

## ENRICHMENT OF SPINAL CORD CELL CULTURES WITH MOTONEURONS

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### ABSTRACT

Spinal cord cell cultures contain several types of neurons. Two methods are described for enriching such cultures with motoneurons (defined here simply as cholinergic cells that are capable of innervating muscle).

In the first method, 7-day embryonic chick spinal cord neurons were separated according to size by 1 g velocity sedimentation. It is assumed that cholinergic motoneurons are among the largest cells present at this stage. The spinal cords were dissociated vigorously so that 95–98% of the cells in the initial suspension were isolated from one another. Cells in leading fractions (large cell fractions: LCFs) contain about seven times as much choline acetyltransferase (CAT) activity per unit cytoplasm as do cells in trailing fractions (small cell fractions: SCFs). Muscle cultures seeded with LCFs develop 10–70 times as much CAT as cultures seeded with SCFs and six times as much CAT as cultures seeded with control (unfractionated) spinal cord cells. More than 20% of the large neurons in LCF-muscle cultures innervate nearby myotubes.

In the second method, neurons were gently dissociated from 4-day embryonic spinal cords and maintained in vitro. This approach is based on earlier observations that cholinergic neurons are among the first cells to withdraw from the mitotic cycle in the developing chick embryo (Hamburger, V. 1948. *J. Comp. Neurol.* **88**:221–283; and Levi-Montalcini, R. 1950. *J. Morphol.* **86**:253–283). 4-Day spinal cord-muscle cultures develop three times as much CAT as do 7-day spinal cord-muscle plates, prepared in the same (gentle) manner. More than 50% of the relatively large 4-day neurons innervate nearby myotubes.

Thus, both methods are useful first steps toward the complete isolation of motoneurons. Both methods should facilitate study of the development of cholinergic neurons and of nerve-muscle synapse formation.

**KEY WORDS** motoneurons · cell separation · in vitro development

Neurons dissociated from embryonic chick spinal cords survive for several weeks in sparse cell culture in the absence of suprasegmental or pe-

ripheral inputs (11). These primary cell cultures are simplified from an electrophysiological point of view compared to the intact tissue or to small spinal cord fragments maintained in vitro as explant cultures. Nonneuronal spinal cord cells proliferate in vitro; but if the cultures are treated with

mitotic inhibitors or if they are plated at low cell density, the neurons do not become encased in glia or satellite cells (11). Neuronal perikarya, fine nerve processes, and individual synaptic boutons can be visualized in sparse cultures. However, spinal cord cell cultures are complex in that several types of neurons are present (7, 8, 11).

We are interested in the formation of neuromuscular junctions and in the development of motoneurons. In previous work, neurons were dissociated from 7-day embryonic spinal cords because, at this stage, the cords are easy to dissect, and many of the cells survive in culture. However, less than 10% of the 7-day neurons innervate nearby muscle fibers (8, 9). While this heterogeneity may indicate that a degree of selectivity in synapse formation is preserved in cell culture, it presents an obstacle to description of the earliest events in nerve-muscle synapse formation. In this paper, we describe two ways in which spinal cord-muscle cultures can be enriched with motoneurons, i.e., with cells that synthesize acetylcholine and are capable of innervating skeletal myotubes.

The first method involves sedimentation of 7-day spinal cord cells in a 1 g field according to methods described by Miller and Phillips (25). This approach depends on the fact that freshly dissociated 7-day cells are stripped of axons and dendrites and appear as spheres that vary in diameter between about 6  $\mu\text{m}$  and 30  $\mu\text{m}$ . The sedimentation rate,  $s$ , of a sphere of radius  $r$  under the influence of gravity ( $g$ ) is given by:

$$s = \frac{2(\rho - \rho')}{9\eta} gr^2, \quad (1)$$

where  $\rho$  and  $\rho'$  are the densities of the cell and fluid, respectively, and  $\eta$  is the coefficient of viscosity. Ignoring differences in density between the cells, a significant separation on the basis of size is to be expected. We assumed that motoneurons are among the largest cells in 7-day embryonic cords, just as they are in adult spinal cords. A similar technique has been used by Barkley et al. (1) to separate Purkinje neurons from a suspension of cerebellar cells, and by Lam (19) to isolate turtle photoreceptors. The survival and growth of the separated neurons (receptors) in cell culture was not pursued in these studies.

The second method is based on the observation that motoneurons are "born" relatively early in the chick neural tube. Large cells destined for the ventral horn undergo their final mitosis between

day 2 and day 5 of embryonic life whereas ventral and dorsal horn interneurons and glia appear at later times (16, 13, 20). Embryonic neuronal precursor cells apparently do not divide or do not differentiate in vitro under usual conditions (33, 37), so we expected that cultures seeded with 4-day spinal cord cells would contain relatively more motoneurons than cultures seeded with 7-day cells.

The results were evaluated by following two measures of cholinergic function: the activity of choline acetyltransferase and the ability of individual neurons to form functional connections (synapses) on co-cultured muscle fibers. Synaptic interactions between dissociated spinal cord neurons and muscle are mediated by acetylcholine (9). By these criteria, both procedures led to a significant enrichment of cell cultures with cholinergic neurons. Preliminary accounts of some of this work have appeared previously (3, 10).

## MATERIALS AND METHODS

### *Preparation of Spinal Cord (7-Day and 4-Day)-Muscle Cultures*

Mononucleated myogenic cells were mechanically dissociated from pectoral muscles of 11-day embryos without the use of enzymes. Minced muscle fragments were incubated in a  $\text{Ca}^{++}$ -,  $\text{Mg}^{++}$ -free salt solution (Puck's, D<sub>1</sub>G) for 30 min at 37°C, concentrated by centrifugation at 200 g for 8 min, resuspended in complete culture medium (containing serum; see below) and disrupted by repeated passage (40  $\times$ ) through a fire-polished Pasteur pipette. The triturated suspension was filtered through a double layer of lens paper. Cells were counted in a hemocytometer, and  $2.0 \times 10^6$  cells were added to collagen-coated (4) 35-mm dishes. After 48 h, the cultures were fed with medium containing  $10^{-5}$  M cytosine arabinoside (ara C). This drug eliminates rapidly dividing fibroblasts but does not affect postmitotic myoblasts or multinucleated myotubes (9). After an additional 48-h period, the medium containing cytosine arabinoside was removed and the cultures were inoculated with  $2.0 \times 10^6$  spinal cord cells in fresh medium.

Spinal cords from 7-day and 4-day embryos were routinely dissociated in the same manner except that the minced fragments were incubated in Puck's D<sub>1</sub>G containing 0.1% crude trypsin (Microbiological Associates, Walkersville, Md.) for 30 min at 37°C. 7-Day cords were stripped of dorsal root ganglia and meninges before mincing (9, 11). Small bulges along the dorsal edge of 4-day cords, which may be rudimentary sensory ganglia, could not always be removed. Most "7-day" embryos corresponded to Hamburger-Hamilton (18) Stage 31. A few were closer to Stage 30 or 32. Most "4-day"

embryos corresponded to Stage 24, but a few Stage 23 and 25 embryos were used as well.

### *Velocity Sedimentation of 7-Day Spinal Cord Cells*

The dissociation protocol described above results in a cell suspension in which 85–90% of the cells are clearly isolated from one another. The remainder are found in clusters of 2–5 cells or in larger clumps. These mini-aggregates are acceptable in routine cell cultures, but they complicate attempts to separate cells by velocity sedimentation. Gentle pipetting may not completely dissociate the cells. In addition, the presence of  $\text{Ca}^{++}$  and serum during trituration in complete medium may promote reaggregation, especially when one is dealing with suspensions that contain more than  $10^6$  cells/ml. Therefore, the following protocol in which spinal cord fragments are vigorously triturated in a  $\text{Ca}^{++}$ -,  $\text{Mg}^{++}$ -free salt solution was employed before velocity sedimentation. Nine 7-day spinal cords were minced and incubated for 30 min in 3.6 ml of Puck's  $\text{D}_1\text{G}$  supplemented with Eagle's essential amino acids and 0.5 mM pyruvate containing 0.1% (wt/vol) trypsin and 0.05% DNase (wt/vol) at 37°C. To avoid the use of serum, a purified preparation of trypsin (L-(tosylamido 2-phenyl)ethyl chloromethyl ketone, TPCK) was used and soybean trypsin inhibitor (8 mg) was then added to terminate enzymatic digestion, and the fragments were then dispersed by vigorous pipetting (40–50  $\times$ ) through a narrow-bore (about 0.2 mm tip) Pasteur pipette. The suspension was filtered through a 20- $\mu\text{m}$  nylon screen (Nitex, Tekto, Inc., Elmsford, N. Y.). The filtrate contained about  $10^7$  cells/ml, and 95–98% were present as single cells (see Fig. 3). Substitution of pronase or collagenase for TPCK trypsin or mechanical disruption without prior exposure to enzymes resulted in a lower yield of viable cells or in more cell clusters.

