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EFFECTS OF INNERVATION ON THE DISTRIBUTION OF ACETYLCHOLINE RECEPTORS ON CULTURED MUSCLE CELLS

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SUMMARY

1. Myotomal muscle cells from embryos of *Xenopus laevis* were cultured as a monolayer either alone or together with neural tube cells from the same embryos.

2. Spontaneous twitching and contractions evoked by electrical stimulation of neuronal perikarya were observed only in nerve-contacted muscle cells, and could be abolished by curare or α-bungarotoxin.

3. Within 2 days in culture muscle cells not contacted by nerve developed one or more discrete patches of acetylcholine (ACh) receptors as revealed by staining with fluorescent conjugates of α-bungarotoxin. Similar patches were also seen when staining was carried out after paraformaldehyde fixation, suggesting that they were not induced by the dye-toxin conjugate.

4. Radioautography after labelling with [^{125}I]α-bungarotoxin revealed patches with grain densities approximately twenty-five-fold greater than over the remainder of the cell.

5. Fluorescent stain on innervated cells was restricted to the path of nerve-muscle contact and sometimes extended for greater lengths than the largest patches seen on non-contacted muscle cells.

6. Similar long bands of stain associated with nerve–muscle contacts were observed when cultures were grown in high concentrations of curare and carbachol which prevented spontaneous twitching. They were also seen in cultures in which the addition of neural tube cells was delayed for 2–3 days.

7. It is concluded that innervation caused receptors to accumulate at sites of nerve-muscle contact and that this process can operate independently of muscle activity.

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INTRODUCTION

It has been known for many years that in skeletal muscle sensitivity to ACh is localized almost exclusively at the region of innervation (Langley, 1907; Kuffler, 1943). The iontophoretic technique (Nastuk, 1953; del Castillo & Katz, 1955) and the subsequent application of Nomarski optics (Peper & McMahon, 1972) have greatly improved the spatial resolution of such measurements and it is now clear that ACh sensitivity is restricted largely to the subsynaptic membrane. For example, sensitivity has been found to decline fiftyfold within 2 μm of the site of nerve contact (Kuffler & Yoshikami, 1975) and a thousandfold or more within a few hundred microns (Miledi, 1960; Dreyer & Peper, 1974). Other investigations, in which ACh receptors have been labelled with conjugates of α-bungarotoxin and their distribution examined by radioautography, have further indicated that receptor density is very much higher in the subsynaptic membrane than in the remainder of the sarcolemma (Barnard, Wieckowski & Chiu, 1971; Hartzell & Fambrough, 1972; Albuquerque, Barnard, Porter & Warnick, 1974; Fertuck & Salpeter, 1974, 1976). Regions of high chemosensitivity have also been observed at sites of synaptic contact on parasympathetic neurones in the amphibian heart (Harris, Kuffler & Dennis, 1971; Roper, 1976) and at excitatory and inhibitory synapses in arthropod muscle (Takeuchi & Takeuchi, 1964, 1965; Usherwood, Machili & Leaf, 1968). On the basis of these findings, it seems likely that a high receptor density will prove to be a common characteristic of many chemical synapses.

The manner in which receptors become localized at synapses during the course of development is an intriguing question. It has been established that at early stages of development, shortly after innervation has occurred, skeletal muscle fibres have a high sensitivity to ACh along their entire length (Diamond & Miledi, 1962; see also Berg, Kelly, Sargent, Williamson & Hall, 1972). A number of experimental procedures also lead to the development of a widespread sensitivity in mature muscle fibres. Since these procedures restore the muscle fibre's ability to accept new innervation, it has been suggested that sensitivity to ACh may be a prerequisite for the establishment of nerve–muscle synapses (Katz & Miledi, 1964; Fex, Sonesson, Thesleff & Zelená, 1966). More recently it has been found that avian and rat myotubes in cell culture develop not only a widespread distribution of receptors, but also patches of even greater density (Vogel, Sytkowski & Nirenberg, 1972; Fischbach & Cohen, 1973; Sytkowski, Vogel & Nirenberg, 1973; Hartzell & Fambrough, 1973; Vogel & Daniels, 1976). This has raised the possibility that receptor patches are potential sites of innervation which growing nerve fibres seek out (Sytkowski *et al.*

1973; Fischbach & Cohen, 1973). On the other hand, the distribution of ACh receptors before innervation has not been determined for the case of normal embryonic development. It is therefore necessary to consider the alternative possibility that innervation itself induces a high density of ACh receptors at the site of contact, just as it is known to induce junctional folds and cholinesterase (Zelená & Szentagothai, 1957; Miledi, 1962; Couteaux, 1963).

We have explored these possibilities in cell cultures of myotomal muscle and neural tube derived from embryos of *Xenopus laevis*. The muscle cells and neural tube cells develop rapidly in culture and establish many functional synaptic contacts. The cultures have other useful features; they can be maintained under relatively simple conditions at room temperature without antibiotics, and with little contamination by other cell types. In addition the muscle cells remain mononucleated and do not proliferate. It is of interest to note that the first indication of nerve–muscle synapse formation *in vitro* is found in R. G. Harrison's (1910) classical study in which he used explants of these tissues to demonstrate axonal growth.

In the present study we have examined the distribution of ACh receptors with fluorescent conjugates of α-bungarotoxin (Anderson & Cohen, 1974). This paper describes the rapid and dramatic changes in receptor distribution which occur when the muscle cells become innervated. The following paper considers the manner in which these changes are brought about (Anderson & Cohen, 1977). Brief accounts of this work have been reported (Anderson & Cohen, 1975; Anderson, Cohen & Zorychta, 1976).

