CHOLINE ACETYLTRANSFERASE ACTIVITY OF SPINAL CORD CELL CULTURES INCREASED BY CO-CULTURE WITH MUSCLE AND BY MUSCLE-CONDITIONED MEDIUM

E. L. GILLER, Jr., J. H. NEALE, P. N. BULLOCK, B. K. SCHRIER, and P. G. NELSON

From the National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT

Activity of the enzyme choline acetyltransferase (CAT), which mediates the synthesis of the neurotransmitter, acetylcholine, was increased up to 20-fold in spinal cord (SC) cells grown in culture with muscle cells for 2 wk. This increase was directly related to the duration of co-culture as well as to the cell density of both the SC and muscle involved and was not affected by the presence of the acetylcholine receptor blocking agent, α-bungarotoxin. Glutamic acid decarboxylase (GAD) activity was often markedly decreased in SC-muscle cultures while the activities of acetylcholinesterase and several other enzymes were little changed.

Increased CAT activity was also observed when SC cultures were maintained in medium which had been conditioned by muscle cells or by undifferentiated cells from embryonic muscle. Muscle-conditioned medium (CM) did not affect the activities of SC cell GAD or acetylcholinesterase. Dilution or concentration of the CM directly affected its ability to increase SC CAT activity, as did the duration and timing of exposure of the SC cells to the CM. The medium could be conditioned by muscle cells in the presence or the absence of serum, and remained effective after dialysis or heating to 58°C. Membrane filtration data were consistent with the conclusion that the active material(s) in CM had a molecular weight in excess of 50,000 daltons. We conclude that large molecular weight material that is released by muscle cells is capable of producing a specific increase in CAT activity of SC cells.

Neurons and their target cells appear to interact in a complex reciprocal manner. These interactions have been most readily established in the neuromuscular system, where denervation results in numerous changes in muscle cell physiology (1, 6), in the visual system, where spatial and temporal relationships influence retinotectal innervation patterns (8, 23), and in the sympathetic nervous system, where the postganglionic axonal pathways appear to subserve bidirectionally significant regulatory processes (2, 7, 20). While these types of interactions may be mediated by neurotransmitters such as acetylcholine, or by larger molecules, such as nerve growth factor, the constraints imposed in vivo by the close physical association between cells whose interactions are demonstrable, militate against characterization of the trophic molecules involved. In contrast, the study of interacting cell systems in vitro obviates this difficulty and, as a result, a variety of techniques—circumfu-
 leased by muscle into its culture medium. The objectives of such approaches have included the identification of significant interactions between different cell types and the characterization of the molecular events mediating these interactions. Because of the possibility that responses observed in vitro result from requirements peculiar to the cell culture conditions, the ultimate significance of such studies may be derived from an extension of these observations, once defined, to in vivo systems.

Using the co-culture approach to study the interactions between murine spinal cord (SC) cells and skeletal muscle, we have observed (4) an increase with time in the number of detectable synaptic interactions between nerve and muscle cells and marked increases in the activity of choline acetyltransferase (CAT, EC 2.3.1.6), the enzyme which mediates the synthesis of the neuromuscular transmitter, acetylcholine. The increased CAT activity was shown not to be due to a general facilitation of cell survival since the activity of the four other enzymes assayed as well as total culture protein and DNA were relatively unaffected by the co-culture conditions. The survival of morphologically identified presumptive neurons was similar whether the cells were grown alone or with muscle (4). The objective of the present study was to examine this interaction between cultured SC and muscle in terms of (a) the quantitative relationships between muscle and nerve, (b) the effects of co-culture on another neurotransmitter-related enzyme, glutamic acid decarboxylase (GAD), and (c) the stimulation of a similar increase in SC cell CAT activity by a factor(s) released by muscle into its culture medium.

MATERIALS AND METHODS

Cultures

Pregnant C57Bl/6 mice were obtained from the National Institutes of Health colony or from Microbiological Associates (Bethesda, Md.). SC cultures were produced from mouse embryos at 12-14 days in utero; heart (atrium) and skeletal muscle cell cultures were made from term embryos. The adult mice were anesthetized with CO2 or chloroform and sacrificed by cervical dislocation (17), extract addition (5, 10, 12), and co-culture (13, 15, 21)—have been successfully applied to cultured cells. The objectives of such approaches have included the identification of significant interactions between different cell types and the characterization of the molecular events mediating these interactions. Because of the possibility that responses observed in vitro result from requirements peculiar to the cell culture conditions, the ultimate significance of such studies may be derived from an extension of these observations, once defined, to in vivo systems.