Vigorously dissociated 7-day cells were allowed to settle through a column of Eagle's Minimum Essential Medium (MEM) containing a shallow gradient of Ficoll. The cylindrical part of the column was 5.55 cm in diameter. The bottom was a cone that tapered to a narrow (0.5 cm) opening fitted with a stainless steel baffle (see Fig. 2). The chamber with attached reservoirs and tubing was autoclaved and then mounted on an antivibration platform in a cold room. All manipulations were carried out at 4°C. The gradients were formed, and the chamber was loaded from the bottom by gravity flow as described by Miller and Phillips (25). The profile of the loaded column from top to bottom was as follows: (a) 12 ml (5.0 mm) of Puck's  $\text{D}_1\text{G}$ ; (b) 2.2 ml (1 mm) of supplemented (see above) Puck's  $\text{D}_1\text{G}$  containing  $2.2 \times 10^7$  cells; (c) 5 ml (2 mm) containing a 0.2–1.0% Ficoll gradient and a 0.0–1.8 mM  $\text{Ca}^{++}$  gradient constructed from Puck's  $\text{D}_1\text{G}$  containing 0.2% Ficoll and Eagle's MEM containing 1.0% Ficoll; (d) 60 ml (34 mm) on MEM containing a 1.0–2.2% Ficoll gradient.

The MEM was supplemented with 3 gm of glucose/liter (total = 4 g/liter), penicillin (50 U/ml) and streptomycin (50  $\mu\text{g}$ /ml). In some experiments, Liebovitz's L-15 medium was used instead of MEM.

The relatively steep, "buffered step" Ficoll gradient immediately beneath the cell layer prevented "streaming" observed at high cell concentrations (25). The short  $\text{Ca}^{++}$  gradient was designed to prevent the reaggregation of cells as they migrated into the column. The more gradual Ficoll gradient (1–2%) in the major portion of the column prevented convective currents without introducing a significant density gradient, i.e.  $\rho'$  in Eq. 1 can be considered constant. Preliminary experiments in which heat-inactivated horse serum gradients (7–30%) rather than Ficoll gradients were used resulted in a greater degree of cell reaggregation.

Cells were allowed to settle for 3 h at 4°C. Fractions (2.4 ml) were then collected in sterile tubes with a Gilson FE-4 microfractionator (Gilson Medical Electronics, Inc., Middleton, Wis.).

### *Choline Acetyltransferase (CAT)*

CAT was assayed by measuring the synthesis of [ $^3\text{H}$ ]acetylcholine (ACh) from [ $^3\text{H}$ ]acetyl CoA, using a modification of the method described by Fonnum (12). Cultures were scraped with a Teflon pad, and the cells were collected in 0.10 ml of 0.05 M  $\text{NaPO}_4$ , pH = 7.5, containing 0.20 M NaCl, 0.5% Triton X-100, and 5 mg/ml bovine serum albumin (BSA) (homogenate buffer). Each dish was rinsed with an additional 0.10 ml of buffer, and the pooled scrapings were homogenized in a glass tube with a Teflon pestle. Duplicate assays were performed as follows. Aliquots (0.010–0.025 ml) were incubated at 37°C in a final vol of 0.030 ml of homogenate buffer containing 1.25 mM choline chloride, 0.11–0.23 mM [ $^3\text{H}$ ]acetyl CoA and 0.1 mM neostigmine. Carrier-free [ $^3\text{H}$ ]acetyl CoA was purified by passage over Dowex 50W-X8 resin (35) and was mixed with unlabeled acetyl CoA to give specific activities of 0.2–0.9 Ci/mmol. After 20 min, the reaction was stopped by the addition of chilled 0.05 M  $\text{NaPO}_4$ , pH = 7.5 (3.5 ml) containing 0.25 mg/ml acetylcholine bromide to act as carrier. [ $^3\text{H}$ ]ACh was then extracted by adding 0.5 ml of a freshly prepared solution containing 15 mg/ml tetraphenylboron in heptanone. After vigorous mixing, the suspension was centrifuged at 1,100 g for 8 min to separate the organic phase from the aqueous phase. An aliquot of the organic phase (0.3 ml) was added to Aquasol (5 ml) and counted in a Packard liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). This extraction procedure recovered 95–100% of the ACh present.

Assays were linear with enzyme concentration over the range examined (0.01–7.5 pmol ACh/min/assay tube). Omission of BSA from the reaction mixture resulted in inhibition of CAT activity at low enzyme concentrations (38, 6). ACh synthesis was not strictly

linear with time over the entire 20-min incubation period, but corrections for nonlinearity were small (10% or less) and were independent of enzyme concentration.

### *Lactic Dehydrogenase (LDH)*

LDH was assayed spectrophotometrically by following the conversion of NADH to NAD in the presence of pyruvate at room temperature. A unit of LDH activity is defined as a decrease of 1 U/min in the absorbance of light at 340 nm using a path length of 1 cm.

### *Carnitine Acetylase*

Carnitine acetylase was assayed with the standard CAT reaction mixture by substituting 1.25 mM carnitine chloride for the choline chloride. Less than 1% of the acetylcarnitine present is extracted into the organic phase under normal conditions, so the amount of acetylcarnitine synthesized was determined by paper electrophoresis. At the end of an incubation, a 0.020-ml aliquot of the reaction mixture was mixed with 0.010 ml of electrophoresis buffer (1.4 M acetic acid plus 0.47 M formic acid, pH = 2) containing 5 mg/ml ACh and 5 mg/ml acetylcarnitine. Samples (0.02 ml) were applied to Beckman "thin" paper strips and electrophoresed for 2.5 h at 400 V in a Beckman electrophoresis cell (Durrum type, Durrum Instrument Corp., Sunnyvale, Calif.). The strips were dried, stained with iodine vapor for quaternary amines, cut into 1 × 3 cm pieces, eluted with 0.5 ml of 0.10 M HCl, and counted in 5 ml of Aquasol.

### *DNA*

The general method of Burton (5) was used to measure the amount of DNA in embryonic chick spinal cords. Dissected cords were homogenized, and the DNA was recovered by acid precipitation (7% perchloric acid at 0°C). Salmon sperm DNA was used as a standard.

### *Synaptic Potentials*

Nerve-muscle junction formation was assayed by recording from individual muscle fibers with intracellular microelectrodes (50–100 MΩ when filled with 3 M KCl or 4 M K(C<sub>2</sub>H<sub>4</sub>O<sub>2</sub>)) while stimulating nearby nerve cell bodies or nerve processes with a blunt (2–3 μm tip), fire-polished, extracellular electrode filled with 1.0 M NaCl. Electrophysiological experiments were performed on the stage of an inverted microscope (Leitz, Diavert Leitz, Inc., Rockleigh, N. J.) equipped with phase-contrast optics. Cultures were perfused with oxygenated MEM maintained at 37°–36°C and pH 7.2–7.4. After suitable preamplification (WPI-M4) synaptic potentials were displayed on a Tektronix storage oscilloscope (Tektronix, Inc., Beaverton, Oreg.) and photographed with a Grass C-4 camera (Grass Instrument Co., Quincy, Mass.).