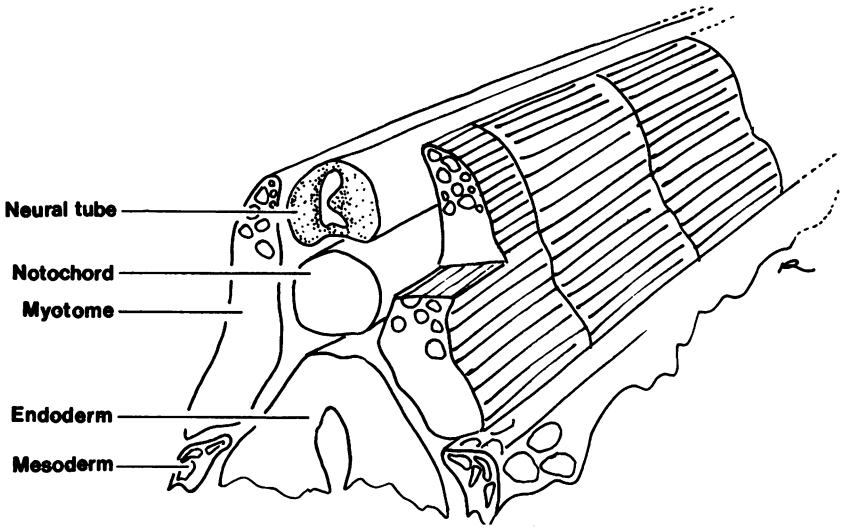
METHODS

Preparation of cultures

Mating was induced in mature *Xenopus laevis* toads by injection of chorionic gonadotrophin (Ayerst) into the dorsal lymph sacs as described by Nieuwkoop & Faber (1956). Approximately 24 hr after fertilization normally developing embryos were transferred to a sterile dissecting medium and thereafter all procedures were carried out under sterile conditions. Dorsal portions of stage 22–23 embryos (Nieuwkoop & Faber, 1956) were isolated by dissection and incubated for 30–60 min in a collagenase solution in order to facilitate the separation of neural tube, myotomal muscle and notochord (Text-Fig. 1). Either of the following three types of culture was then prepared. (1) Mixed nerve and muscle cultures: isolated neural tubes and myotomal muscle were bathed in a trypsin-EDTA solution for about 1 hr and then gently agitated in order to enhance their dissociation. The cells were then plated directly in the same culture chamber. One day later the plating medium was replaced by a maintenance medium, and thereafter the cultures were left undisturbed until required for experimentation. (2) Muscle cultures, free of nerve: in this case only isolated muscle was dissociated and plated. Otherwise, the procedures were the same as for the mixed cultures. (3) Muscle cultures to which neural tube cells were added after 2–3 days: Muscle cultures were prepared as above and isolated neural tubes were stored at 4–10° C in plastic Petri dishes (Falcon) containing plating

medium. After 2–3 days the neural tubes were dissociated and added. As described below the maintenance medium was modified immediately before neural tube cells were added in order to facilitate their adhesion to the culture dish.

All procedures were carried out at room temperature (21–24° C).



Text-fig. 1. Diagram illustrating a portion of the dorsal region of a stage 22–23 *Xenopus* embryo after removing the skin. On either side of the neural tube and notochord there are nine to twelve myotomes only three of which are shown. Each myotome consists of about 100 muscle cells, approximately 100 μm long and 10 μm in diameter. The cells are laden with yolk granules and innervation is just beginning (Nieuwkoop & Faber, 1956; Muntz, 1975). Treatment with collagenase allows the myotomes and neural tube to be separated from each other and from the notochord.

Solutions and culture media

Dissecting medium: L-15 (Gibco), 60% (v/v); dialysed horse serum (Gibco), 5% (v/v); Mycostatin (Gibco), 50 u./ml.; gentamycin (Schering), 50 μg/ml. The latter two agents are effective in eliminating the fungal and bacterial contamination normally associated with *Xenopus* eggs (Laskey, 1970).

Collagenase (Type 1, Sigma) was used at approximately 1 mg/ml. in 60% L-15.

Dissociation medium: a solution (Gibco) of trypsin (5 mg/ml.) and EDTA (2 mg/ml.) was diluted to 20% (v/v) with a Ringer solution containing 67 mM-NaCl, 1·6 mM-KCl and 8 mM-HEPES buffer (Gibco).

Plating medium: L-15, 60%; dialysed horse serum, 5%; Holmes' α-1-protein (Gibco), 0·2 μg/ml.

Maintenance medium: L-15, 60%; Holmes' α-1-protein, 0·2 μg/ml. Serum was omitted because its continued presence at a concentration of 5% caused myotomal muscle cells to degenerate within 3–4 days. However, some serum was found to be essential for the attachment of cells to the culture dish. As a result when neural tube cells were added to 2- and 3-day-old muscle cultures the maintenance medium was supplemented at the same time with up to 1% dialysed horse serum. These

levels of serum caused granulation to occur in some muscle cells, but they remained viable for at least another 4 days.

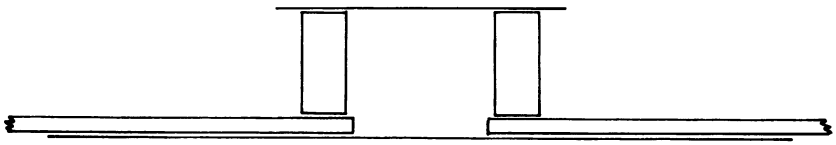
For some experiments the plating and maintenance media also contained carbachol chloride (Mann) 10^{-5} to 10^{-4} g/ml. or D-tubocurarine chloride (Sigma) 10^{-4} g/ml.

All of the above media were prepared using sterile distilled water (Gibco) and when necessary pH was adjusted to about 7·2 with NaOH or HCl. Solutions of non-sterile agents were sterilized by double filtration through Millipore membranes (pore size: 0·22 μm).

Frog Ringer contained, in m-mole/l.: NaCl, 111; KCl, 3; $CaCl_2$, 1·8; Tris maleate buffer adjusted to pH 7·4 with NaOH, 5.

Culture chambers

When cultures were to be fixed after fluorescent staining a simple chamber was frequently used which consisted of a glass ring on a collagen-coated glass coverslip. After filling with medium (volume about 1·8 ml.) and adding dissociated tissue, the chamber was sealed by placing another glass cover-slip on top of the glass ring. All connexions were made with heat-sterilized silicone grease (Dow Corning). After staining and fixing the cultures, the collagen-coated cover-slip was removed and mounted on a glass slide for examination in the fluorescence microscope.



Text-fig. 2. Diagram illustrating a side view of the culture chamber. The floor consists of a collagen-coated cover-slip which covers a hole (15 mm in diameter) in a glass slide. A glass ring (9 mm high) rests on the slide and surrounds the hole. After filling the chamber with medium and adding tissue to it the top is covered with a second cover-slip. All connexions are sealed with silicone grease. The diagram is not drawn to scale.