The hindlimb suspension was enriched for myoblasts by preplating for 20 min in 60-mm diameter plastic culture dishes. Unattached cells were then filtered through 130-µm-pore nylon mesh (Nitek, Silk Screen Supplies, Inc., Brooklyn, N. Y.) to remove undissociated tissue. At this stage of preparation the tissue suspensions contained predominantly single cells with rare 2- to 10-cell clumps. The cells were more than 90% viable as determined by nigrosin stain exclusion. Except as noted in the individual experiments, 3 X 10⁶ SC cells or 0.7-0.8 X 10⁶ skeletal muscle cells were inoculated per 60-mm dish, while 1 X 10⁶ SC cells or 0.25 X 10⁶ muscle cells were plated on 35-mm dishes. Heart cells were not counted; 3 ml of the suspension (equivalent to three embryonic hearts) were plated per 60-mm dish. All culture dishes were coated with a thin, air-dried film of acid-soluble, calf skin collagen (A grade, Calbiochem).

Myoblast-fibroblast cultures were prepared from muscle cultures by multiple subculturings (using trypsin) to remove the myoblasts. In some experiments, cells from established muscle cultures were added to SC cultures or pooled to produce cultures with more myoblasts per dish. For these experiments, the myoblast cultures were removed from the dishes by incubation for 1/2 h at 37°C in DISGH containing 10% heat-inactivated horse serum and 1 mg/ml collagenase (Worthington Biochemical Corp., Freehold, N. J., 449 U/mg). This procedure, in contrast to subculture with trypsin, did not destroy the myoblasts. All cultures used for a given experiment came from the same cell suspension.

Medium

The medium used for a given set of cultures was either DMEM: the high glucose formation (Grand Island Biological Co., Grand Island, N. Y.) in DISGH. The medium used for a given set of cultures was either DMEM: the high glucose formation (Grand Island Biological Co., Grand Island, N. Y.) in DISGHcontaining 10% heat-inactivated horse serum and 1 mg/ml collagenase (Worthington Biochemical Corp., Freehold, N. J., 449 U/mg). This procedure, in contrast to subculture with trypsin, did not destroy the myoblasts. All cultures used for a given experiment came from the same cell suspension.
gle's medium with 10% heat-inactivated (55°C for 30 min) horse serum (selected lots from Grand Island Biological Co. or Colorado Serum Co.), or MEM: Eagle's minimal essential medium with final concentrations of 30.3 mM glucose and 44 mM NaHCO3 similarly supplemented with horse serum. The media also included 10% fetal calf serum (Grand Island Biological Co. or Colorado Serum Co.) for the first 6 days of culture. The cultures received 20 μg/ml of 5-fluoro-2'-deoxyuridine (FUdR) (a gift from Hoffman-LaRoche) and 50 μg/ml uridine from day 6 to day 8 to prevent overgrowth by background cells. The culture medium was changed every 2-3 days. The cultures were maintained at 36°C in a 10% CO2-90% air, water-saturated atmosphere. Muscle cell cultures were 10 days-2 wk old before nerve cells were added, and the medium of the muscle cultures was changed immediately before the addition of neuronal cells.

Conditioned medium (CM) was taken from the appropriate cultures after 2-3 days of incubation and transferred to the experimental cultures. The CM was left on the recipient cultures until the next medium change, when the process was repeated. In some experiments, muscle CM was dialyzed at 6°C for 36 h (three changes) against 12 vol of medium with serum and then filtered through a 0.2-μm-pore diameter membrane (Nalge Co., Rochester, N.Y.) before use on SC cell cultures. To estimate the molecular weight of the active factor, we filtered muscle CM through Diaflow XM-50 membrane filters under nitrogen pressure. The filtrate and an equal volume of CM concentrated above the filter were tested for activity on SC cell cultures.

**Harvest and Assay**

At appropriate intervals after their initiation, the cultures were rinsed 3 x with Hanks' balanced salt solution and stored at -70°C until harvest. Cells were scraped from dishes, pooled in some instances, sonicated, and diluted for radiochemical assay of CAT and GAD activities (18). Enzyme activities were determined in triplicate. Protein was assayed according to a modification of the method of Lowry et al. (11). Both Nomarski differential interference and phase contrast optics were employed for photomicrography of SC cells grown on collagen-coated glass cover slips.

**RESULTS**

**SC and Muscle Cell Cultures**

Cell cultures of dissociated mouse hindlimb muscle closely resembled those obtained from chick (3, 19) and rat (9, 22). Myoblast division and fusion resulted in the development of multinucleate myotubes within a few days of inoculation. Spontaneous contractile activity was seen by day 7. The myotubes rested on a layer of flat undiffer entiated cells which continued to divide until the time of FUdR treatment.

Cells cultures of dissociated mouse SC contained phase-bright neuronal cells with extensively branching processes. The neurons could be grouped into three broad morphologic categories: (a) large multipolar cells (Fig. 1 a and b and Fig. 2); (b) large, spherical, often pseudo-unipolar cells (Fig. 1 c) which were observed only when the dorsal sensory ganglia were included in the culture; and (c) small, less well-defined cells which were often observed in clusters (Fig. 1 b). The cultures also contained a variety of other cell types, including fibroblast-like cells in a basal layer, phase-dark cells with extensive branching processes (Fig. 1 d), and other less common morphologic types. The development and physiologic characteristics of SC cultures, similar to those used in these experiments, were previously described in work from this laboratory (14, 16). Neurons in these cultures have been found to innervate skeletal muscle. Quite similar morphologic and enzymatic characteristics were observed in cultures from the same inoculum of pooled, dissociated spinal cords. This reliability within each experiment was in contrast to the large variation in CAT activity of control SC cultures often observed between different cell preparations. The results in this report are accordingly presented as comparisons of the effects of various co-culture or CM treatments within groups of cultures obtained from the same inoculum.