### *Media*

Culture medium consisted of Eagle's MEM supple-

mented with 2 mM glutamine, 10% (vol/vol) horse serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and either 2% (vol/vol) or 5% (vol/vol) chick embryo extract. Cells were plated in 1.5 ml of medium containing 5% embryo extract and subsequently fed with 1.5 ml of medium containing 2% embryo extract at 2–3-day intervals. Horse serum, MEM, L-15, glutamine, penicillin, streptomycin and essential amino acids were purchased from Grand Island Biological Co. (Grand Island, N. Y.). Embryo extract was prepared each week by homogenizing 10-day chick embryos in an equal volume of Earle's balanced salt solution. Embryo extract and heat-inactivated horse serum were centrifuged for 90 min at 27,000 g before use. Puck's D<sub>1</sub>G contains 137.0 mM NaCl, 5.36 mM KCl, 0.20 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.22 mM KH<sub>2</sub>PO<sub>4</sub>, and 1 g/liter glucose.

### *Chemicals*

Cytosine arabinoside, acetylcholine bromide, acetylcarnitine chloride, choline chloride, neostigmine methylsulfate, acetyl CoA, tetraphenylboron, NADH, pyruvate, diphenylamine, bovine serum albumin, and acetylcholinesterase were purchased from Sigma Chemical Co. (St. Louis, Mo.). Naphthyl(vinyl) pyridine (NVP) was purchased from Calbiochem (San Diego, Calif.), heptanone from Eastman, Kodak Co., (Rochester, N. Y.) Ficoll from Pharmacia Fine Chemicals (Division of Pharmacia, Inc., Piscataway, N. J.), Dowex 50W-X8 from Bio-Rad Laboratories (Richmond, Calif.), and [<sup>3</sup>H]acetyl CoA from New England Nuclear (Boston, Mass.). Trypsin was purchased from Microbiological Associates (17-160F). TPCK trypsin, DNase (DP) and soybean trypsin inhibitor were purchased from Worthington Biochemical Corp. (Freehold, N. J.).

## RESULTS

### *Comparison of 7-Day and 4-Day*

#### *Embryonic Spinal Cords*

There is a great increase in spinal cord size over the 72-h period between embryonic day 4 and day 7. Whole spinal cords (tail-to-brainstem) from 4-day and 7-day embryos contained about 6 μg and 15 μg of DNA, respectively. 1.0–1.5 × 10<sup>6</sup> intact cells could be liberated from 4-day cords and 3.5–4.0 × 10<sup>6</sup> cells could be liberated from 7-day cords by mild trituration of trypsinized tissue fragments, so the relative cell yield (number of isolated cells per microgram of DNA) was similar at the two ages.

Most cells dissociated from 4- or 7-day cords measured between 7 μm and 10 μm in diameter. However, the ranges of cell sizes in the two populations were quite different. In the experiment shown in Fig. 1, the distribution of 4-day

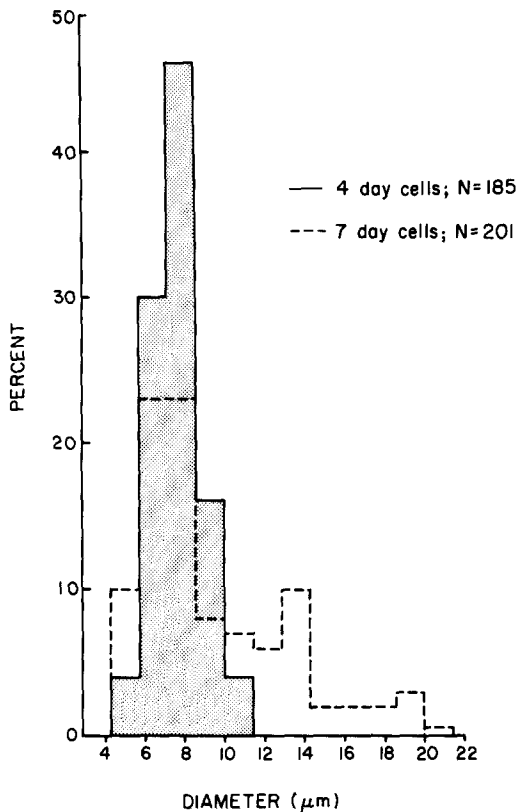


FIGURE 1 Normalized histograms of diameters of freshly dissociated 4-day and 7-day spinal cord cells. The cells were examined and measured under phase-contrast illumination at  $500\times$  magnification.

diameters was symmetric with a mean of  $7.7\ \mu\text{m}$ . No cells larger than  $12\ \mu\text{m}$  were observed. In contrast, the distribution of 7-day cell diameters were skewed to the right. The arithmetic mean was only  $9.3\ \mu\text{m}$ , but 23% of the cells were larger than  $12\ \mu\text{m}$  in diameter. Our first experiments were designed to isolate and culture large 7-day spinal cord cells.

### Separation of Large 7-Day Embryonic Spinal Cord Cells by Velocity Sedimentation

**CELL SEPARATION:** 7-Day spinal cord cells dissociated by vigorous trituration in supplemented D<sub>1</sub>G were allowed to settle through a shallow, "buffered-step" Ficoll gradient made up in MEM as described in Materials and Methods (Fig. 2). After 3 h at  $4^\circ\text{C}$ , the majority of cells had migrated about 1 centimeter. In different experiments, between 50 and 80% of the cells

applied to the column were recovered in the collected fractions. As shown in Fig. 3, leading fractions contained many large cells whereas trailing fractions were essentially devoid of large cells. Each 2.4-ml fraction occupied 1.0 mm on the column, so the smallest and largest cells shown in Fig. 3 were separated by 11 mm. Histograms of cell diameters in two leading and two trailing fractions are shown in Fig. 4. Assuming that the cells are spheres, the mean cell volume in leading fractions was about three times that of cells in trailing fractions. Thus, velocity sedimentation was effective in separating cells according to size.

In four experiments,  $52 \pm 8\%$  (mean  $\pm$  SEM) of the cells in leading fractions were larger than  $12\ \mu\text{m}$  while only  $7 \pm 2\%$  of the cells in control (vigorously dissociated) suspensions fell in this class. The discrepancy between the relative number of large cells found in the population of vigorously dissociated cells (7%) and that found in the population dissociated by more gentle means (23%) presumably reflects the rigors of dissociation and the fact that the former cells were kept at  $4^\circ\text{C}$  for 3 h before measurement.

Since the number of cells in leading and trailing fractions was small, 2-3 fractions were pooled for most experiments. Pooled leading fractions and pooled trailing fractions will be called large cell fractions (LCFs) and small cell fractions (SCFs), respectively. Some resolution was sacrificed by this procedure: mean cell volumes for LCFs and SCFs differed by twofold.

If LCFs are enriched with motoneurons, they should contain a higher specific activity of choline acetyltransferase (CAT) than do SCFs. LCFs and

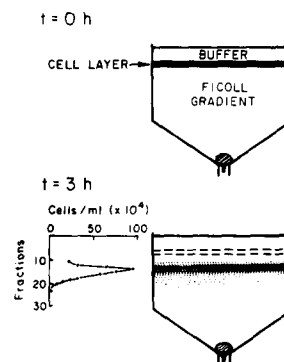


FIGURE 2 Schematic representations of the 1g velocity sedimentation column immediately after loading ( $t = 0$  h) and after 3 h at  $4^\circ\text{C}$  ( $t = 3$  h). Cell counts are shown on the left. Each fraction (2.4 ml) represents 1 mm along the column.