When fluorescent staining was to be examined in living cultures a more complex chamber was used (Text-fig. 2). The floor of the chamber also consisted of a collagen-coated glass cover-slip but in this case it covered a hole (15 mm diameter) in a glass slide. On top of the slide, surrounding the hole, was a glass ring. The chamber was held together with silicone grease and sealed as above. After fluorescent staining the glass ring was removed and a cover-slip set directly over the hole in the glass slide.

Electrical stimulation

Cultures were placed on the fixed stage of an inverted phase-contrast Zeiss microscope and grounded by an agar-Ringer electrode. Electrical pulses of about 1 msec duration were delivered through a glass micro-electrode filled with frog Ringer and having a bevelled tip of about 2 μm diameter. Stimulus intensities required to evoked muscle contraction by direct stimulation were at least fivefold greater than for evoking contractions by stimulation of neuronal cell bodies.

Fluorescent staining

α-Bungarotoxin and its conjugates with fluorescein isothiocyanate (BBL) and tetramethylrhodamine isothiocyanate (BBL) were prepared as previously described

(Anderson & Cohen, 1974), except that the final solutions were equilibrated with frog Ringer instead of with 0·05 M ammonium acetate buffer. Stock solutions (10^{-4} to 10^{-3} g/ml.) were stored frozen at $-40°$ C and thawed when required. Rhodamine-toxin conjugates had a potency of 5–10 % relative to native toxin, as determined by tests on *Xenopus* tadpole tails (see Anderson & Cohen, 1974). The potency of fluorescein-toxin conjugates was 1–3 %.

Cultures were stained by exposing them to fluorescent toxin (10^{-5} g/ml. in plating medium) for 20–30 min at room temperature. After rinsing with 60 % L-15 the cultures were either fixed by immersion in cold ($-16°$ C) 95 % ethanol or examined alive. Ethanol-fixed cultures were rehydrated briefly with frog Ringer. When fluorescein staining was to be observed the fixed cultures were mounted in 90 % glycerol containing either 0·1 M sodium carbonate (pH 9·5) or 0·1 M Tris buffer (pH 9·0). When only rhodamine staining was to be observed the cultures were mounted in pure glycerol. Staining appeared brighter in fixed, glycerol-mounted, cultures than in living cultures but the staining patterns were similar.

Fluorescence microscopy

In early experiments fluorescent staining was observed with a Zeiss microscope using transmitted dark-field illumination as previously described (Anderson & Cohen, 1974). More recently we have used incident illumination, employing the Zeiss RSIII epi-condenser. With this latter system the same field can also be examined directly with phase contrast optics. For excitation of fluorescein the light from an HB0200/W2 lamp was passed through an LP455 filter and a KP500 interference filter. For rhodamine KP600 and BP546 interference filters were used. The corresponding barrier filters were an LP520 for fluorescein and a Kodak No. 23A for rhodamine. Living cultures were always examined with incident illumination and for rhodamine staining an extra BP 546 filter was placed in the excitation path.

Kodak Tri-X 35 mm film, processed to ASA 1600 in Acufine Developer, was used in preparing black and white micrographs. For colour micrographs High Speed Ektachrome, processed to ASA 400, was used.

Radioautography

α-Bungarotoxin was conjugated with carrier-free ^{125}I (New England Nuclear) by chloramine T oxidation as described by Berg *et al.* (1972) and separated from the reaction mixture by chromatography on Sephadex G-25. The labelled toxin had an initial specific activity of 20,000 cpm/ng. Radioactivity was measured in a gamma scintillation spectrometer (Nuclear Chicago, Model 1185).

Cultures were exposed to the radioactive toxin (10^{-6} g/ml. in plating medium) for 20 min. After rinsing they were fixed with 3 % glutaraldehyde in 0·1 M sodium phosphate buffer (pH 7·0), rinsed with buffer, dehydrated in ethanol, degreased in xylene and then returned to ethanol. The coverslips containing the cultured cells were dried in air at room temperature, coated with Kodak NTB-2 emulsion and incubated in the dark at 4° C (Kopriwa & Leblond, 1962). Radioautographs were developed in Kodak D170 at 18° C and fixed with sodium thiosulphate, 0·24 g/ml.

Grains were counted at a magnification of 1250 times, and areas were determined with an eye-piece grid. For patches containing a high grain density an area of 64 μm^2 or more was counted. For grains over the rest of the cell the areas that were counted were at least fifteen times larger. To estimate the area of a dense patch relative to the remainder of the cell photographic prints were cut into the two respective parts and weighed.

RESULTS

General description of cultures

Muscle cultures

Dissociated muscle cells attached to the collagen substrate and began to elongate within 12 hr. The majority reacquired a bipolar configuration, usually with several projections emerging along their length, but others developed a more stellate appearance (Pl. 1). Striations, having a periodicity of about 1·9 μm, were observed within 1 day and increased in prominence during the following 3–4 days. Over the same period most of the yolk granules originally present in the cells were consumed and the cells grew in size. Bipolar cells attained lengths of up to 300 μm and widths of up to 40 μm. The cells also increased in thickness and after about 4 days began to lose some of their finer processes. Throughout the period that the cultures were maintained (up to 1 week) the muscle cells remained mononucleated and did not migrate on the culture dish.

Each culture contained several hundred muscle cells as well as a few other cells, such as fibroblasts and an occasional melanocyte. Contamination by neural tube cells was rare, occurring in less than 10 % of the more than forty muscle cultures examined in this study. These contaminated cultures never contained more than four nerve cells, and were not included in further studies.

Muscle contraction did not occur spontaneously but could be evoked by direct electrical stimulation and by application of ACh and carbachol. Both D-tubocurarine and α-bungarotoxin were effective in blocking the action of the cholinergic agonists but did not affect contractions evoked by electrical stimulation. Contractions of sufficient intensity caused the cells to detach from the substrate, resulting in irreversible damage.

Mixed cultures

Neural tube cells in culture occurred either in isolation or in small clusters, and had spherical perikaraya which were 10–20 μm in diameter. During the first day processes began to emerge from them in the manner originally described by Harrison (1910). These nerve processes continued to grow for 2–3 days and contacted many muscle cells (Pl. 1*B*). However, after the third day nerve growth appeared to cease. In fact fewer nerve processes and nerve-muscle contacts were observed in 4- to 5-day-old cultures than in 2- to 3-day-old ones. This retraction of nerve processes was related to the absence of serum in the culture medium after the first day (see Methods). Although the continued presence of serum facilitated the growth of nerve processes it also caused the myotomal muscle cells to

degenerate within 3–4 days. By withdrawing serum from the cultures after the first day the muscle cells remained viable for at least a week.