**SC-Muscle Cell Co-cultures**

Morphological maturation of SC cells during 2 wk of culture without muscle was accompanied by a moderate increase in CAT activity (Fig. 3). When replicate inocula of these SC cells were co-cultured with hindlimb muscle, a marked stimulation of CAT activity was apparent, beginning on the second day; at 14 days, the activity in co-culture exceeded that of SC cultured alone by 20-fold and the difference was still increasing. In other co-cultures the CAT activity continued to increase for at least 3 wk, the longest interval tested. These data suggest that both the time-course of CAT development and the total activity achieved were affected by culture with skeletal muscle cells. Because of our inability to assess reliably the relative contribution of SC and muscle to the DNA or protein content of co-cultures, the activities in most instances are expressed per culture. This is justified because the range of total
Figure 1  Phase contrast photomicrographs of SC cells after 14 days in culture. Large multipolar cells (a and b) have morphologic characteristics similar to those of motoneurons. Their physiology has been described [14, 16] and similar cells innervate muscle in co-cultures. Other phase-bright presumptive neurons are present singly and in clusters (b). Cells resembling pseudo-unipolar dorsal root ganglion cells are seen in c. Smaller cells with extensive processes (d) are a further feature of SC cultures. Underlying the more apparent phase-bright cells is a continuous sheet-like network of flat cells. Bars, 50 μm. Scale in a and b is the same as in c.
the increase continued at a slower rate in SC cultures but a marked decrease occurred in the co-cultures. The CAT activity of these same co-cultures increased steadily through day 21, at which point it was 10-fold greater than in the SC cells cultured alone. This finding supported our previous conclusion (4) that there was considerable specificity in the stimulation of CAT activity when compared to that of other muscle and nervous system enzymes.

Cells from dorsal sensory ganglia were included in most SC cultures because of the extra dissection time required to remove the ganglia which were closely associated with the spinal cords of 12- to 13-day embryos. In one experiment, however, these ganglia were carefully removed during the dissection and the SC cells from the spinal cords alone were cultured with or without muscle cells. After 21 days of culture, SC cell cultures contained CAT activities of 35 pmol/min per dish while the co-cultures contained activities of 498 pmol/min per dish. From this result we conclude that the presence of dorsal sensory ganglion cells is not necessary for the muscle-mediated stimulation of CAT activity.

The data presented in Figs. 5 and 6 were obtained from replicate SC cell cultures and, as previously shown (4), normalization of the CAT activity to total culture protein or DNA confirmed the dramatic nature of the co-culture effect. In the experiment presented in Fig. 3, the cultures were also assayed for the activities of acetylcholinesterase, creatine phosphokinase, phosphoglucomutase, myokinase, and phosphorylase. As we have found previously (4), these enzyme activities were not substantially affected by the co-culture condition relative to the magnitude of the CAT activity increase.

The effects of co-culture on SC cell CAT activity contrasted sharply with those on the activity of GAD (EC 4.1.1.15), the enzyme which mediates the synthesis of gamma-aminobutyric acid (Fig. 4). GAD activity increased at similar rates in control and co-cultures through day 7, after which it increased at a slower rate in SC cultures but a marked decrease occurred in the co-cultures. The CAT activity of these same co-cultures increased steadily through day 21, at which point it was 10-fold greater than in the SC cells cultured alone. This finding supported our previous conclusion (4) that there was considerable specificity in the stimulation of CAT activity when compared to that of other muscle and nervous system enzymes.

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Effect of muscle cells on SC cell GAD activity development. These data were obtained from GAD assays on the homogenates reported in Fig. 1 of reference 4 in which was shown >11-fold induction of CAT activity by day 21 of co-culture. Pooled SC cells from three litters of 13- to 15-day mouse embryos were inoculated (1.4 x 10^5 cells per cm^2 surface area) onto 2-wk-old myotube cultures (SC-M, squares) or on collagen-coated dishes (SC, circles), harvested on the days shown, and assayed for GAD activity and protein. Data represent assays of duplicate homogenates (SC-M) for days 7, 14, and 21, or of single homogenates (SC) of the contents of two or three replicate dishes. Homogenates of muscle control cultures harvested on day 21 after SC cell addition had GAD activity of 0.019 nmol/min per dish.

Obtained from replicate muscle cultures which were inoculated with increasing quantities of SC cells from a single cell suspension. At all doses and all time-points, an increase in the inoculation dose of SC cells resulted in an increase in CAT activity (Fig. 5a and b). Proportionality between dose and response was obtained when the inoculum was increased from 1 x 10^5 to 2 x 10^5 cells. At lower SC cell densities, the increase in CAT activity was greater than proportional; above 2 x 10^5 SC cells, the increase was markedly diminished. These data are consistent with the conclusion that the capacity of muscle cell cultures to stimulate SC cell CAT activity was saturable with SC cells. In these same cultures (Fig. 6), it was found that the capacity of muscle cells to decrease GAD activity was limited and could be partially overcome by large inocula of SC cells.