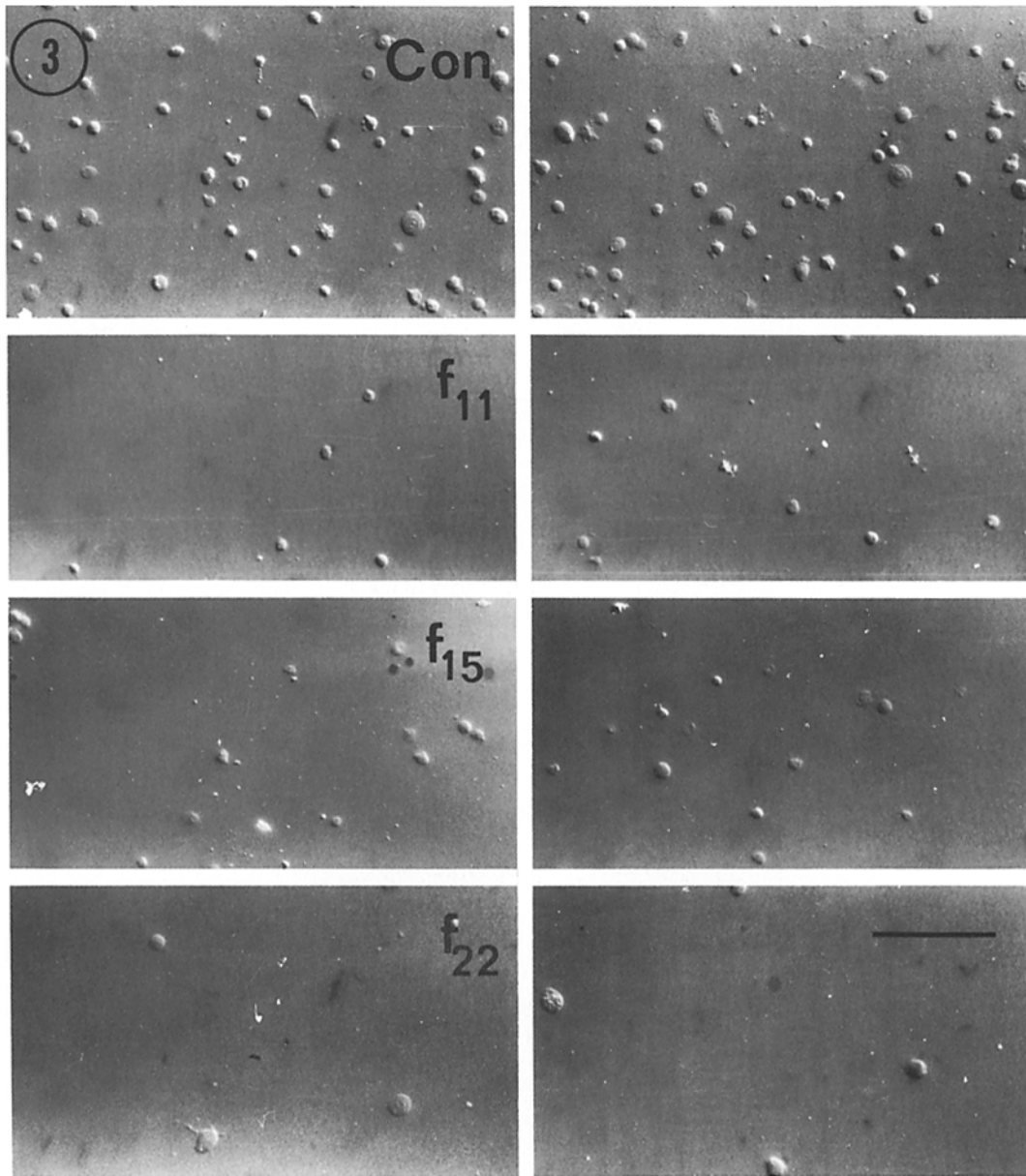


FIGURE 3 Representative 7-day spinal cord cells in various fractions from the same experiment shown in Fig. 2. *Con*: control (vigorously dissociated) cells before sedimentation;  $f_{11}$ ,  $f_{15}$ , and  $f_{22}$ : cells from fractions 11, 15 and 22, respectively. Interference contrast optics. Bar, 100  $\mu\text{m}$ .

SCFs were concentrated by low-speed centrifugation, and the recovered cell pellets were assayed for CAT activity. Differences in mean cell volume were taken into account by dividing the measured CAT activity by the amount of recovered lactic dehydrogenase (LDH), a soluble cytoplasmic enzyme. In one experiment, the CAT/LDH ratio in

LCFs was 2.53, and in SCFs it was 0.27. In another experiment, the LCF ratio was 2.62, and the SCF ratio was 0.72. Thus, on the average the specific activity of CAT (CAT/LDH) was sevenfold higher in LCFs than in SCFs, and this striking difference supports the notion that leading fractions are enriched with cholinergic neurons. Fur-

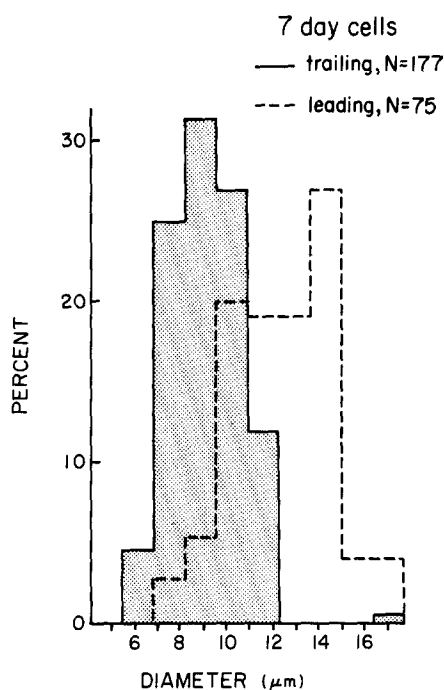


FIGURE 4 Normalized histograms of diameters of 7-day spinal cord cells in two trailing ( $f_{11}$  and  $f_{18}$ ) and two leading ( $f_{20}$  and  $f_{22}$ ) fractions from the same experiment shown in Figs. 2 and 3. The cells were photographed under phase-contrast illumination, and measurements were made from projected negatives.

ther support for this interpretation came from the subsequent development of LCFs and SCFs in cell culture.

**DEVELOPMENT IN CULTURE:** Neurons survived vigorous dissociation and sedimentation at 4°C; more than 95% of the cells in each fraction excluded trypan blue. Aliquots (0.5-1.0 ml) of LCFs and SCFs containing  $2 \times 10^5$  cells were usually added directly to ara C-treated muscle cultures in 35-mm culture dishes containing 1.0 ml of complete medium. Thus, the cultures contained a small amount of Ficoll until the first feeding 2-3 days later. Control experiments indicated that growth in this amount of Ficoll did not affect neuronal survival or the level of CAT activity.

Differences in cell size between LCFs and SCFs were maintained in culture. Several days after plating, LCF-muscle cultures contained many large, refractile, multipolar neurons whereas small-phase-dark bipolar and tripolar cells predominated in SCF-muscle cultures. Typical examples are shown in Figs. 5 and 6. At this time,

spinal-cord, fibroblast-like cells were either few in number or absent altogether. In a few platings, these non-neuronal cells apparently proliferated more rapidly and, ultimately, formed a confluent mat. We cannot account for this variability but, when present, non-neuronal cells were found in LCF and SCF cultures in about equal number. Failure to segregate proliferating non-neuronal cells by velocity sedimentation may reflect variation in cell size in different phases of the mitotic cycle. Alternatively, a few non-neuronal cells may aggregate or adhere to large and small neurons.

**CAT ACTIVITY:** CAT activity in LCF-muscle cultures, assayed 1 wk after plating, was about 6 times higher than in control spinal cord-muscle cultures and 10-70 times higher than in SCF-muscle plates (Fig. 7). The difference in initial mean cell volume between LCFs and SCFs can account for only a twofold difference in CAT activity. Although estimates of CAT activity in different cultures from the same plating were in close agreement, the level of CAT varied considerably from plating to plating. Nevertheless, the striking difference between LCF, SCF, and control cultures was evident in each experiment (Fig. 7). The specific activity of CAT was not measured since, in spinal cord-muscle co-cultures, most of the protein is located within mature, striated muscle fibers. CAT activity per culture was used as an index instead because the muscle plates were seeded with the same number of neurons (LCF, SCF, or control) and, at the time of assay, each plate contained about the same number of neurons (estimated by counting perikarya under phase-contrast illumination).

The radioactive product recovered in the organic phase of the Fonnum CAT assay was ACh. Omission of choline from the reaction mixture or substitution of acetylcholinesterase (7 U) for neostigmine reduced the extracted counts to background levels. The amount of radioactivity that co-migrated with carrier ACh on paper electrophoresis was equal to that extracted in the Fonnum assay.

