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THE FORMATION OF SYNAPSES BETWEEN CHICK EMBRYO SKELETAL MUSCLE AND CILIARY GANGLIA GROWN *IN VITRO*

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SUMMARY

1. Chick embryo ciliary ganglia (explanted) and skeletal muscle (dissociated) were grown together *in vitro* for up to 3 weeks. Nerve processes sprouted from the ganglia and contacted neighbouring myotubes and striated muscle fibres.

2. Spontaneous action potentials and subthreshold e.p.p.s were recorded from muscle fibres with intracellular micropipettes. Similar potentials could be evoked by electrical stimulation of the ganglion. The pharmacological effects of curare and tetrodotoxin were identical to those observed at adult vertebrate neuromuscular junctions.

3. The amplitude, but not the frequency, of the spontaneous potentials was affected by changing the muscle fibre membrane potential. The reversal potential of evoked synaptic potentials occurred at a membrane potential of about 0 mV.

INTRODUCTION

In vitro cultures of cells that are capable of forming functional synaptic connexions offer certain advantages for studies of the cellular mechanisms involved in synaptogenesis. For instance, in primary cultures of embryonic muscle and spinal cord it is possible to observe the establishment of contacts between individual nerve terminals and muscle cells, and to position micro-electrodes with great accuracy for electrophysiological studies. Experiments employing both extracellular (Crain, 1964; Crain, Alfei & Peterson, 1970) and intracellular (Fischbach, 1970; Robbins & Yonezawa, 1971; Kano & Shimada, 1971) recording techniques have demonstrated that functional neuromuscular synapses do in fact form in such cultures, and so it is becoming feasible to study cellular events which occur during

synaptogenesis. However, one limiting complexity of these cultures is their heterogeneous population of neurones. The presence of non-cholinergic spinal neurones can complicate the interpretation of experimental results. For instance, Fischbach (1972) has shown that, in cultures of embryonic chick muscle and spinal cord, only 5–10% of the visible nerve–muscle contacts are functional. One possible explanation for this low ratio is that many of the spinal neurones are non-cholinergic and so their processes release transmitters incapable of exciting the muscle fibres. Therefore, experiments might be simpler to interpret if a uniform population of cholinergic neurones were cultured with the muscle.

The preparation described in this paper was designed to provide a uniform culture of cholinergic neurones together with skeletal muscle. The source of neurones was the embryonic chick ciliary ganglion, a part of the parasympathetic nervous system, whose neurones normally innervate the intrinsic eye muscles. It is generally believed that ACh is the sole parasympathetic transmitter, and pharmacological and biochemical studies on the avian ciliary ganglion are entirely consistent with this view. The ciliary ganglion of the embryonic chick consists of about 6000 neurones (Landmesser & Pilar, 1974). Carpenter (1911) described two morphologically distinct populations of neurones, which have been named choroidal and ciliary (Marwitt, Pilar & Weakly, 1971). There are approximately equal numbers of each type (Marwitt *et al.* 1971). The choroidal neurones innervate the smooth muscle fibres of the choroid, where synaptic transmission is cholinergic muscarinic (unpublished observations cited by L. Landmesser & G. Pilar, 1970). The ciliary neurones innervate muscle fibres of the iris and lens, the former muscle being striated in birds (Hess, 1966). Most of the muscle fibres of the lens are circularly arranged, and so mediate constriction of the pupil. Synaptic transmission here is cholinergic nicotinic (Pilar & Vaughan, 1969). A few of the muscle fibres of the iris mediate dilatation of the pupil; transmission at these synapses is blocked by curare (G. Pilar, personal communication), and so presumably transmission at these synapses is also cholinergic. Synapses on lens muscle fibres have apparently not been studied in birds, although in mammals they are certainly cholinergic. Biochemical studies have demonstrated that the ganglionic neurones and their axons contain appreciable amounts of ACh (Pilar, Jenden & Campbell, 1973). Finally, fluorescence histochemical techniques have failed to detect any catecholamine-containing nerve cell bodies in the ganglion (Ehinger, 1967; Cantino & Mugnaini, 1974). Thus, it seems reasonable to conclude that certainly most, and probably all, of the neurones in the ciliary ganglion are cholinergic.