The contrasting results between the CAT and GAD activities in this experiment (Figs. 5 and 6) argue against nonspecific facilitation either of SC cell plating efficiency or of long-term survival as the source of the increase in co-culture CAT activity. The decrease in GAD activity during co-culture with muscle occurred in three of the five preparations in which GAD assays were performed. Analysis of the records from these five experiments revealed that GAD decreases were found in co-cultures for which SC cells were prepared from 12- to 13-day embryos, but not in co-cultures which were prepared from 14- to 15-day embryonic spinal cords.

The degree of stimulation of CAT activity, for replicate SC cell inocula, also varied with the dose of muscle cells used for co-culture (Fig. 7). The relationship was essentially linear when co-cultures were made in the usual manner by inoculation of SC cells onto muscle cultures of different densities (Fig. 7a). After 2 wk of co-culture, CAT activities in the cultures with the largest dose of muscle were 10-fold greater than in cultures without muscle. There was no evidence that the capacity for stimulation of CAT activity in these SC cells had been saturated by this amount of muscle. This muscle dose experiment was also done in a different way, with similar results. Myotubes were removed intact with collagenase treatment from 2-wk-old muscle cultures and placed on SC cell cultures 2 days after SC cell inoculation. In such co-cultures, after 1-2 days, the myotubes lay beneath the SC cells and the cultures were indistinguishable from the standard co-cultures. The assays (Fig. 7b) showed that CAT activity was stimulated nearly 10-fold at the highest muscle dose, and that lesser doses of muscle gave less stimulation. Since SC cells normally were adherent to the dishes before day 2, when the myotubes were added, this experiment demonstrated that the CAT stimulation effect was not dependent upon an increase in initial plating efficiency of SC cells due to the presence of muscle cells.

**CM Effects**

The CAT activity of SC cell cultures could also be stimulated as much as 10-fold by chronic treatment with culture medium which had been conditioned by muscle cell cultures for 2-3 days. The data of Table I show similar increases with the use...
Figure 5 Effect of SC cell dose on CAT activity increase induced by muscle. SC cells from (one litter each) 12-, 12½-, and 13-day mouse embryos were combined and inoculated at the densities shown into 60-mm dishes (0.24-1.4 x 10⁶ cells per cm² surface area) with or without replicate 2-wk-old myotubes grown for 7-21 days as described in Materials and Methods, harvested, and assayed for CAT and GAD activities (see also Fig. 6). Each datum shows the activity per dish for a single homogenate made from the contents of two replicate cultures. Muscle cultures without SC cells had no detectable CAT activity (4). Panel a: time-course of development of CAT activity. Panel b: CAT activities after 21 days in culture in combined cultures (SC-M, circles) and cultures of SC cells alone (SC, squares). The protein contents of the cultures after 21 days of SC culture were 0.07-0.65 mg/dish, 2.2-3.4 mg/dish, and 2.2 mg/dish for SC alone, SC-M, and muscle alone, respectively.

of muscle CM and co-culture. The "myoblast-fibroblast" cells of this experiment were obtained by repeated trypsinization and sub-culture of muscle cultures which had not been treated with FUdR; this resulted in the growth of morphologically undifferentiated cells (myoblasts or fibroblasts) in cultures devoid of mature myotubes. Medium conditioned by these cells caused the CAT activity of SC cell cultures to increase twofold, substantially less than the increase in CAT activity in replicate SC cell cultures which was produced by CM from muscle cultures of similar cell density. The extent to which these undifferentiated cells possessed the chemical characteristics of muscle and, alternatively, the degree to which cells and tissues other than muscle may affect SC cell CAT activity remain to be determined. In this experiment and others presented here often indicate a lesser effect on CAT activity than do the data when expressed as enzyme activity per culture. This is due to the fact that the CM treatment often resulted in increases in the protein content of SC cell cultures. This was presumably related to the resumption of cell division by some cells as well as to a continued increase in cell size after the completion of FUdR treatment. Since neurons presumably did not increase in number after inoculation, increases in the protein content of control or experimental cultures appeared to reflect primarily an increase in the number of non-neuronal cells.

SC cells co-cultured with skeletal muscle or cardiac muscle had a substantially increased CAT activity which was greater than that of SC cells cultured alone (Table 1, Exp B). In these cultures, both GAD and acetylcholinesterase (AChE) were relatively unaffected. When these SC cells were maintained for 8 days in muscle CM, both the total and specific activities of CAT were increased.
nearly threefold. No increases in GAD or AChE were detected in the SC cells treated with muscle CM. Growth of SC cells for 12 days in medium conditioned by other SC cultures resulted in increased CAT activity and small increases in GAD and AChE. The use of different treatment intervals in this experiment makes difficult a direct comparison of the effectiveness of these CM sources, as data below will show that early application of CM is an important determinant in the degree of CAT activity stimulation. However, the CM data in this table show that cells other than muscle cells probably can condition medium with a factor or factors which accomplish this stimulation.