In some tissues, ACh synthesis reflects the activity of carnitine acetyltransferase rather than CAT (39, 34). In the absence of carnitine, this enzyme can utilize choline as a substrate. Carnitine acetyltransferase was assayed in LCF-muscle, SCF-muscle and control muscle plates by substituting 1.25 mM carnitine for choline and identifying the products by paper electrophoresis. High levels of activity were present, but the activity was

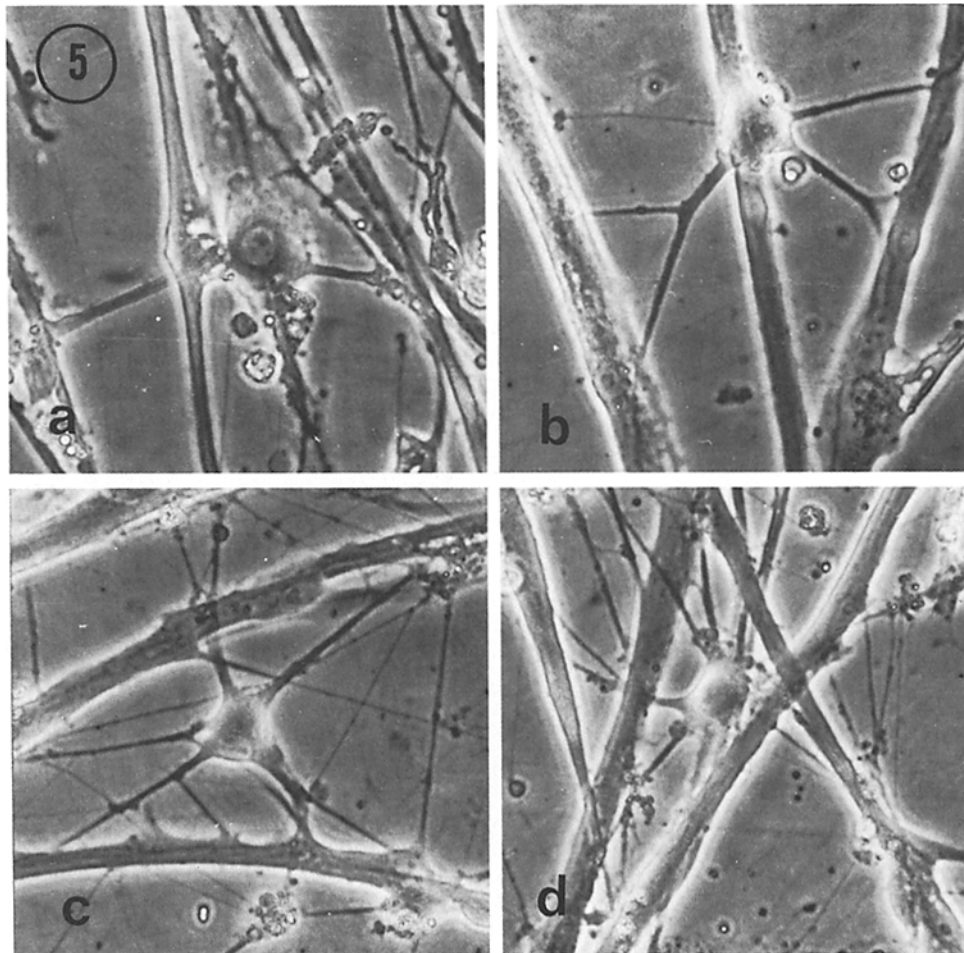


FIGURE 5 7-day LCF neurons in vitro. Phase-contrast micrographs of typical large refractile neurons in LCF-muscle co-cultures 12 days (*a*) and 7 days (*b-d*) after plating the spinal cord cells. Smaller, phase-dark neurons (not shown) were also present in these cultures. Bar (in Fig. 5*b*), 50  $\mu$ m, applies to all.

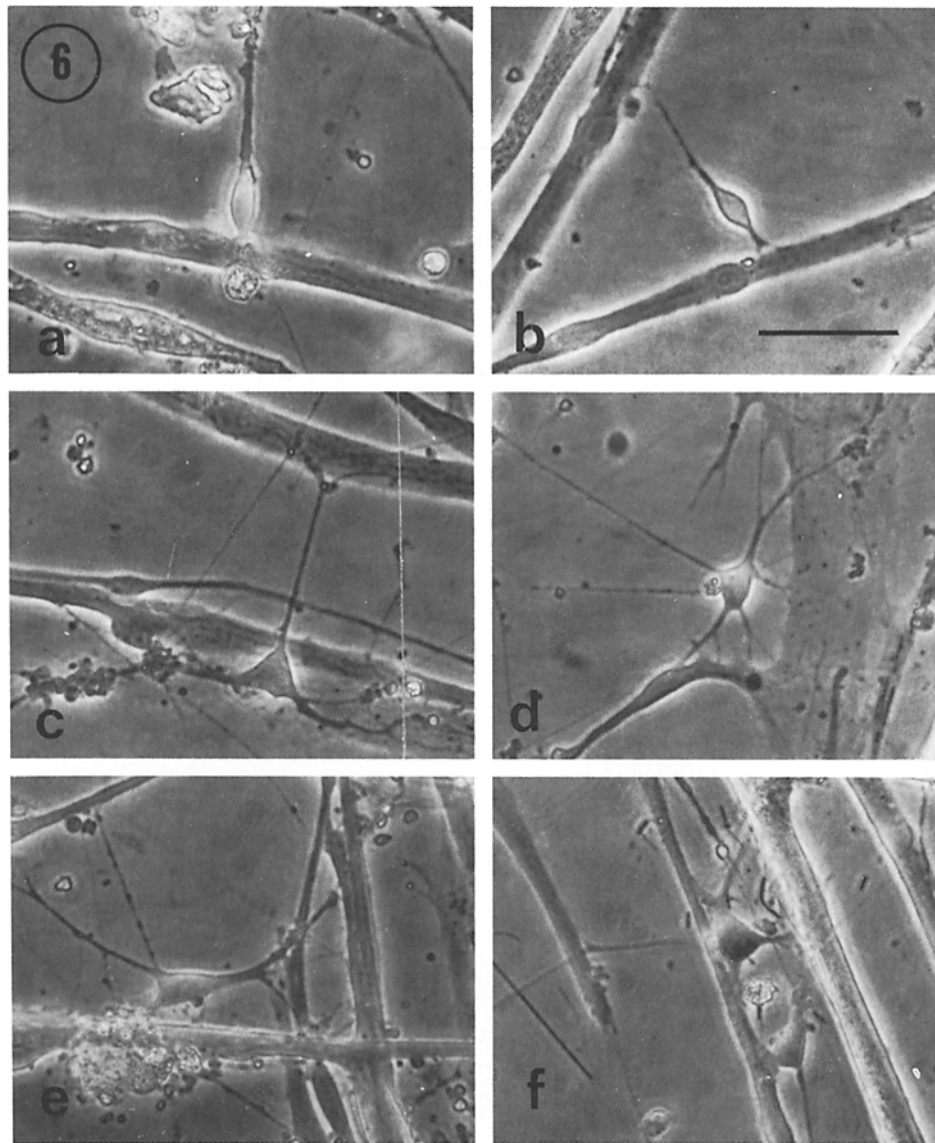
the same in LCF-muscle and SCF-muscle plates and in muscle cultures that contained no neurons. Addition of carnitine to the normal CAT assay (containing 1.25 mM choline) did not depress the amount of ACh synthesized, and under these conditions, 0.5 mM naphthyl(vinyl)-pyridine (NVP), a specific inhibitor of CAT (38), reduced the synthesis of ACh by 85-90% without inhibiting the synthesis of acetylcarnitine (Fig. 8). Thus, ACh synthesis in LCF-muscle and SCF-muscle extracts reflects CAT activity.

It is unlikely that the low levels of CAT activity in SCF-muscle culture homogenates is due to the presence of an enzyme inhibitor unique to cells in trailing fractions. No inhibition of CAT was observed when extracts of SCF-muscle cultures were

mixed with extracts of LCF-muscle cultures. It is also unlikely that LCFs contain a cell type that can induce CAT activity in all spinal cord neurons. Cells from LCFs and SCFs were mixed together before plating and after 1 wk the CAT activity in these cultures was compared to that in LCF-muscle and SCF-muscle cultures plated with the same number of cells. In one experiment, LCF-muscle, SCF-muscle and (LCF + SCF) muscle cultures contained 5.34, 0.14, and 2.69 U of CAT activity, respectively, and, in another, the results were 2.32, 0.30, and 1.13. Thus, the CAT activity in mixed cultures is simply the average of activities found in unmixed plates.

