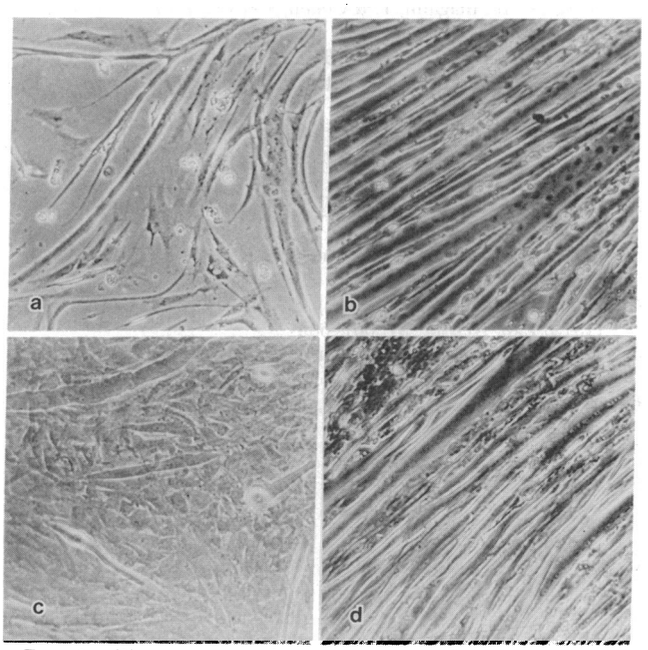

FIG. 6. Phase contrast photomicrographs of chicken embryonic muscle cells in culture. (*a*) Control culture grown for 6 days in the standard culture medium. (*b*) Muscle culture grown for 6 days in the presence of active sciatic nerve protein. (*c*) Control culture maintained for 5 weeks in the standard culture medium. (*d*) Muscle culture maintained for 5 weeks in the presence of active sciatic nerve protein.

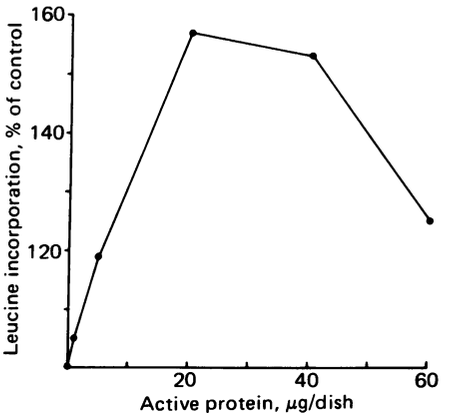

FIG. 7. Dose–response curve for biological activity. After fusion of myoblasts had occurred, cultures of immature myotubes were treated with active protein for 24 hr. Cultures were then pulse-labeled with 1 μCi of [^{14}C]leucine for 18 hr. After the cultures were washed, protein was precipitated with ice-cold, 10% trichloroacetic acid, and acid-precipitable radioactivity was determined in triplicate by scintillation counting (19). Values represent the mean; $n = 6$.

mitogenic effect of the active protein since this stimulation occurs at a time when all muscle cells are in the postmitotic, myotube stage. Inhibition of protein synthesis from the maximum attainable level was observed when higher levels of active protein (40–60 μg) were added to muscle cultures. Thus, the protein exhibited a narrow saturation range for maximal stimulation of cell protein synthesis.

DISCUSSION

Previous studies have demonstrated that soluble extracts of nervous tissue exert neurotrophic effects on skeletal muscle in cell culture (12, 18–21, 32, 33) and organ culture (10, 14–16, 34). However, biochemical characterization of a putative "neurotrophic substance" has not yet been accomplished. In the present study, we found that fractionation of citrate-soluble peripheral nerve protein by DEAE-cellulose ion-exchange chromatography followed by gel filtration on Sephadex G-100 superfine yielded a substantially purified protein fraction with neurotrophic activity. This protein fraction not only enhanced the rate and degree of morphological maturation of muscle cells in culture, but also maintained noninnervated muscle fibers in a well-differentiated state for more than 5 weeks, a time when control cultures had completely degenerated (Fig. 6). Thus, this protein fraction can duplicate at least some of the effects of innervation upon the further maturation and long-term maintenance of muscle cells in culture (13). Although the active fraction obtained by gel filtration was not homogeneous (Fig. 4), evidence indicates that biological activity is associated with an acidic major protein band of M_r 84,000 and not with three minor protein bands. Since the three minor protein bands were enriched in gel filtration fractions that were biologically inactive (Fig. 5), these proteins appear to be contaminants of the active protein fraction.

In analyzing the constituent proteins of the active gel filtration fraction, it should be noted that the protein "doublet" migrating at M_r 68,000–70,000 on $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 4) has a molecular weight similar to that of the neurofilament protein of peripheral nerve (35). Similarly, the protein of M_r 45,000 migrates in a manner similar to that of actin, another component of peripheral nerves (36). In a study of the constituent proteins of peripheral nerve axoplasm of the rat, an 85,000-dalton protein was observed in $NaDodSO_4$ electrophoretic profiles (36). Whether this protein

from the rat is related to the purified protein from the chicken described in the present report remains to be determined.

A consideration of the physical characteristics of the active protein appears to rule out the possibility that this protein is identical to any of the known trophic or "maintenance" factors that have been described. The protein of M_r 84,000 is appreciably heavier than epidermal growth factor (37), fibroblast growth factor (38), β-nerve growth factor (39), insulin (40), nonsuppressible insulin-like activity (41), or various other growth factors isolated from plasma or non-neuronal tissues (see review, ref. 42). However, it is possible that biological activity could be associated with a small polypeptide absorbed to a heavy molecular weight carrier, as in the case of nonsuppressible insulin-like activity (42). Further experiments would be needed to investigate this possibility. Interestingly, the protein does have similar biological activities and physical characteristics to myoblast growth factor, a factor that was described in brain extracts (43) but never further characterized.

Conclusive evidence from *in vivo* experiments has shown that a neurohumoral factor participates in the neurotrophic maintenance of skeletal muscle (refs. 44–46; for review, see ref. 9). The concept that muscle activity can solely regulate muscle metabolic properties is no longer a tenable hypothesis (45, 46). The isolation, identification, and partial purification of a neurotrophic protein from peripheral nerve should greatly aid in assessing the interaction between muscle activity and neurotrophic mechanisms in the control of muscle metabolic properties *in vivo*. Finally, the availability of this protein should help in characterizing the intracellular disposition and mechanism of action of this macromolecule *in situ*.

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