We found that the increase in CAT activity in SC cell-muscle co-cultures which were also treated with muscle CM was almost precisely equal to the sum of the increases produced by the two treatments administered separately (Table II, Exp A). If the muscle CM and co-culture were acting to increase CAT activity by the same mechanism, such additivity might have been the result of failure to saturate that mechanism by either treatment. Alternatively, these data are also consistent with the possibility that the two treatments were working through different mechanisms. Since we have as yet been unable to saturate the effect of either CM or muscle co-culture treatments, a common mechanism cannot be ruled out. Consistent with a similarity in the mode of action of these treatments was the finding that functional neuromuscular transmission was not required in co-cultures in order to obtain stimulation of CAT activity. Although we had previously shown that there was a temporal relationship between the formation of functional neuromuscular junctions and the development of CAT activity in co-cultures, we found (Table II, Exp B) that the increase in enzyme activity occurred equally well in co-cultures which were chronically maintained in \( (8.5 \times 10^{-9} \text{ M}) \alpha\)-bungarotoxin, a snake venom polypeptide which binds to and blocks the muscle acetylcholine receptors. We found that this treatment blocked neuromuscular transmission in these cultures.

The increase in CAT activity was dependent upon the concentration of CM with which the cultures were treated. When SC cells were treated with muscle CM diluted variably with fresh medium (Fig. 8), the increase in enzyme activity which resulted was linearly related to the dose of CM. As mentioned above, we have not yet been able to saturate the CM effect. The increase in CAT activity was also dependent on the length of treatment and the time after initiation of SC cultures at which the CM was first applied (Table III, Exp A). Cultures treated from day 3 to day 10 and given regular medium from day 11 to day 13 had lower CAT activity than did cultures treated with CM continuously from day 3 to day 13. Starting the CM treatment later, after culture inoculation, results in a substantial decrease in the responsiveness of the SC cells, as measured by the increase in CAT activity per day of treatment. Similarly, when either muscle cells or muscle CM was applied to SC cells after several weeks in culture (Table III, Exp B), no increase in CAT activity was detected above that of SC cells cultured alone.

Attempts were made to determine some of the physical characteristics of the factor(s) present in muscle CM which was responsible for increased SC cell CAT activity. Since the horse serum present in the culture medium was routinely heated to 58°C before its use, this temperature was selected to test the thermal stability of the factor(s) in muscle CM. A 20-min treatment of muscle
FIGURE 7 Effect of muscle cell density on CAT activity increase. In each experiment the amount of muscle in the dishes was calculated by subtracting the total protein in control dishes (no muscle) from that of the combined cultures. Panel a: culture dishes (60 mm) were inoculated with varying amounts of muscle inoculum and grown to myotubes for 9 days before SC cells (0.95 × 10⁶ per cm² surface area) were added. The combined cultures, as well as SC and muscle alone controls, were grown for 21 days more and then harvested and assayed for CAT and protein content. Protein content of the SC control dish was 0.60 mg of protein. Each datum represents a single homogenate made from the contents of a single dish. Panel b: mouse myotubes, after 2 wk of culture were removed from dishes by treatment with collagenase, and re-inoculated in varying amounts onto 60-mm dishes which contained 2-day-old cultures of SC cells (1.4 × 10⁶ cells/cm² surface area) from 14-day mouse embryos. Combination cultures were then grown for an additional 12 days, harvested, and assayed for CAT and GAD activities and protein content of the dishes. In this experiment GAD activity was not decreased in cultures with muscle. Each datum represents the activity in one homogenate made from the contents of three replicate dishes. The SC control dishes contained an average of 0.64 mg of protein.

CM at that temperature before its application to SC cell cultures did not destroy the activity (Table IV, Exp A); 69% of the effect obtained with unheated CM was still present after heating. Myoblast-fibroblast CM (data not shown) gave similar results. Dialysis of muscle CM for 48 h against three changes of fresh medium also failed to reduce substantially its effectiveness relative to otherwise similarly treated, but nondialyzed, muscle CM (Table IV, Exp B). In this experiment, both the dialyzed and nondialyzed CM were filtered through a membrane with a pore size of 0.2 μm to ensure postdialysis sterility. We have observed in other experiments that such filtration reduced the effectiveness of the CM for increasing CAT activity; this effect was most significant when the CM was obtained from very dense muscle cultures.