**NERVE-MUSCLE SYNAPSES:** Neuromuscular junction formation in LCF-muscle and SCF-mus-



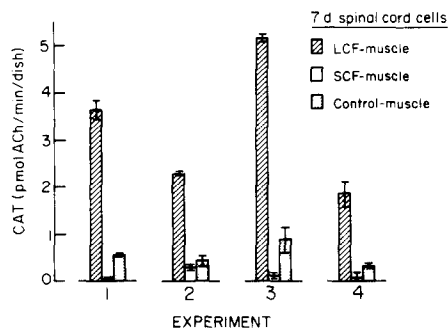


**FIGURE 6** 7-day SCF neurons in vitro. Phase-contrast micrographs of typical bipolar and multipolar cells in SCF-muscle co-cultures 11 days after plating the spinal cord cells. The neurons shown in (e) and (f) are among the largest found in SCF-muscle plates. Bar (in Fig. 6b), 50  $\mu\text{m}$ , applies to all.

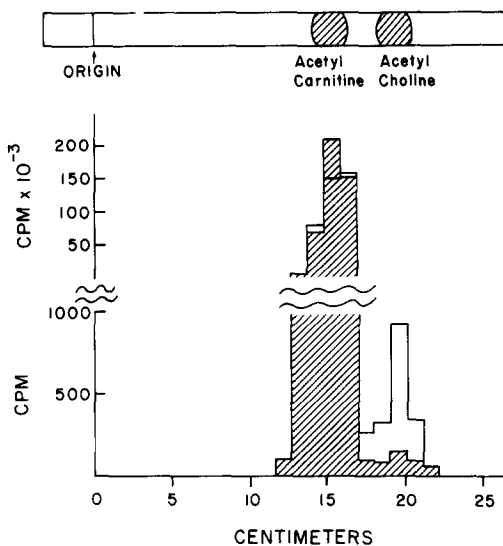
cle cultures was compared by determining the percentage of neurons that innervated nearby myotubes and also by determining the percentage of myotubes that exhibited spontaneous synaptic potentials.

In the first assay, a single neuron was stimulated with an extracellular electrode (2–3  $\mu\text{m}$  tip) while an intracellular electrode was used to record from one or more myotubes contacted by a process of

that neuron. Effective stimuli ranged between 0.5 and 5.0  $\mu\text{A}$  and 0.5 and 2.0 ms. When the electrode was moved 5  $\mu\text{m}$  away from the target neuron the evoked response was lost, so it is unlikely that other neurons were directly excited by the stimulating current. A series of evoked synaptic, “endplate” potentials (epps) is shown in Fig. 9. Some epps were large enough to trigger muscle action potentials. However, in every case,



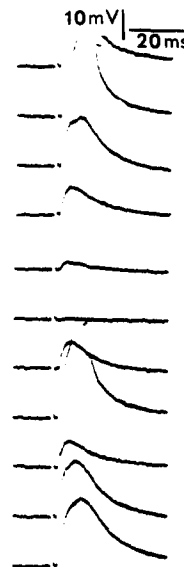
**FIGURE 7** CAT activity in LCF-muscle, SCF-muscle and control 7-day spinal cord-muscle cultures assayed 1 wk after addition of neurons. Control cells were vigorously dissociated but not sedimented. Each experiment represents a separate dissociation, sedimentation, and plating. Background values observed in muscle cultures without added spinal cord neurons were equivalent to 0.2–0.4 pmol ACh/min/culture and were subtracted in each case. We did not determine whether the background radioactivity was in fact ACh. Each column represents the mean of duplicate cultures. Vertical lines indicate ranges.



**FIGURE 8** Selective inhibition of ACh synthesis by NVP. Paper electrophoresis of an LCF-muscle culture homogenate incubated with 1.25 mM choline, 1.25 mM carnitine and [<sup>3</sup>H]acetyl Co-A (see Materials and Methods). The upper strip indicates the site of sample application (origin; cathode) and the final positions of unlabeled acetylcholine and acetylcarnitine standards. The distribution of label determined by scintillation counting is shown in register below. Hatched area—with 0.5 mM NVP added to the reaction mixture. Clear area—without the inhibitor. Note the break in the ordinate.

the size of successive epps fluctuated, and several failures were recorded. One explanation for this variability is that the mean number of quanta released per impulse was low (cf. reference 9).

In different LCF-muscle cultures, between 10 and 50% of the neurons tested evoked synaptic potentials in one or more muscle fibers. The overall incidence was 22% (Table I). In contrast, none of the neurons stimulated in SCF-muscle cultures evoked synaptic potentials in contacted



**FIGURE 9** Oscilloscope traces of consecutive synaptic potentials (endplate potentials: epps) recorded in a myotube while a nearby LCF neuron was stimulated. Stimuli were delivered through an extracellular electrode at a rate of 0.5 Hz. The break in each record immediately before the epp is the stimulus artifact. There was one "failure" in this short series, and 3 epps evoked muscle action potentials that disappear off the screen.

**TABLE I**  
*Nerve-Muscle Synapses in 7-Day LCF-Muscle and SCF-Muscle Cultures*

Culture	Evoked	Spontaneous
LCF-muscle	20/91 (22%)	28/86 (33%)
SCF-muscle	0/77 (0%)	2/71 (3%)

*Evoked:* The number of stimulated neurons that generated synaptic potentials in at least one muscle fiber/the total number of stimulated neurons.

*Spontaneous:* The number of myotubes that exhibited spontaneous synaptic potentials/the total number of myotubes tested. The data were pooled from seven cultures in each category examined between 6 and 12 days after addition of neurons.

muscle cells. A few experiments in cultures seeded with vigorously dissociated but unsedimented cells indicated that less than 5% of the neurons tested innervated nearby myotubes. These estimates are biased somewhat in that the smallest neurons were usually ignored, and neurons larger than 15–20  $\mu\text{m}$  in diameter with more than two processes were selected. However, the same type of neuron was selected in LCF and control cultures, and the largest neurons present in SCF cultures were sought out.

In the second assay, muscle fibers were selected at random, and the field of view was shifted after each trial to ensure that the same myotube was not penetrated twice. Large and small spontaneously occurring synaptic potentials recorded in a muscle fiber in a LCF-muscle culture are shown in Fig. 10. Spontaneous synaptic potentials were detected in 2 of 71 muscle fibers co-cultured with SCF cells, but this was far less than the incidence of spontaneous activity observed in LCF-muscle cultures (Table I). Thus, both assays indicate that LCFs contain many more neurons that are capable of innervating muscle fibers than do SCFs.

#### Four-Day Embryonic Spinal Cord Cells

**DEVELOPMENT IN CULTURE:** Four-day embryonic spinal cord cells survived enzymatic digestion and gentle mechanical dissociation. More than 95% of the cells excluded trypan blue, and the plating efficiency of 4-day cells was comparable to that of 7-day cells dissociated in the same gentle manner. Freshly dissociated, 4-day cells are smaller than 7-day cells on the average (see Fig. 2), and this size disparity remained evident 1–2 days after plating. However, after 1–2 wk in vitro, many large refractile multipolar neurons (Fig. 11) were present in 4-day spinal cord-muscle cultures.

**CAT ACTIVITY:** Cultures seeded with 4-day cells had about two to three times as much CAT activity as cultures seeded with the same number

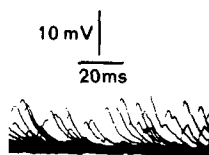


FIGURE 10 Superimposed oscilloscope traces showing spontaneous synaptic potentials recorded in a myotube in an LCF-muscle culture. Large and small potentials probably reflect spontaneously occurring epps and mepps, respectively (9).

of 7-day cells. This difference was evident at the earliest time tested (3 days) and at the latest (2 wk). The results obtained 1 wk after addition of neurons are shown in Fig. 12. The same variation in CAT activity between platings encountered with vigorously dissociated 7-day cells was found in these experiments as well.