Nerve–muscle contacts seemed to have no preferred location or orientation (see Pls. 5 and 6). Frequently the nerve grew along the edges of muscle cells but in many cases it coursed over the muscle cell, either on the surface facing the collagen substrate of the culture dish or on the opposite free surface. Some of the contacts were oriented longitudinally with respect to the muscle cell axis whereas others had a transverse orientation. They occurred in virtually any region of the muscle cell and varied greatly in length.

Single electrical stimuli applied to nerve cell bodies evoked synchronous contractions in many of the contacted muscle cells. Spontaneous contractions were also observed in some of the nerve-contacted muscle cells. Their frequency was usually low but could be quickly and reversibly augmented by the addition of 1–2 % serum. Both spontaneous and evoked contractions were reversibly blocked by D-tubocurarine (10^{-5} g/ml.) and by magnesium (10 mM), and were irreversibly blocked by α-bungarotoxin (10^{-6} g/ml. for 20 min). These findings indicate that muscle cells had become functionally innervated, as was previously shown to be the case in explant cultures of these same tissues (Cohen, 1972).

Patterns of fluorescent staining

Muscle cultures

One or more patches of fluorescent stain were observed on virtually every muscle cell in fourteen cultures examined 2–5 days after plating. Actual counts were made in seven cultures, 2–4 days old. In each case about 100 muscle cells were examined, and out of a total of 652 cells only one was observed which did not have a patch of stain. On the other hand, fluorescent stain was never seen on fibroblasts, melanocytes, or other non-muscle cell types.

Several examples illustrating the diversity of the patches of stain are shown in Pls. 2, 3 and 4*A*. Although there was considerable variation from cell to cell in the location, number, size and configuration of these patches some generalizations can be made. Their most common location was at or near the ends of cells, or cell processes, on the surface facing the collagen substrate (Pls. 2*A*, *B*, *E*, *F* and 3*A*, *B*). Another common location was on the free surface in more central regions of the cell (Pls. 3*C*, *D* and 4*A*). However patches on the free surface sometimes occurred near the ends of cells (Pl. 3*E*, *F*) and those on the collagen surface also occurred in central regions. Less frequently patches were also situated on the edges of cells and could not be visualized face-on (Pl. 2*C*, *D*). Patches on the same cell often belonged to more than one of these categories (Pls.

2*C*, *D* and 4*A*). Large cells tended to have more and larger patches and in some instances as many as seven distinct patches were seen on a single cell. However, even on the largest cells patches usually extended for less than 20 μm and never more than 40 μm. When they occurred in central regions of cells they never extended from one edge of the cell to the other.

Individual patches of stain had fairly distinct boundaries but the intensity within the patch was frequently non-uniform. Thus many patches appeared to be compound structures composed of numerous lines or spots. Their substructure varied according to their location on the cell. Patches on the collagen surface were usually composed of a series of fine lines of alternating intensity generally oriented parallel to each other (Pl. 2*E*, *F*). On the other hand, patches on the free surface consisted of either compact arrays of small spots (Pls. 3*C*, 4*A*) or complex arrangements of thin lines sometimes oriented in a stellate pattern (Pl. 3*E*). Often there appeared to be little if any fluorescent stain between the lines or spots.

Mixed cultures

Forty mixed cultures ranging in age from 2 to 5 days were examined in this study. In six of the cultures which were 3 days old some of the innervated muscle cells were first identified on the basis of spontaneous twitching or contractions evoked by electrical stimulation of the appropriate nerve cell bodies; they were then stained and examined alive.

In every one of seventy-eight identified innervated muscle cells staining was restricted to the path of the innervating nerve process and there were no patches of stain elsewhere on the cell (Pl. 5). Indeed the distribution of stain in most of these examples was dramatically different from that found either on the cells in muscle cultures or on non-contacted muscle cells in these mixed cultures. Such distinctive staining patterns were seen on many of the nerve-contacted muscle cells in all mixed cultures, and a variety of examples are shown in Pls. 4*B*, 5 and 6. The following description is based on the staining patterns seen in all forty mixed cultures of varying ages.

Stain was associated with nerve–muscle contacts independently of their location on the muscle cell. In some cases the staining appeared as a fine line along the edge of the cell (Pls. 5*E*, 6*C*, *E*) and in other cases it appeared as a narrow band either on the surface facing the collagen substrate or on the opposite free surface. These face-on examples seemed to have no preferred orientation with respect to the long axes of the muscle cells or their processes. Thus in some instances the bands of stain had a longitudinal orientation (Pls. 5*A*, 6*D*) whereas in others they extended across the cell (Pls. 5*G*, *I*, 6*D*) or even across several cells in succession (Pls. 4*B*, 6*A*). Interestingly the bands and lines of stain were frequently located in the

central regions of muscle cells. This is in contrast to the situation *in vivo* where the myotomal muscle cells are innervated exclusively at their ends (Lewis & Hughes, 1960).

Viewed at high magnification it was apparent that the distribution of stain along the path of nerve contact varied considerably. Occasionally the staining continued without interruption along the entire length of the contact (Pl. 6*A*, *B*). Most often, however, the bands were discontinuous and consisted of a series of small irregular regions of stain (Pls. 5, 6*C*, *D*). In still other examples the stain was limited to only a portion of the contact. Some bands of stain had intense borders parallel to the path of the nerve (Pls. 4*B*, 6*A*), but in the majority of examples this was not the case. Nevertheless the bands of stain always had well defined boundaries where the staining intensity changed abruptly rather than gradually. Their widths were variable, even along the path of a single contact, and usually had a range of 2–5 μm. However, the bands of stain often extended beyond the diameters of the corresponding nerve process (Pl. 5*A*, *B*, *I*, *J*), suggesting that the stain was associated mainly if not entirely with the muscle cells. Indeed fluorescent stain was never found on nerve processes as they coursed over the culture dish or over other cell types such as fibroblasts.

The lengths of the lines and bands of stain were also highly variable. Thus, while many were less than 20 μm some extended for more than 40 μm and occasionally for as much as 100 μm (Pls. 5*A*, *E*, 6*C*, *D*). This finding is of particular significance since the patches on non-contacted muscle cells were never more than 40 μm in their greatest dimension. In addition, unlike the patches on non-contacted cells, the bands of stain sometimes extended entirely across the central regions of muscle cells (Pls. 4*B*, 5*I*, 6*A*). These examples therefore cannot be explained simply in terms of the nerve having contacted pre-existing receptor patches. Instead they indicate that the localization of at least some of the receptors along the path of contact was neurally induced.