In the experiments reported to this point, SC cultures had been maintained for 11–21 days with and without several changes of CM. Although this approach produced impressive differences between control and CM-treated cultures, it represented a slow, expensive, and inefficient assay method for continued studies of the factor(s) in CM. To obtain a more efficient experimental procedure for bioassay of muscle CM, we began to assay the SC cells after one treatment of 4 days with or without CM. Thus, the data in Table IV show much smaller increases than do those obtained in previous experiments. Using this more rapid assay method, we found that CM which would produce an increase in CAT activity could be obtained from muscle cultures maintained in the absence of horse serum. We had previously determined that horse serum could be omitted from muscle culture medium for at least 3 days without cessation of spontaneous contractions of the myotubes. A single dose of this medium, to which serum was added after condi-
### Table I

**Effects of Co-Culture and Conditioned Media on the Activities of CAT, GAD, and AChE in SC Cell Cultures***

| SC cell culture conditions | Days of treatment | Protein per dish (mg) | Protein per dish (pmol/min) | Fold increase | GAD activity (nmol/min) | AChE activity (nmol/min) |
|---------------------------|-------------------|----------------------|-----------------------------|--------------|------------------------|-------------------------|
| Exp A                     |                   |                      |                             |              |                        |                         |
| Alone                     | 0-21              | 0.52                 | 56                          | –            | 5.4                    | –                       |
| Myoblast-fibroblast CM    | 3-21              | 0.51                 | 123                         | 2.2          | 3.7                    | 5.5                     |
| Muscle CM                 | 3-21              | 1.08                 | 591                         | 11           | 30                     | 5.4                     |
| On muscle cells           | 0-21              | 1.72                 | 567                         | 10           | 24                     | 1.2                     |
| Exp B                     |                   |                      |                             |              |                        |                         |
| Alone                     |                   | 0.60                 | 34                          | –            | 4.8                    | 25                      |
| Muscle CM                 | 6-14              | 0.74                 | 99                          | 2.9          | 8.1                    | 4.9                     |
| On skeletal muscle cells  | 0-14              | 1.74                 | 270                         | 8            | 17                     | 6.6                     |
| On cardiac muscle cells   | 0-14              | 1.35                 | 641                         | 19           | 43                     | 7.0                     |
| SC cell culture CM        | 2-14              | 0.74                 | 89                          | 2.6          | 4.6                    | 5.7                     |

* SC cells were cultured alone in freshly prepared medium, in medium conditioned for 2-3 days by other cultured cells, or co-cultured with muscle. Cultures were harvested after 21 (Exp A) or 14 (Exp B) days and the enzyme activities determined, in triplicate, in the pooled contents of two 60-mm culture dishes. The increase per day of treatment is the difference in the CAT activity between SC cultured alone and the SC cultured under the various conditions divided by the number of days of treatment.

### Table II

**Effects of Conditioned Media, Co-Culture, and α-Bungarotoxin on CAT Activity in SC Cultures***

| SC cell culture conditions | Per dish (pmol/min) | Fold increase | Per mg protein (pmol/min) |
|---------------------------|---------------------|---------------|--------------------------|
| Exp A                     |                     |               |                          |
| Alone                     | 70                  | –             | 78                       |
| Muscle CM                 | 282                 | 4.0           | 390                      |
| On muscle cells           | 765                 | 11            | 249                      |
| On muscle cells + muscle CM | 1,080               | 15            | 548                      |
| Exp B                     |                     |               |                          |
| Alone                     | 34                  | –             | 57                       |
| On muscle cells           | 557                 | 16            | 229                      |
| On muscle cells + α-Btx   | 515                 | 15            | 238                      |

* SC cells, as described in Materials and Methods, were harvested after 14 days of culture, and each datum represents a determination, in triplicate, of the activity from pooled contents of two cultures in 60-mm dishes. Bungarotoxin (α-Btx) in Exp B was included in the culture medium at 8.5 x 10^-6 M.

Detoxification of serum by muscle cell cultures. The fact that CM (no serum) was effective in the assay enabled us to fractionate such CM by filtration on membranes of known pore size without concomitant concentration of serum macromolecules. The muscle CM components which passed through a filter with an approximate molecular weight exclusion limit of 50,000 daltons were inactive in increasing SC cell CAT activity. The CM components which did not pass through this filter were approximately twofold concentrated for high molecular weight molecules and produced an increase in CAT activity per culture that was nearly twice that obtained with untreated muscle CM. The SC cells in this experiment were not treated with FUDR, in contrast to our normal protocol, and, as a result, nonneuronal cell division continued in all of these cultures. Cell growth was apparently stimulated by the CM treatments since the total protein content of these treated cultures was increased. Despite this increase in protein, the CAT specific activity was significantly higher in cultures treated once with CM concentrated for >50,000-dalton macromolecules than in cultures treated with CM from which the high molecular weight components had been removed. These results demonstrate the efficacy of both the single dose bioassay for muscle CM factor(s) and the fractionation...
of medium conditioned in the absence of serum as useful approaches to the identification of the trophic substance(s) released by muscle which increases CAT activity in SC cell cultures.