**NERVE-MUSCLE SYNAPSES:** Nerve-muscle synapse formation was assayed by recording stimulus evoked and spontaneous synaptic activity as described above. After 1 wk, about 50% of the relatively large neurons in 4-day spinal cord-muscle cultures evoked synaptic potentials in nearby muscle fibers, whereas only about 10% of the large neurons in 7-day spinal cord-muscle plates innervated nearby muscle cells (Table II). The difference in the incidence of spontaneous synaptic potentials in randomly chosen myotubes between cultures seeded with 4- and 7-day cells, was not as striking. This reflects the unusually high (in our experience) proportion of innervated myotubes in the sample drawn from 7-day cultures.

#### Nerve-Nerve Synapses

Intracellular recordings from large neurons in LCF and in 4-day spinal cord cultures 1 wk after plating showed that these cells received synaptic input. An example of a depolarizing synaptic potential evoked by stimulating a small bouton overlying on LCF nerve cell body is shown in Fig. 13. Hyperpolarizing potentials were detected in some cells (Fig. 14). Synaptic potentials were also detected on intracellular recording from typical SCF neurons like those shown in Fig. 6c–f. Thus, although few, if any, SCF neurons formed synapses on muscle fibers, they were able to innervate one another. No attempt was made to quantitate interactions between these cells. We did not record successfully from small bipolar cells like those shown in Fig. 6a, and b, so it is not certain whether they are neurons.

#### DISCUSSION

Relatively large neurons can be separated from a suspension of 7-day chick embryonic spinal cord cells by 1-g velocity sedimentation. Even though leading (and trailing) fractions were pooled into LCFs (and SCFs) with some loss of resolution, muscle cultures seeded with LCFs contained 10–70 times as much CAT activity as SCF-muscle cultures, and 6 times as much CAT as cultures seeded with 7-day cells that were treated in the same manner but not subjected to velocity sedi-

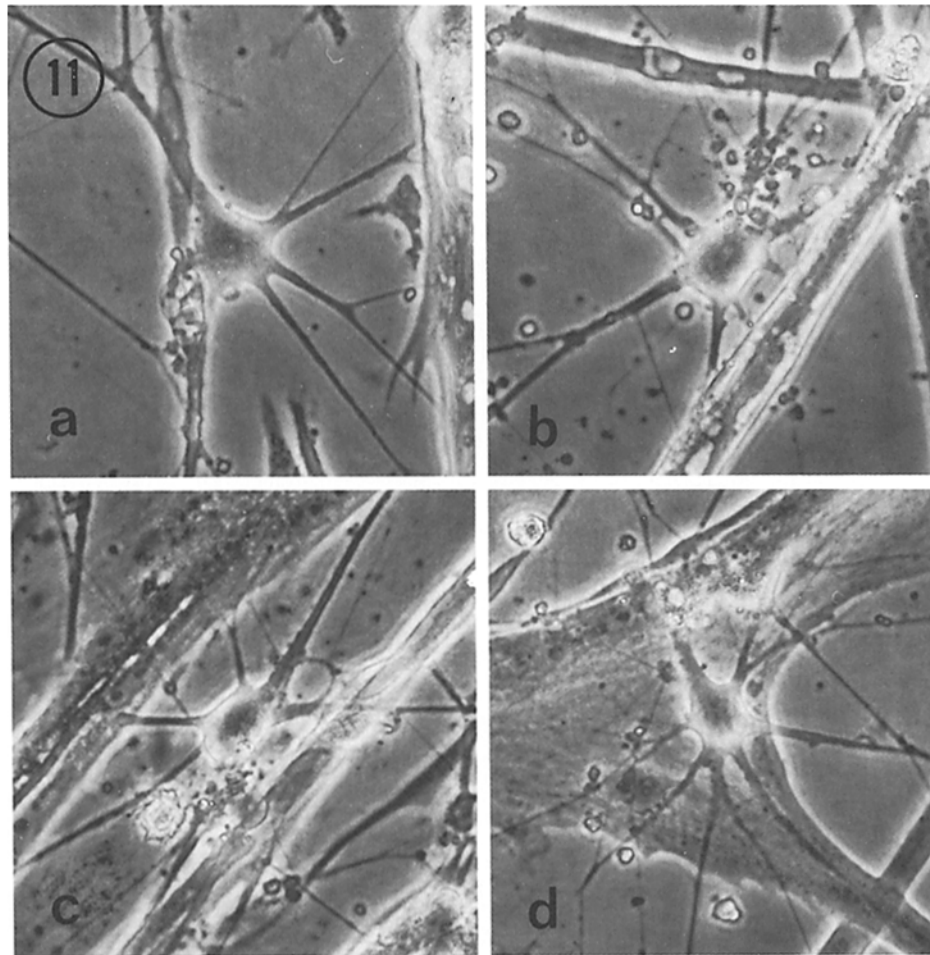


FIGURE 11 4-day spinal cord neurons. Phase-contrast micrographs of typical large neurons in 4-day spinal cord-muscle cultures 7 days after plating the spinal cord cells. Some small phase-dark neurons (not shown) were also evident in these cultures. Bar in Fig. 11 *b*, 50  $\mu\text{m}$ , applies to all.

mentation. The incidence of nerve-muscle synapse formation determined electrophysiologically in LCF-muscle and SCF-muscle cultures paralleled the distribution of CAT activity.

The simplest interpretation of these data is that large cells in leading fractions include a functionally distinct, e.g., cholinergic, class of neurons. The fact that the activity of CAT per unit cytoplasm is sevenfold higher in large cells than in small cells before plating is consistent with this interpretation. An alternative explanation, that cell size, CAT activity and nerve-muscle synapse formation are simply measures of health or maturity rather than markers for cholinergic function, is less likely. Neurons in SCF-muscle cultures are healthy and mature in the sense that they extend

processes and grow in size over the period observed, and at least some of the multipolar cells receive synaptic input. In addition, more than 50% of the SCF neurons concentrate GABA by a high-affinity transport mechanism (Farb, D., D. K. Berg, and G. D. Fischbach, unpublished observations).

In order to completely dissociate 7-day spinal cord cells before velocity sedimentation, the minced tissue fragments were subjected to vigorous trituration in a  $\text{Ca}^{++}$ -,  $\text{Mg}^{++}$ -free medium. This plus the 3-h period at  $4^\circ\text{C}$  probably accounts for the fact that fewer cells greater than 12  $\mu\text{m}$  in diameter were found in vigorously dissociated control suspensions than in suspensions prepared by gentle trituration in complete medium. The

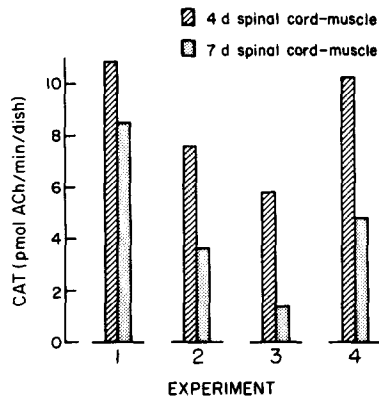


FIGURE 12 CAT activity in 4-day spinal cord-muscle and 7-day spinal cord-muscle cultures assayed 1 wk after addition of neurons. The spinal cords were dissociated in parallel by relatively gentle means compared to the vigorous techniques used in the experiments shown in Fig. 7 (see Materials and Methods). Each column represents the mean of two to three plates. Background values and ranges were comparable to those shown in Fig. 7.

severity of the dissociation procedure probably also explains why cultures seeded with vigorously dissociated 7-day cells developed less CAT than cultures seeded with gently dissociated 7-day cells (cf. Figs. 7 and 12) and why a slightly lower

percentage of the relatively large neurons innervated nearby myotubes (5% vs. 12%). This, of course, does not compromise the utility of 1-g velocity sedimentation for achieving a relative increase in the number of cholinergic neurons. On the average, about 20% of the relatively large neurons in LCF-muscle cultures innervated nearby myotubes. This represents approximately a twofold increase compared to gently dissociated 7-day spinal cord cells. It seems likely that improvement in cell dissociation and sedimentation techniques will result in an even greater recovery of large cholinergic neurons.