In the experiments described in this paper, explanted ganglia were

grown with dissociated embryonic chick skeletal muscle, and morphological and electrophysiological evidence was obtained for the formation of functional neuromuscular synapses.

METHODS

The techniques for culturing muscle were similar to those described by Shimada, Fischman & Moscona (1969) and Fischbach (1972). Small pieces of pectoral muscles were excised from 11-day chick embryos, incubated for 30 min in Ca-free minimal Eagle's Medium (M.E.M., Difco) containing 0·1% trypsin (Sigma). Then this suspension was centrifuged for 4 min at about 2000 rev/min; the supernatant was discarded and the cell clumps were resuspended in M.E.M. The clumps were then triturated with Pasteur pipette, filtered through a double layer of lens paper, and counted in a haemocytometer. Cells were plated on collagen-coated glass cover-slips at a density of about 25,000 cells/cm^2 culture surface. The plating medium consisted of M.E.M. with 10% chick embryo extract (C.E.E.), 10% horse serum (Grand Island Biochemicals), 0·29 g glutamine/l., 0·52 g streptomycin/l., 17 mg penicillin/l., 2 g $NaHCO_3$/l., and 0·2 g $CaCl_2$/l. (1·8 mM). Cultures were incubated at 37° C in a water-saturated atmosphere of 95% air–5% CO_2. The mononucleated myoblasts fused to form myotubes in about 2 days, at which time C.E.E. concentration was reduced to 2% and 2 μM cytosine arabanoside (Sigma Chemical Co.) was added to the culture medium in order to reduce the number of proliferating fibroblasts; 36–48 hr later the cultures were washed free of arabanoside and ciliary ganglia were added. The ganglia were dissected from 6- to 7-day-old chick embryos and placed on the cover-slips. In order to give the ganglia time to attach to the cultures, they were kept in the dissection hood for 4–6 hr on a metal plate heated to 37° C, which was covered and perfused with a 95% O_2–5% CO_2 gas mixture. After the ganglia had attached, the cultures were returned to the incubator.

Electrophysiological experiments were performed on the stage of a Reichert inverted phase-contrast microscope with Nomarski interference-contrast optics. The cover-slip on which a ganglion and muscle cells were growing was placed in a chamber, covered with mineral oil, and kept at 37° C by means of a platinum heating loop immersed in the oil. Intracellular micropipettes were filled with 4 M-K acetate and had resistances of 80–120 MΩ. Extracellular stimulating electrodes had tip diameters of 2–10 μm and were filled with 1·5 M-NaCl. Electrically controlled micromanipulators were used to position micropipettes.

RESULTS

Microscopic observations. Explanted ganglia attached to the cover-slips in about 4 hr and, within a day, thin, branched processes could be seen growing from them. Pl. 1*A* shows a typical low-power (100×) view of a ganglion (upper right) and myotubes 1 day after the ganglion was plated. Some nerve processes are present, but they are more easily seen in Pl. 1*B* (from a different culture, 2 days after plating the ganglion). Pl. 1*C* is a high-power (630×) view of a single striated muscle fibre with several branching processes coursing over its surface. Most electrophysiological experiments were performed at this magnification.

In addition to the nerve axons, other cell types, presumably fibroblasts,

capsule cells and Schwann cells, grew out from the ganglion, and after 2 weeks covered the muscle fibres in the vicinity of the ganglion, making it difficult to visualize nerve processes and impale muscle fibres. The mononucleated myoblasts fused with each other during the first 2–3 days in culture, and the resulting myotubes became striated during the next week. Synapses formed mostly during the first week after the ganglion was plated (3 to 10-day cultures). The ganglia were spherical when plated, but became flattened in 4–5 days, and as non-neuronal cells grew radially from them, the ganglia became less distinct. In cultures over 2–3 weeks old, it was sometimes difficult to identify the ganglion unequivocably.