DISCUSSION
We have found that growth of SC cells with cultured muscle cells resulted in markedly increased CAT activity. We have previously reported (4) that co-culture of SC and muscle cells did not increase either the number of identifiable neurons or the activity of several other enzymes. Indeed, the transmitter-related enzyme, GAD, was decreased under some conditions of co-culture. The increase in SC cell CAT activity was directly dependent on the muscle and SC cell density in culture, as well as the duration of co-culture. We did not obtain a saturation of the response by increasing the quantity of muscle in co-culture with SC. Muscle CM also increased SC cell CAT activity by as much as 10-fold within 3 wk, while it did not affect the activity of GAD or acetylcholinesterase. The CAT activity increase produced by muscle CM was directly dependent upon the CM concentration and the interval of exposure. The factor(s) in muscle CM responsible for increasing the SC cell CAT activity was heat stable at 58°C and nondialyzable; membrane filtration data were consistent with the factor's having a molecular weight greater than 50,000 daltons.

It seems reasonable to suppose that muscle grown in co-culture conditioned the medium as did muscle grown alone; and, thus, a substantial part of the increase in CAT activity observed in co-cultures was probably produced by mechanisms similar to those in the experiments with CM. It is valid to ask whether any additional factors are provided by the contiguity of SC cells and muscle in co-cultures. Physiologically effective synaptic interaction between nerve and muscle is not involved in the increased CAT activity in co-cultures, since the increase was not affected by the presence of α-bungarotoxin. Our experiments provide no evidence for any effect on CAT activity requiring some other aspect of nerve-muscle contact, but such an effect cannot be excluded as a basis for some component of the increase seen with nerve and muscle co-cultures.

The experiments with CM demonstrated that one or more macromolecules released by myogenic cells act to increase the CAT activity of SC cells. Several questions naturally arise with regard to this phenomenon. (a) Are myogenic cells the only source for the active molecules or can more than one tissue serve as a source? (b) Is CAT activity of the nerve cell culture affected in a relatively specific manner or is the effect a general one on culture growth? (c) Is the stimulation of CAT activity the effect of a single interaction between CM factor and recipient cell, or is it due to multiple small additive effects? (d) Does

**Figure 8** Effect of dose of muscle CM on CAT activity in SC cell cultures. SC cells (1.35 × 10⁶ cells/cm² surface area) from 14½-day mouse fetuses were inoculated into collagen-coated 35-mm dishes and grown as described in Materials and Methods, except that on days 2, 5, 7, 9, 11, and 13 the medium changes included the indicated proportions of CM (unprocessed, from mouse myotube cultures which had been in culture for 57 days), diluted with the usual culture medium (this regimen included 1 day of treatment with FUDR on day 8). Cultures were harvested on day 14 and assayed for CAT activity and protein. Each datum represents CAT activity per dish (circles; presented as equivalent activities for 60-mm diameter dishes) and per milligram of protein (squares) in single homogenates from the contents of 4, 2, 3, or 3 dishes containing 0, 25, 50, and 100% CM, respectively.
### Table III

**Effects of Time and Duration of CM Treatment and Co-Culture on CAT Activity in SC Cultures***

| SC cell culture conditions | Days of treatment | CAT activity increase per day of treatment | Fold increase |
|---------------------------|-------------------|------------------------------------------|---------------|
|                           |                   | pmol/min                                 |               |
|                           |                   | Per mg protein                           | Per dish      |               |
| Exp A                     |                   |                                          |               |
| Alone                     | –                 | 337 (±24)                                | 57 (±4)       | –             | –             |
| Muscle CM                 | 3-10              | 894 (±51)                                | 202 (±27)     | 3.5           | 20.7 (±3.2)   |
| Muscle CM                 | 3-13              | 1,545 (±105)                             | 298 (±9)      | 5.2           | 24.1 (±0.9)   |
| Muscle CM                 | 5-13              | 780 (±100)                               | 187 (±26)     | 3.3           | 16.4 (±3.2)‡  |
| Muscle CM                 | 7-13              | 565 (±23)                                | 144 (±3)      | 2.5           | 14.5 (±0.7)§  |
| Exp B                     |                   |                                          |               |
| Alone                     | –                 | 199                                      | –             | –             |
| Muscle cells added        | 2-14              | 784                                      | 4.9           | 49            |
| Muscle cells added        | 15-29             | 160                                      | 0.8           | 0             |
| Muscle cells added        | 19-33             | 243                                      | 1.2           | 3.1           |
| Muscle CM                 | 19-33             | 216                                      | 1.1           | 1.2           |

* SC cells were cultured alone in freshly prepared medium, in medium conditioned for 2-3 days by muscle cells, or co-cultured with muscle. Cultures were harvested at the end of the treatment intervals and the CAT activity was assayed independently in cells pooled from 2-4 dishes (35 mm) in Exp A (expressed as mean ± SD) or from two 60-mm culture dishes (Exp B). The increases in CAT activity were calculated as in Table I.

‡ Differ from 3-13 at P > 0.02.

§ Differ from 3-13 at P > 0.001.

a similar regulatory interaction occur in vivo? The present work provides some information with regard to the first two questions.