We are unaware of previous attempts to culture neurons dissociated from 4-day neural tubes. Our

TABLE II  
*Nerve-Muscle Synapses in Muscle Cultures Seeded with 4-Day and 7-Day Spinal Cord Cells*

Culture	Evoked	Spontaneous
4-day SC-muscle	37/72 (51%)	35/63 (56%)
7-day SC-muscle	6/50 (12%)	11/40 (28%)

Entries are as described in Table I. Cultures were examined 6–12 days after addition of either 4-day or 7-day (gently dissociated) embryonic spinal cord cells to muscle cultures. Data were pooled from eight 4-day cultures and from five 7-day cultures.

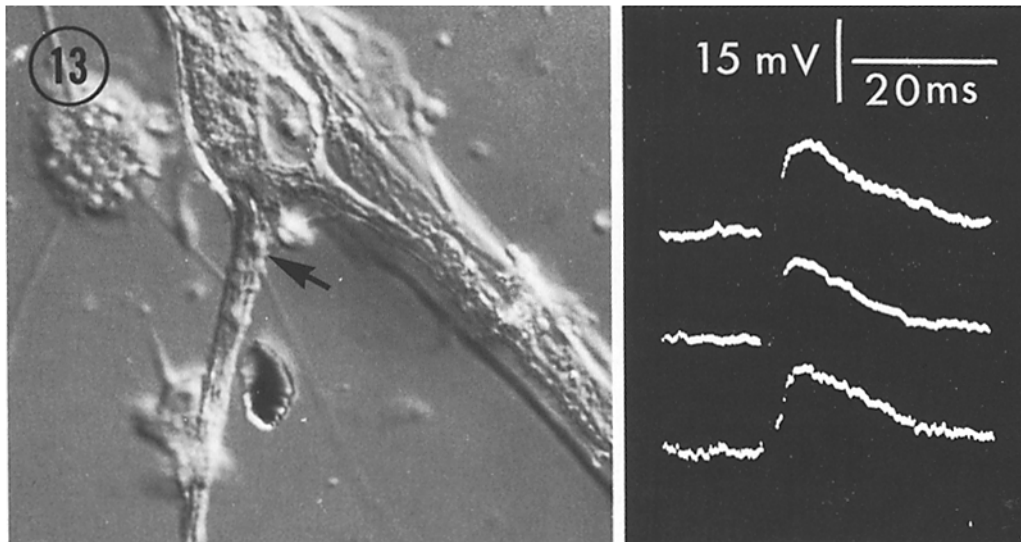


FIGURE 13 Interneuronal synaptic potentials. An interference contrast micrograph of LCF neuron partly overlying a myotube is shown on the left. A fine, incoming neurite appears to terminate in a small bouton at the base of one of the thick processes (arrow). Focal extracellular stimulation at that site evoked the sequence of depolarizing synaptic potentials shown on the right. The initial breaks in the rising phases indicate the stimulus artifacts.

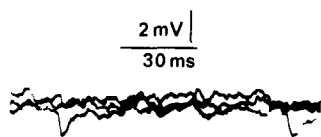


FIGURE 14 Superimposed oscilloscope traces showing spontaneously occurring, hyperpolarizing synaptic potentials recorded in a relatively large 4-day spinal cord neuron.

results indicate that the neurons are apparently not completely dependent for survival on the *in vivo* environment at this stage. On the contrary, 4-day cells grew rapidly in size *in vitro* and, after 1 wk, many were as large as the largest 7-day spinal cord cells prepared in the same (gentle) way and plated at the same time. Muscle cultures seeded with 4-day cells developed more than twice as much CAT activity as cultures seeded with gently dissociated 7-day cells, and they contained more than four times the number of neurons that innervated muscle. It is quite possible that an even greater enrichment with motoneurons can be achieved by plating cells from even younger neural tubes.

The relative increase in cholinergic function observed in cultures seeded with 4-day embryonic spinal cord cells compared to 7-day spinal cord cultures can be accounted for if it is assumed that only those neurogenic cells that have already undergone their final mitosis *in vivo* survive and mature *in vitro*. Motoneurons, like other large neurons throughout the neural tube, are among the first to withdraw from the mitotic cycle and migrate into the mantle layer (16, 13, 20, 36). Several observations indicate that newborn motoneurons in 4-day spinal cords have already achieved some degree of differentiation. Bursts of action potentials have been recorded in brachial segments of 4-day cords with extracellular electrodes (30). At this time, some of the brachial motoneurons have extended axons outside of the neural tube (24) and established functional contacts on skeletal muscle cells (17, 31). Contractions within the hindlimb muscle mass after stimulation of lumbar spinal nerves have been observed in 5.5-day (Stage 27) chick embryos (22). It is likely that synapses had been established in this region at an earlier time. Few data are available concerning the ability of proliferating neuronal precursor cells to survive *in vitro*.

Even if one assumes that neurons which innervate skeletal muscle *in vitro* were specified for

cholinergic function *in ovo*, it is not certain whether they are all  $\alpha$ -motoneurons derived from the motor columns in the anterior horn. In the chick, visceral preganglionic neurons appear at about the same time as somatic motor neurons, and by day 7 *in ovo* they have formed distinct "motor" columns in thoraco-lumbar segments (23). These preganglionic neurons are cholinergic and, in other species, they have been shown to be capable of innervating skeletal muscle (21, 2). In more mature animals, preganglionic neurons are small compared to somatic motoneurons, but it is possible that some of the large 4-day embryonic spinal-cord or 7-day LCF cells derived from this source.

Although survival of "committed" cholinergic neurons can account for our results, the development of CAT and the number of cholinergic synaptic interactions may be influenced by the culture conditions. Media that are conditioned by skeletal myotubes or by fibroblasts can induce an increase in CAT activity in cultured mouse spinal cord cells after 2-3 wk (15, 26). Media conditioned by non-neuronal cells from a variety of sources can induce rat sympathetic neurons (that would otherwise synthesize norepinephrine) to synthesize and store ACh (28, 29, 32). Synaptic interactions between such induced sympathetic cells are mediated by ACh (27, 14). We did not observe a consistent difference in CAT activity between chick spinal cord cells (4 day or 7 day) grown with or without muscle fibers after 1 wk *in vitro*. Further studies at longer times after plating are needed.

It should be emphasized that while 7-day LCFs and suspensions prepared from 4-day cords are enriched with cholinergic neurons, they are not pure populations. A few small 7-day cells appeared in leading fractions collected from the 1-g column, and small bipolar and tripolar neurons were present in LCF cultures. Small neurons were also found in 4-day spinal cord cultures. In addition, the fact that 7-day LCF and 4-day neurons received synaptic input indicates the presence of noncholinergic neurons in established cultures. Few, if any, of the large spinal cord neurons are sensitive to iontophoretically applied ACh (Fischbach, G. D., unpublished data), so the majority of the synaptic potentials presumably are mediated by some other transmitter. It might be argued that the interneuronal synaptic activity originated entirely within the population of small neurons and that the large neurons are all cholin-

ergic. However, other evidence indicates that this is not the case. The majority of large neurons failed to evoke epps in nearby myotubes. This assay might underestimate the number of motoneurons, but it seems unlikely that false negatives can account for all of the failures. This impression is supported by the finding that the percentage of myotubes that exhibited spontaneous synaptic activity in the various cultures paralleled the percentage of motoneurons. Another argument for heterogeneity within the populations of large cells is that many large 4-day and LCF neurons (but none which innervate nearby myotubes) concentrate  $^3\text{H}$ -GABA by a high-affinity uptake mechanism (7).

In any case, 1-g velocity sedimentation of 7-day spinal cord cells and the plating of 4-day cells provide useful first steps in attempts to purify cholinergic neurons that are capable of innervating skeletal muscle. Even without additional manipulations, these procedures should facilitate studies of the development of cholinergic spinal cord neurons and of neuromuscular junctions. A unique virtue of separation of 7-day cells is that a population of small neurons essentially devoid of cholinergic neurons can be obtained. A practical virtue of the use of 4-day embryos is the relative ease of the technique.

We are grateful for the expert technical assistance of Ruth L. Siegel.

This work was supported by United States Public Health Service Research grant NS 11160 from the National Institute of Neurological and Communicative Disorders and Stroke and by a Medical Foundation (Massachusetts) Fellowship.

Received for publication 27 June 1977, and in revised form 9 December 1977.

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