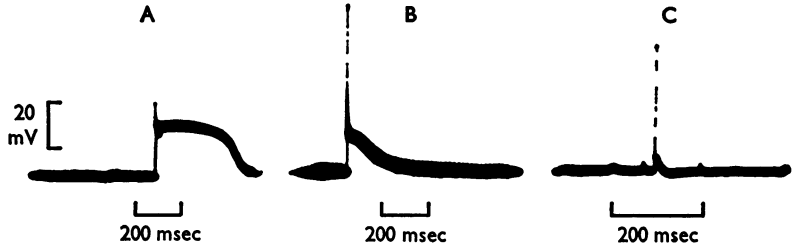
In young cultures, spontaneous twitching of muscle fibres provided a reliable index of functional innervation. That is, muscle fibres remote from the ganglion (and all muscle fibres in nerve free cultures) rarely twitched spontaneously in culture less than 7–10 days old. Twitching near a ganglion usually began in 3- to 4-day-old cultures (1–2 days after plating the ganglion), and was invariably associated with electrophysiological signs of synapse formation. In older cultures, non-innervated striated muscle fibres sometimes fibrillated, thereby masking the synaptically evoked twitches. The point is simply that spontaneous twitching near the ganglion in young cultures was the chief criterion for selecting cultures for experiments; young cultures which showed no signs of spontaneous twitching near the ganglion were discarded.

Electrophysiology. Intracellular recordings were obtained from innervated muscle fibres between 4 and 20 days after plating (2–18 days after adding ganglia). Resting potentials ranged up to 75 mV; cells with resting potentials below 30 mV were considered damaged and were not studied. In cultures older than 5–6 days, spontaneous depolarizing potentials were observed in nearly all muscle fibres near the ganglion, and were subsequently identified as action potentials and subthreshold end-plate potentials (e.p.p.s).

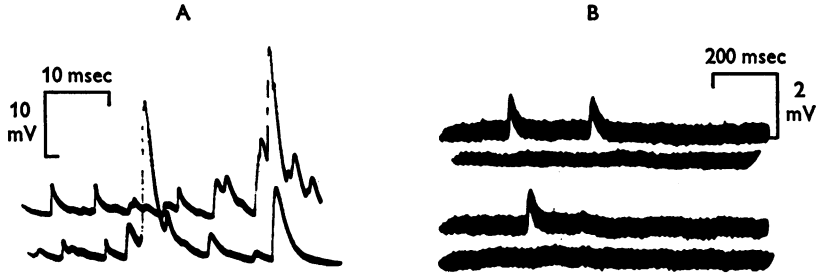
Action potentials had rise times of about 1 msec, and usually, but not always, the membrane potential reversed sign (the cell became inside-positive) at the peak. Presumably the smaller, non-overshooting action potentials were the result of damage at the site of impalement or conduction failure at branch points in the myotubes.

As illustrated in Text-fig. 1, the duration of the action potentials changed with age of the culture. In young myotubes (Text-fig. 1*A*) the initial spike was typically followed by a 'plateau' phase of variable amplitude lasting from several hundred milliseconds up to 3 sec in different fibres. As muscle fibres matured, the duration of the plateau decreased (Text-fig. 1*B*), and striated muscle fibres repolarized quickly, except for a small residual depolarization which resembled the negative after potential

characteristics of adult skeletal muscle (Text-fig. 1*C*). The progressive shortening of the action potential during development is similar to that observed by Kidokoro (1973), who showed that, in cultures of a cloned muscle cell line, the shortening is associated with the development of delayed rectification in the muscle fibre membrane.