Lines and bands of stain sometimes appeared to extend beyond the region of nerve contact and in a few cases were also found on muscle cells which had no apparent nerve contact. These latter examples were seen only in regions of the culture which contained neural tissue. They can most likely be explained either by difficulty in resolving very fine nerve processes in the light microscope (see also Fambrough, Hartzell, Rash & Ritchie, 1974), by mechanical displacement of the nerve process during the staining procedure, or by spontaneous retraction of the nerve process. Some retraction clearly did occur with aging of the cultures and an example of retraction associated with a modified pattern of stain is given in the following paper (see Pl. 6 in Anderson & Cohen, 1977).

Although innervation was invariably correlated with a localization of stain along the path of nerve contact and with an absence of stain elsewhere on the cell, such distinctive patterns of stain were not associated with all nerve-contacted cells. Instead some muscle cells had little or no stain along the path of nerve contact. In these cases there was almost always additional stain on other regions of the cell. This staining sometimes consisted of the characteristic patches associated with non-contacted muscle cells. On the other hand the staining pattern was sometimes markedly different and consisted of a scattering of small fluorescent spots over a large portion of the cell. This latter form of staining was observed more frequently in cultures where addition of neural tissue was delayed for 2–3 days (see below).

Addition of neural tube cells to 2- and 3-day-old muscle cultures

The results described so far indicate that contact of a growing nerve fibre with a pre-existing patch of high receptor density cannot account for all of the staining that was associated with nerve–muscle contacts. Indeed since the nerve and muscle cells were plated simultaneously the receptor distribution prior to innervation was unknown and patches may never have been present. It was therefore of interest to determine whether the nerve would induce a similar localization of receptors if innervation was delayed until patches had already developed on all muscle cells. This possibility could be readily tested because all the muscle cells in 'pure' muscle cultures developed characteristic receptor patches within 2 days of plating. Muscle cultures were therefore allowed to develop for 2–3 days during which time they were carefully examined to ensure that there was no neural contamination. Freshly dissociated neural tube cells were then plated. Within one day growing nerve processes contacted many of the muscle cells, some of which began to twitch spontaneously, indicating that innervation had occurred. Altogether six such cultures were stained with fluorescent toxin 1–2 days after the addition of neural tube cells.

In each of these cultures the staining patterns on nerve-contacted cells were similar to those described for cultures where neural tube cells were present from the start. Most significantly lines and bands of stain were associated with paths of nerve-muscle contact, and some of these extended for more than 40 μm (Pl. 7*A*, *C*) or crossed the entire width of the cell. In such cases there were usually no characteristic patches elsewhere on the cell. One interesting difference, however, between these cultures and those in which neural tissue was present from the start, was that in many more instances there was a widespread speckling of small spots of fluorescent stain (Pl. 7*A*, *E*), particularly on the first day after adding neural tissue. This speckling was usually most extensive on those muscle cells

which had relatively little stain along the path of nerve–muscle contact. Furthermore, it was never seen on non-contacted muscle cells in regions of the culture devoid of neural tube cells, or in two additional cultures which were treated in the same manner (see Methods) except that neural tube cells were not added. Instead the non-contacted muscle cells had only the characteristic patches of stain.

These observations therefore indicate that the nerve can cause both a rapid accumulation of receptors at sites of contact, and the development of scattered receptor clusters, on muscle cells which have receptor patches before contact is made.

Mixed cultures grown in curare and carbachol

Previous studies have indicated that neuromuscular connexions can become established in the presence of agents which block neuromuscular activity (Harrison, 1904; Crain & Peterson, 1971; Cohen, 1972; Jansen & Van Essen, 1975; Giacobini-Robecchi, Giacobini, Filogamo & Changeux, 1975) although maturation of the junctions may be retarded (Giacobini, Filogamo, Weber, Boquet & Changeux, 1973; Gordon, Perry, Tuffery & Vrbová, 1974). The occurrence of high sensitivities to acetylcholine at sites of contact between cloned muscle and nerve cells is likewise not prevented by such blocking agents (Steinbach, Harris, Patrick, Schubert & Heinemann, 1973). On the other hand suppression of neuromuscular activity in adult muscle results in the appearance of extrajunctional ACh receptors (Thesleff, 1960; Lømo & Rosenthal, 1972; Berg & Hall, 1975*a*; Chang, Chuang & Huang, 1975; Lavoie, Collier & Tenenhouse, 1976; Pestronk, Drachman & Griffin, 1976). Studies of synapse formation in cell culture have led to the suggestion that contractile activity may also be required for nerve contacts to bring about changes in the distribution of extrajunctional receptors during development (Fischbach & Cohen, 1973; Cohen & Fishbach, 1973). It was therefore of interest to determine whether the spontaneous neuromuscular activity in the present experiments was necessary for the development of the distinctive staining patterns on nerve-contacted cells. For this purpose mixed cultures were grown from the start, for 3–4 days, in the presence of either D-tubocurarine chloride (10^{-4} g/ml.; three cultures) or carbachol chloride (10^{-5} to 10^{-4} g/ml.; six cultures). In addition, neural tube cells were added to 3-day-old muscle cultures in the presence of D-tubocurarine chloride (10^{-4} g/ml.; three cultures) and allowed to develop for 1–2 days. As in drug-free cultures, nerve processes developed rapidly and within 1 day many could be seen in contact with muscle cells. However, as expected, contractions did not occur spontaneously under these conditions and could not be evoked by electrical stimulation of neural tube cells. On the other hand, both

spontaneous and nerve-evoked contractions were observed within 10–20 min after withdrawing the drug. When such cultures were stained after rinsing out the drug the usual patterns of fluorescence were observed. Muscle cells not contacted by nerve had characteristic patches of stain, whereas some of those which were contacted had the distinctive lines and bands without any patches elsewhere (Pl. 8*D*, *F*). Likewise, muscle cells which had little or no stain along the path of nerve contact sometimes had a widespread scattering of small fluorescent spots. As in cultures grown in the absence of the drugs, this feature was more common in cultures where the addition of neural tube cells was delayed. These results therefore indicate that nerve-induced changes in the distribution of ACh receptors occurred by a mechanism which is independent of contractile activity. This finding is extended in the following paper which demonstrates similar patterns of stain even when cultures are grown in the presence of α-bungarotoxin (Anderson & Cohen, 1977).