We have found that medium conditioned by SC cells, differentiated skeletal muscle, and undifferentiated cells of myogenic origin increased CAT activity of SC cell cultures. Because of the differences between these cultures in cell type, size, and density, and because of the uncertainty as to the amount of effective protein secreted by the cultures into the medium during conditioning, it is difficult to assess the relative efficacy of the different cell cultures in the production of the CM factor. It seems clear, however, that skeletal muscle is not the only source of material that can produce an increase in CAT activity in SC cell cultures.

The co-culture and CM effects exhibited considerable specificity with regard to the enzyme changes produced in the SC cultures. In the experiment in Fig. 3, we assayed for protein, DNA, CAT, acetylcholinesterase, creatine phosphokinase, phosphoglucomutase, myokinase, and phosphorylase. Of these, only CAT activity was substantially increased, while in that experiment and some others, GAD activity was reduced in co-cultures (Figs. 4 and 6 and Table I). When SC cells were treated with muscle CM, CAT activity was substantially increased while the activities of GAD and acetylcholinesterase were not. It is this specificity in enzyme response of SC cells to coculture or treatment with CM which distinguishes the interaction reported here from a relatively nonspecific promotion of cell survival (15). The data on enzyme activities, total culture DNA, and culture protein indicate neither a substantial nonspecific increase in plating efficiency with coculture nor a facilitation of long-term survival with CM. The specific increase in CAT activity produced by both of these conditions indicates either that survival of a population of CAT-producing cells is selectively increased or that cell survival is unchanged and CAT production per cell is increased. Some combination of these processes might of course occur. The fact that CAT activity in co-culture continued to increase well after the activity in control cultures had become constant (Fig. 3 and Reference 4), argues against an increased survival in co-culture of a population of neurons in which the CAT activity increased at the same rate as that of surviving control cells. We previously reported that differences in the number of identifiable neuron-like cells were not detected between cultures of SC cells grown alone and SC grown with muscle, while 20-fold more CAT activity was present in the co-cultures (4). Thus, our results suggest that the increase in CAT activity that occurred in co-
### Table IV

Characteristics of the Trophic Factor(s) in Muscle CM*

| SC cell culture conditions  | Days of treatment | no. of Treatments | CAT activity (pmol/min) | Fold increase | Per mg protein |
|---------------------------|-------------------|-------------------|-------------------------|---------------|---------------|
| Exp A‡                     |                   |                   |                         |               |               |
| Alone‡                    |                   |                   | 29 (±8)                 |               | 126 (±21)     |
| On muscle                 | 0-11              |                   | 92 (±2)                 | 3.2           | 2            |
| Muscle CM                 | 2-11              | 4                 | 102 (±29)               | 3.5           | 412 (±35)     |
| Heated muscle CM          | 2-11              | 4                 | 79 (±18)                | 2.7           | 298 (±29)     |
| Exp B§                    |                   |                   |                         |               |               |
| Alone§                    |                   |                   | 73                      |               | 333          |
| On muscle                 | 0-14              |                   | 505                     | 7             | 790          |
| Muscle CM                 | 3-14              | 4                 | 200                     | 2.7           | 685          |
| Dialyzed muscle CM        | 3-14              | 4                 | 172                     | 2.4           | 589          |
| Exp C||                   |                   |                   |                         |               |               |
| Alone||                    |                   |                   | 42 (±2)                 |               | 222 (±6)     |
| Muscle CM (with serum)    | 6-10              | 1                 | 62 (±5)                 | 1.5           | 280 (±18)    |
| Muscle CM (no serum)      | 6-10              | 1                 | 63 (±7)                 | 1.5           | 255 (±5)     |
| Muscle CM (<50,000)       | 6-10              | 1                 | 37 (±6)                 | 0.88          | 195 (±28)    |
| Muscle CM (>50,000)       | 6-10              | 1                 | 81 (±2)                 | 1.9           | 276 (±19)    |

* SC cells were cultured in replicate 35-mm dishes, harvested, and assayed for CAT activity and protein as described in Materials and Methods.
‡ Harvested on day 11. Data are means (±SD) from three to four independently assayed cultures. For heat treatment, see text.
§ Harvested on day 14. Data are from single homogenates made from two cultures each.
|| Harvested on day 10. Data are means (±SD) from three independently assayed cultures. Muscle CM without serum had horse serum (to 10%) added before CM was placed on SC cultures. Filtration of CM without serum was performed as in Materials and Methods and horse serum was added to both fractions before use. The CAT activities (per milligram protein) of all SC cell cultures treated with CM were significantly (P > 0.01) greater than the activity in SC alone, with the exception of the cells treated with CM <50,000.

Cultures resulted from an increase in the net production of this enzyme per cell. Until the cell of origin of the CAT activity is established, however, other mechanisms cannot be excluded.

The specificity of the effect of both co-culture and CM on CAT activity suggests some relevance to trophic interactions in the neuromuscular or other cholinergic systems. Since this enzyme mediates the synthesis of acetylcholine at the synaptic junction between spinal motoneurons and skeletal muscle, the interaction in culture may reflect a significant neuromuscular regulatory process. The nature of the regulatory material(s) and its mechanism of action merit further investigation.

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