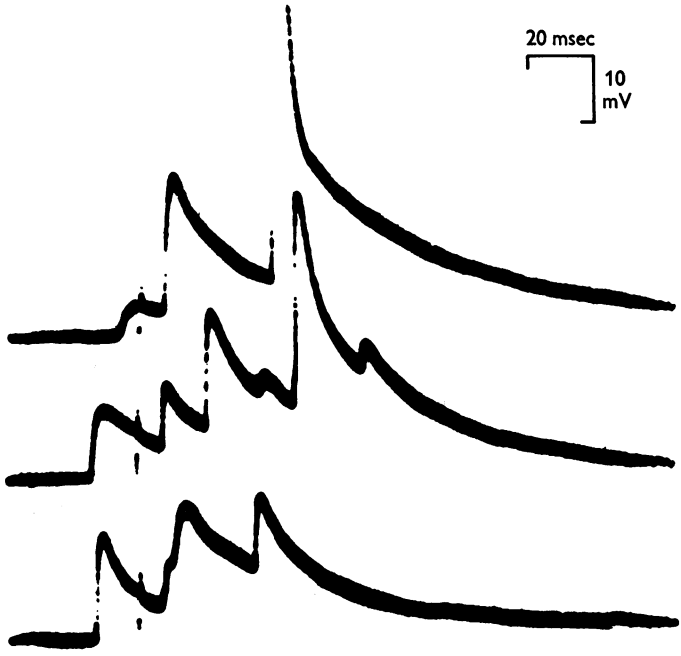
Text-fig. 1. Examples of spontaneous action potentials recorded from innervated muscle fibres of different ages. *A*, 4-day cultures; *B*, 8-day culture; *C*, 11-day culture. The duration of the 'plateau' following the initial spike became progressively shorter in older cultures. Resting potentials: 55, 67 and 50 mV, respectively. Calibration: 20 mV, 200 msec.



Text-fig. 2. *A*, spontaneous synaptic activity recorded from a muscle fibre near a ganglion which had been plated 3 days earlier. Calibration: 10 mV, 10 msec. *B*, spontaneous activity after perfusing the chamber with a solution containing tetrodotoxin, 1 μg/ml. The frequency and mean amplitude of spontaneous potentials were greatly decreased, leaving only the smallest potentials, of relatively constant amplitude. Resting potential: 64 mV. Calibration: 2 mV, 200 msec.

Subthreshold depolarizing potentials were recorded from most fibres which gave action potentials. Examples are shown in Text-fig. 2*A*. These subthreshod potentials were shown to be spontaneous synaptic potentials by several simple tests. First, as shown in Text-fig. 2*B*, tetrodotoxin, 1 μg/ml., reversibly abolished only the largest potentials; small potentials, about 0·5–2 mV in amplitude, persisted in the presence of tetrodotoxin. Secondly, curare, 5 μg/ml., reversibly abolished all spontaneous potentials, including action potentials. The simplest interpretation of these results is

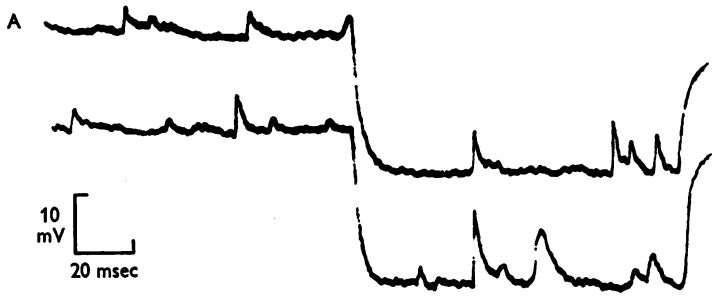
that the neurones spontaneously generated action potentials which caused the release of relatively large amounts of ACh, thereby generating e.p.p.s and action potentials in the muscle fibres. The action potentials were blocked by tetrodotoxin, but the spontaneous leakage of single quanta of transmitter is unaffected by the drug (Katz & Miledi, 1967), and so the small potentials persisted in the presence of tetrodotoxin. The fact that curare abolished the spontaneous action potentials in the muscle fibres suggests that these were evoked synaptically.



Text-fig. 3. Three examples of synaptic potentials recorded from a muscle fibre, evoked by electrical stimulation of the ganglion. In each trace, a spontaneous synaptic potential precedes the shock artifact. The evoked response consists of multiple, temporally dispersed potentials resembling the spontaneous ones. Resting potential: 54 mV. Calibration: 10 mV, 20 msec.