Control experiments

Specificity of fluorescent staining

In a previous study it was established that α-bungarotoxin retains its specificity for ACh receptors after it has been labelled with fluorescent dyes (Anderson & Cohen, 1974). As indicated in Table 1, specificity tests were also carried in the present study, with similar results. Fluorescent stain was barely detectable when the staining procedure was carried out in the presence of D-tubocurarine (10^{-4} g/ml.) and was not seen when cultures were pretreated with native α-bungarotoxin (10^{-6} g/ml. for 20 min). Carbachol was also tested but in this case the cultures were grown in the drug from the start in order to avoid muscle contracture and damage. At a concentration of 10^{-4} g/ml. it completely inhibited fluorescent staining. As expected the blocking effect of native toxin was not overcome even when the cultures were rinsed extensively with toxin-free medium for up to 1 hr. On the other hand the blocking effects of curare and carbachol were quickly reversible. For example, in one type of experiment cultures were exposed for 30 min to rhodamine-toxin (10^{-5} g/ml.) in the presence of curare (10^{-4} g/ml.). They were then rinsed for 20 min with a medium free of both agents and exposed to fluorescein-toxin (10^{-5} g/ml.) for a further 30 min. Upon examination in the fluorescence microscope only the green stain due to the fluorescein conjugate was observed (Pl. 8*A–C*). Similar results were obtained when carbachol was used instead of curare (Pl. 8*D–F*; Table 1). All of these tests therefore indicate that the staining is specific for ACh receptors.

Receptor staining was readily distinguishable from yolk granules which proved to be autofluorescent. The granules had a smooth appearance (Pl. 3*C*) with no single

plane of focus, and when viewed with fluorescein optics they appeared yellow rather than green. Their presence in large numbers resulted in a bright glow which obscured the fluorescent stain, and because of this we restricted our observations to cultures which were 2 or more days old. Smaller bright refractile spots, also readily distinguishable from receptor staining, were seen in early experiments in which transmitted light was used for excitation (Pl. 4). These spots were not observed with incident light fluorescence.

TABLE 1. Effects of cholinergic agents on fluorescent staining. F and R indicate fluorescein and tetramethylrhodamine respectively. Concentrations are given in text

Culture medium	Staining procedure			Fluorescent staining	
				F	R
Standard	F-toxin	—	—	Bright	None
Standard	R-toxin	—	—	None	Bright
Standard	Toxin	R-toxin	F-toxin	None	None
Standard	Curare	Curare, R-toxin	F-toxin	Bright	Barely visible
Plus carbachol	Carbachol, R-toxin	F-toxin	—	Bright	None
Plus carbachol	Carbachol F-toxin	R-toxin	—	None	Bright

Staining after fixation with paraformaldehyde

Although the patterns of fluorescent stain reflect the distribution of ACh receptors, it is less clear that they also correspond to the distribution of receptors prior to binding with fluorescent toxin. For example, other ligands such as immunoglobulins and lectins can cause their receptors to aggregate in the plasmalemma (Taylor, Duffus, Raff & de Petris, 1971; Rosenblith, Ukena, Yin, Berlin & Karnovsky, 1973). This effect appears to depend upon the fact that these ligands are multivalent and can therefore cross-link their previously dispersed receptors. α-Bungarotoxin on the other hand is a small univalent ligand (Mebs, Narita, Iwanaga, Samejima & Lee, 1971; Lee, 1972) and would therefore not be expected to cause changes in the distribution of ACh receptors. In any event we have attempted to examine this possibility experimentally, and have based the test on the fact that ligand-induced changes in receptor distribution can be prevented if the cells are first fixed with paraformaldehyde (Rosenblith *et al.* 1973; Ryan, Borysenko & Karnovsky, 1974).

For these experiments muscle cultures were fixed at room temperature for 1 hr with 3×10^{-2} g/ml. paraformaldehyde in 0·1 M phosphate buffer at pH 7·0. The cultures were then rinsed with plating medium for at least 30 min and stained with fluorescent toxin. In some cases the rinse and staining were carried out in the presence of carbachol (10^{-4} g/ml.). Upon

examination with fluorescence optics the cells generally appeared brighter than usual, but had characteristic patches similar to those on living cells (Pl. 9*A*, *B*). Cells stained in the presence of carbachol also appeared brighter than usual, suggesting some non-specific uptake of dye-toxin conjugate, but had no patches of fluorescent staining. These results indicate that staining specificity for ACh receptors was retained after paraformaldehyde fixation, and suggest that receptor patches existed on the cells before exposure to the toxin. Similar conclusions have been drawn for the patchy distribution of toxin-binding sites on cultured chick myotubes (Sytkowski *et al.* 1973).

TABLE 2. Radioautography with ^{125}I-labelled α-bungarotoxin in 2-day-old muscle cultures

A, inhibition by pre-incubation with native toxin (10^{-6} g/ml. for 20 min). Isolated cells were chosen at random and all grains over at least 70 % of each cell were counted. Similar areas adjacent to the cells were also counted in order to obtain the background grain density. The results (mean ± S.E.) in each case are based on fourteen cells or culture dish areas. Percent inhibition was calculated after correction for background grains on the culture dish

Grain density (grains/64 μm^2)				
Pre-incubated with native toxin		No pre-incubation		Inhibition by native toxin (%)
Culture dish	Cell	Culture dish	Cell	
0·4 ± 0·04	0·8 ± 0·1	0·4 ± 0·03	5·4 ± 0·4	92

B, distribution of grains. Isolated cells were chosen which had only one patch of high grain density. Grain densities are corrected for background grains on the culture dish

No. of cells	Grain density (grains/64 μm^2)		Grain density in patch relative to rest of cell	Area of patch relative to rest of cell	Grains in patch (%)
	High density patch	Rest of cell			
13	48 ± 2	2 ± 0·2	26 ± 3	0·034 ± 0·004	44 ± 3