In addition to these pharmacological tests, several physiological experiments also suggest that the spontaneous potentials are synaptic in origin. First, similar potentials could be evoked by electrical stimulation of the ganglion. Examples are shown in Text-fig. 3. The ganglion was stimulated with a pipette filled with 1·5 M-NaCl, with a tip diameter of about 5 μm. In each record, a spontaneous potential precedes the shock artifact, and the evoked response consists of multiple, temporally dispersed potentials. The evoked potentials were reversibly abolished by curare (5 μg/ml.) and tetrodotoxin (1 μg/ml.) (not shown).

As a second physiological test, the muscle fibre membrane potential was changed by passing current through a second intracellular micropipette, and the effects of such changes on spontaneous and evoked potentials were measured. In Text-fig. 4*A*, an experiment is illustrated in which a muscle fibre was intermittently hyperpolarized by 23 mV, and the amplitudes and frequency of spontaneous potentials compared at rest and during the hyperpolarization. The results of this experiment are summarized in Text-fig. 4*B*, and show that the average amplitude of the spontaneous potentials increased about 50 % during the hyperpolarizing pulses, but their frequency did not change significantly.



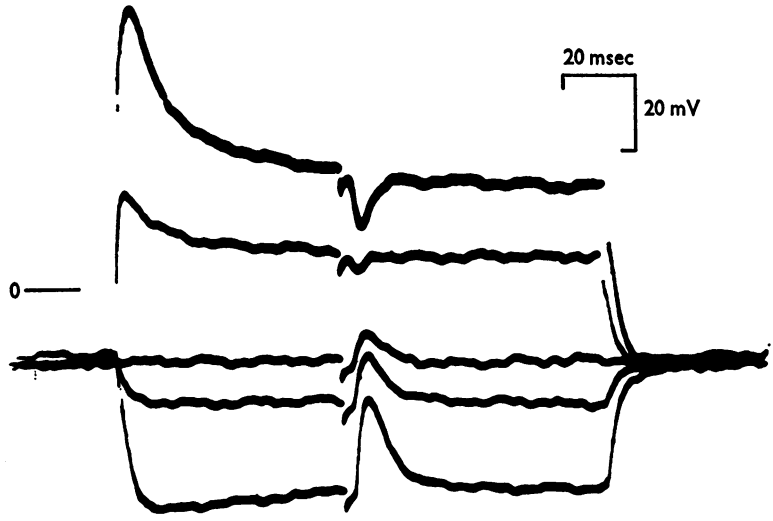
B

Membrane Potential	*N*	Frequency (sec^{-1})	Amplitude (mv)
Resting	239	39·5±20	4·0±1·9
Hyperpolarized (23 mV)	269	44·8±20	6·2±4·4

Text-fig. 4. The effect of hyperpolarization of the muscle fibre on spontaneous synaptic potentials. *A*, example oscilloscope traces. During the second half of each 200 msec sweep, the muscle was hyperpolarized by about 23 mV. The synaptic potential frequencies and amplitudes were measured during sixty sweeps, and the results (mean ± S.D.) are given in *B*. The second column (*N*) gives the total number of observed synaptic potentials in sixty 100 msec observation periods. The mean frequency of potentials during the hyperpolarizing pulses is not significantly different from rest ($0{\cdot}1 < P < 0{\cdot}25$). The mean amplitude, however, increased by about 50 % during hyperpolarization, and the increase is statistically significant ($P < 0{\cdot}001$). Resting potential: 48 mV.

Evoked potentials were also affected by changes in muscle membrane potential. Examples are shown in Text-fig. 5. The potentials increased in amplitude during hyperpolarization, and decreased upon depolarization, with a measured value of reversal potential occurring at about zero

membrane potential. Since the precise location of the synaptic contact was not known, it was impossible to know for certain the potential of the synaptic membrane. Thus, the true synaptic reversal potential may be more negative (but not more positive, since the two intracellular micropipettes were very close to each other) than that observed. The point, however, is simply that the evoked potentials respond qualitatively in a manner similar to end-plate potentials of vertebrate neuromuscular junctions from adult animals (e.g. Fatt & Katz, 1951) and from *in vitro* preparations (Fischbach, 1972).