Radioautography with ^{125}I-labelled toxin

Although fluorescent staining can reveal areas of high receptor density the sensitivity of the method may not be sufficiently great to reveal lower densities of receptors (Anderson & Cohen, 1974). It was therefore not possible to determine by fluorescent staining whether all receptors on muscle cells were localized in patches or whether some were also distributed elsewhere on the sarcolemma. In order to check this possibility experiments were carried out in which receptor distribution was assessed by radioautography using ^{125}I-labelled α-bungarotoxin. For these experiments

muscle cultures were exposed to the radioactive toxin (10^{-6} g/ml.) for 20 min, rinsed, and then processed as described in the Methods. As expected, patches of high grain density were observed which were similar both in size and distribution to the patches of fluorescent staining on the free surface of the muscle cells (Pl. 9*C*, *D*, *E*). In addition to these patches a lower density of grains was observed over the remainder of the cell. Most of the grains appeared to be due to specific binding of radioactive toxin to ACh receptors: thus in a sister culture which was incubated for 20 min with 10^{-6} g/ml. native toxin before being exposed to the radioactive toxin the grain counts on the cells were reduced by 92 % (Table 2). In one culture grain counts were made on thirteen muscle cells having only one obvious patch and the grain densities were found to be about 25 times higher in the patches than on the rest of the cell (Table 2). Estimates based on measurements of relative patch and cell areas further indicated that about 44 % of all the grains on these cells were localized in patches (Table 2). It follows from these experiments that ACh receptors occurred not only in patches but also over the entire cell. The density of receptors in this widespread phase, however, was too low to be detected by the fluorescent staining technique.

DISCUSSION

Patches of ACh receptors on non-innervated cells

The present study has demonstrated distinctive patterns of ACh receptor distribution on myotomal muscle cells cultured with and without nerve. Non-innervated muscle cells develop characteristic patches of receptors which tend to occur in preferred locations, such as near the tips of cell processes facing the collagen substrate and in central regions on the opposite side of the cell. They occupy a small percentage of the cell area but contain a significant fraction of the receptors. Radioautographic experiments indicated that single patches which occupy only about 3–4 % of the cell area contain almost 50 % of the receptors. Presumably these patches reflect regions of sarcolemma where the receptor density is relatively high. This has recently been found to be the case for ACh receptor patches on cultured chick myotubes in experiments which combined an immunoperoxidase technique with electron microscopy (Vogel & Daniels, 1976). The alternative possibility, that receptor density in the sarcolemma is uniform and that the patches reflect regions of extensive membrane folding, seems unlikely. For example bright fluorescent staining was observed on fine cell processes which were less than 1 μm in diameter (see Pl. 3*A*) and which would not be expected to have extensive membrane folding. Furthermore, radioautography revealed patches with grain densities which were on the average twenty-fivefold greater than over the remainder

of the cell. In individual examples the factor was as large as fortyfold. To increase the surface area by such a large factor would require a degree of membrane folding which has never been seen in normal or cultured muscle cells. For example, the complex folds at the mammalian neuromuscular junction are estimated to increase membrane area by only 4–5 times (Andersson-Cedergren, 1959). In view of these considerations it seems likely that the receptor patches on cultured myotomal muscle cells, like those on cultured chick myotubes, reflect regions of sarcolemma containing a high receptor density. Similar patches have also been observed on adult denervated muscle fibres (Ko, Anderson & Cohen, 1977).

Another question is whether the patches of receptors on non-innervated cells are a normal feature of the sarcolemma. This matter is of considerable relevance since other ligands such as immunoglobulins and lectins have been shown to induce the aggregation of their receptors (for reviews see Raff & de Petris, 1974; Nicolson, 1974). However, discrete regions of high ACh receptor density have been revealed in cultures of chick and rat myotubes by the method of iontophoresis which is carried out in the absence of α-bungarotoxin (Cohen & Fischbach, 1973; Hartzell & Fambrough, 1973). Receptor patches were also observed in the present study as well as on chick myotubes (Sytkowski *et al.* 1973) when receptor labelling was carried out after formaldehyde fixation, a procedure which is known to prevent ligand-induced clustering of receptors in other systems (Rosenblith *et al.* 1973; Ryan *et al.* 1974). It therefore appears that ACh receptor patches are a normal feature of the sarcolemma and do not merely reflect an aggregation of receptors induced by toxin binding.

It is possible nevertheless that the formation of ACh receptor patches may reflect processes similar to those which have been implicated in the aggregation of immunoglobulin and lectin receptors. For example, several studies have suggested that the mobility of some membrane receptors is controlled by attachment to cytoskeletal elements composed of microfilaments and microtubules (for reviews see Nicolson, 1974; Raff & de Petris, 1974; Edelman, 1976). Similar cytoplasmic structures may also interact with ACh receptors. The resolution afforded by fluorescent staining has revealed that ACh receptor patches are highly ordered structures with distinctive patterns. Furthermore, the complex substructure of the patches on the surface of the cell in contact with the collagen substrate is distinctly different from that of patches on the free surface. This difference in organization presumably reflects the fact that the sarcolemma is attached in some regions to the substrate. The complexity of receptor patches on the free surface is particularly difficult to rationalize if one assumes the membrane is a simple 'fluid-mosaic' in which integral glycoproteins exist in a state of diffusional equilibrium (see Singer & Nicolson,

1972). These ACh receptor patches usually consist of either an aggregate of small receptor clusters (Pl. 3*C*) or a stellate pattern of thin lines, sometimes interspersed with small clusters (Pl. 3*E*). It is unlikely that the components of these patches are held together in such non-random arrays by simple intermolecular forces between receptor units. Instead it seems more probable that small ACh receptor clusters are anchored to cytoplasmic elements which might then provide a structural basis for the complex patch substructure. In this vein it is interesting also to note that the cable-like bundles of contractile proteins which form part of the cytoskeleton in non-muscle cells have been found to assume complex networks (Lazarides, 1976) with foci not unlike the stellar arrangements of some ACh receptor patches.

Distribution of ACh receptors on innervated cells

The most distinctive feature of the receptor staining on innervated myotomal cells was its localization along the path of nerve contact. Since staining was never seen on nerve processes which were not in contact with muscle cells, and in many cases bands of stain were broader than the corresponding nerve, the pattern of stain at sites of nerve contact almost certainly reflects the development of a high receptor density in the subsynaptic membrane. This conclusion can be drawn most directly from the results of the following paper where similar patterns of stain were found to develop even when muscle cells were exposed to fluorescent toxin prior to the addition of nerve (Anderson & Cohen, 1977). It is also in agreement with other studies which have demonstrated either a high sensitivity to ACh (Harris, Heinemann, Schubert & Tarakis, 1971; Kano & Shimada, 1971; Fischbach & Cohen, 1973) or a high density of ^{125}I-α-bungarotoxin binding sites (Fambrough *et al.* 1974) in the vicinity of nerve–muscle contacts in cell culture.