Text-fig. 5. Superimposed traces of synaptic potentials evoked by electrical stimulation of the ganglion and recorded at different values of muscle membrane potential. The resting potential was abnormally low in this muscle fibre, due to damage inflicted by the current-passing electrode, which was separated from the recording electrode by about 20 μm. The horizontal bar on the left marks zero potential. The e.p.p. amplitude varied linearly with membrane potential, with the reversal potential occurring at about 0 mV. Calibration: 20 mV, 20 msec.

Spontaneous potentials were observed in muscle fibres as early as 15 hr after explanting a ganglion. These earliest potentials were small (1–3 mV) and very infrequent (0·2–2/min). Electrical stimulation of the ganglion or of processes observed to contact impaled muscle fibres failed to evoke such potentials. Larger, suprathreshold synaptic potentials, which produced muscle twitches, were not observed until at least 24–30 hr after a ganglion was plated. This sequence of developmental events is qualitatively similar to that reported for regenerating neuromuscular connexions in adult animals (e.g. Dennis & Miledi, 1974*a*, *b*). The earliest potentials were observed

in muscle fibres which lay directly beneath or adjacent to the ganglion; fibres which lay more than about 50 μm from the ganglion did not become active until 36–60 hr after the ganglion was plated, which doubtless reflects the time required for nerve processes to grow from the ganglion and contact the more remote muscle fibres.

DISCUSSION

The synapses described in this paper were formed in culture between embryonic chick skeletal muscle and ciliary ganglion neurones. The preparation has the usual advantages and drawbacks of tissue and organ cultures. Chief amongst the advantages is the excellent visibility, which greatly facilitates electrophysiological experiments. The major disadvantage is the unnatural growth condition, which may make some of the results of questionable pertinence to normal, *in vivo* development. Despite the novel source of neurones, the synapses formed in these cultures are similar in general respects to those synapses formed between muscle and spinal cord neurones. The preparation has several advantageous features for studying development. Firstly, some synapses form as early as 15 hr after the ganglion is explanted. It is not known how much time is required for the severed ciliary axons to heal, sprout, and contact the muscle fibres, but 15 hr seems a sufficiently short time to entertain the possibility that the growing axons continuously release ACh, and that synaptic potentials commence immediately when the axon contacts a suitably receptive area of muscle membrane. If true, then the initial events in synaptogenesis would require no cellular recognition processes at all (cf. Landmesser & Pilar, 1970). This question could be tested directly by observing the growth of individual nerve processes and recording from muscle fibres at the time the initial contact is made.

A second noteworthy observation is the spontaneous activity in the cultures. In some cultures, muscle fibres around the ganglion twitched vigorously, at frequencies of several twitches per second. The muscle twitches were produced by spontaneous activity of the neurones, which synaptically excited muscle fibres. Whether these neurones were intrinsically active, or were themselves synaptically driven by other neurones has not been established. The neuronal cell bodies in freshly excised ganglia are sensitive to iontophoretically applied ACh (H. Brenner & E. W. Johnson, personal communication), and electrical stimulation of the cultured ganglia sometimes produced complex, multiple e.p.p.s in muscle fibres suggestive of after discharges. Thus the possibility exists that the neurones may develop reciprocal innervation in culture, and that reverberating excitatory connexions give rise to the spontaneous activity.

Such connexions, however, do not exist *in vivo*, and ciliary neurones in a freshly excised ganglion do not fire spontaneously (Martin & Pilar, 1964). Whatever the origin of spontaneous neuronal activity in culture, it raises the possibility that nerve-controlled muscle parameters, such as sensitivity to ACh, may be studied with relative ease in this preparation.

Note added in proof. These results are in substantial agreement with the recent work of J. Hooisma, D. W. Slaaf, E. Meeter and W. F. Stevens (1975).

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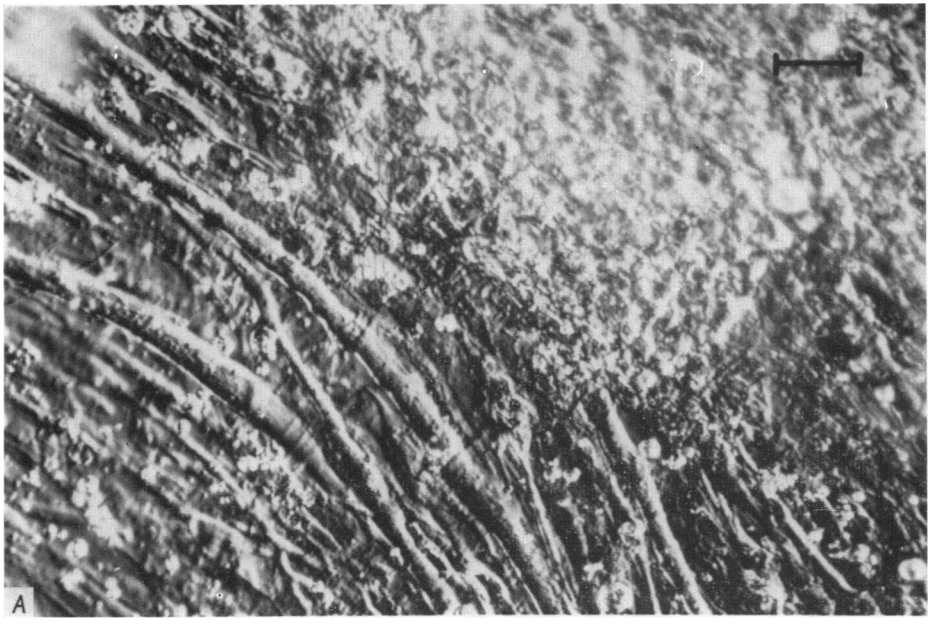
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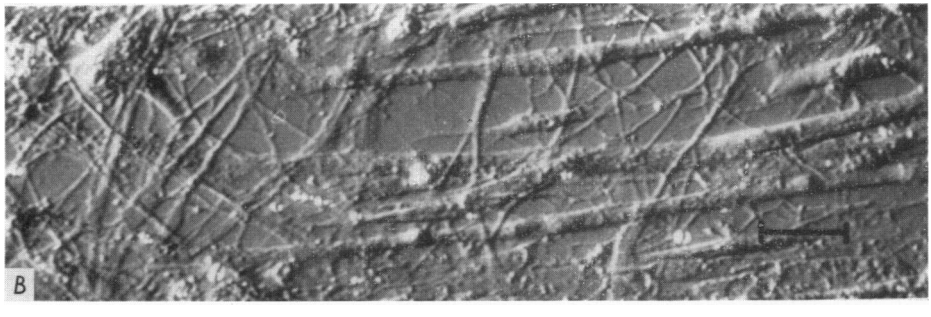
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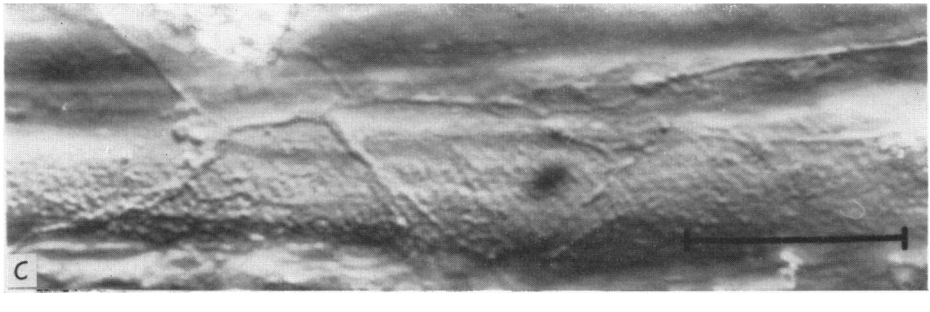
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EXPLANATION OF PLATE

A, low-power (100 ×) view of culture. Ganglion appears as indistinct mass in upper-right quadrant.

B, low-power view of another culture, showing many branched nerve processes contacting muscle fibres.

C, high-power (630 ×) view of a portion of the surface of a striated muscle cell. Thin, branched, varicose nerve fibres have grown across and along the length of the muscle fibre. Calibration marks: 20 μm.