The resolution afforded by fluorescent staining revealed a large diversity in the pattern of receptor distribution along the path of nerve–muscle contact. In some cases the staining along the path of contact was continuous and uniform but in the majority of examples the bands of stain were discontinuous and irregular. Of particular significance is that in all cases there were abrupt changes in staining intensity, and hence in receptor density, along the edges of the bands of stain. Similar abrupt changes in receptor density occur at the border between synaptic and extrasynaptic membrane in normal adult muscle (Peper & McMahon, 1972; Hartzell & Fambrough, 1972; Anderson & Cohen, 1974; Kuffler & Yoshikami, 1975; Fertuck & Salpeter, 1976). The present study has indicated that they can also occur elsewhere on the muscle cells. For example, receptor patches on non-contacted muscle cells had well-defined boundaries, as did the

small receptor clusters which were seen on some of the nerve-contacted cells. It is apparent therefore that abrupt changes in receptor density are not a unique feature which is dependent upon innervation. Instead they are more likely to reflect a mechanism within the muscle cell that can organize ACh receptors into two distinct phases which have markedly different packing densities and are separated by distinct borders.

Since non-contacted muscle cells developed patches of high receptor density analogous to those which have been observed on chick (Vogel *et al.* 1972; Fischbach & Cohen, 1973; Stykowski *et al.* 1973; Vogel & Daniels, 1976) and rat myotubes (Hartzell & Fambrough, 1973), the question can be raised as to whether these structures play a role in synaptogenesis, perhaps by providing a site which growing nerves seek out. To answer this question it would be necessary to determine whether any part of the subneural membrane contained a high density of ACh receptors prior to the establishment of synaptic contact. The results of the present study therefore do not exclude this possibility. However, they do indicate the existence of an alternative mechanism by which receptors become localized at sites of innervation. For example, the lengths of the bands of staining at sites of nerve contact were sometimes too great to be explained simply by the nerve having contacted a pre-existing patch. Even if a patch was contacted, the staining pattern on innervated myotomal cells can thus only be rationalized by assuming that new regions of densely packed receptors formed along the path of the nerve. In fact, such an inductive process could alone account for all the changes in ACh receptor distribution at sites of innervation. Taken together, these considerations imply that the formation of dense receptor patches may not be a requirement for synapse formation. Instead the formation of patches may simply reflect a mechanism in muscle cells which normally mediates the nerve-induced localization of ACh receptors.

The nerve-induced changes in receptor distribution demonstrated in the present study involved not only an accumulation of receptors in the subneural membrane, but also in some cases the development of a widespread scattering of small receptor clusters. Similar changes occurred even when the cultures were grown in high concentrations of curare or carbachol which block all spontaneous twitching. Indeed, the concentration of curare used in these experiments has been shown to abolish all neurogenic electrical activity in cultured myotomal muscle cells (Cohen, 1972). This indicates that the development of new regions of high receptor density at the site of innervation, and the extensive changes in the distribution of 'extrajunctional' receptors, were brought about by some neural factor independent of synaptic or contractile activity. The same conclusion can be drawn from the results of the following study where

corresponding changes in receptor distribution were observed when development took place in the presence of α-bungarotoxin (Anderson & Cohen, 1977). These observations thus indicate that changes in receptor distribution must have been induced either by the release of a neural substance or by direct interaction between the surface membranes at the site of contact.

The inductive process involved in the change in receptor distribution could operate in at least two conceptually distinct ways. In one case nerve contact might provide a spatial component to receptor metabolism, leading to a gradual build-up of receptors synthesized after the contact is made. For example, newly synthesized receptors might be inserted preferentially at the site of contact or receptors in the region of contact might be protected from degradation. The latter possibility is in line with recent studies which have revealed that junctional receptors are more stable metabolically than extrajunctional receptors (Berg & Hall, 1974, 1975*b*; Chang & Huang, 1975; Frank, Gautvik & Sommerschild, 1975). The other principal alternative would be that nerve contact produces a rearrangement of receptors in the sarcolemma such that mobile receptors aggregate at the site of innervation. This possibility is supported by the finding of the present study that the inductive effect of innervation on receptor distribution is not restricted to the vicinity of the nerve contact, but can also lead to the appearance of small receptor clusters over large areas of extrasynaptic muscle membrane. More direct evidence in support of a process of receptor redistribution is provided in the following paper (Anderson & Cohen, 1977).

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EXPLANATION OF PLATES

Except where stated otherwise examples of fluorescent staining were obtained with incident illumination.

PLATE 1

Low-magnification phase-contrast views of living cultures. *A*: a region of a 3-day-old muscle culture. By this time in culture the myotomal muscle cells have developed striations. The cells remain mononucleated. *B*, a region of a 2-day-old mixed, nerve and muscle culture. Nerve processes emanating from a cluster of neural tube cells have contacted several myotomal muscle cells. Most of the muscle cells still have numerous yolk granules in their nuclear region and have less prominent striations than in *A*.

PLATE 2

Patches of fluorescent stain on muscle cells cultured without neural tissue. Cultures were stained with rhodamine-labelled toxin, fixed in ethanol and mounted in glycerol. *A*, *B*: fluorescence and phase-contrast views of the same field in a 5-day-old culture. Patches of stain are on the collagen surface of the cells. The bright profile near the left-hand edge of *A* is autofluorescence associated with the unidentified particle in *B*. Cell outlines (*A*) have been traced in. *C*, *D*: fluorescence and phase contrast of a field in a 3-day-old culture. A patch of stain is located on the edge of the cell. Other patches on the collagen surface of the cell are not in the plane of focus and appear faint. *E*: patches of stain on collagen surface at ends of cell processes. Fluorescence and phase-contrast views are superimposed. Three-day-old culture. Scale as in *D*. *F*, same as *E*, but from another 3-day-old culture. Note the non-uniformities within each patch. Scale as in *B*.

PLATE 3

Patches of fluorescent stain on muscle cells cultured without neural tissue. Four-day-old cultures were stained with rhodamine-labelled toxin and examined alive. Each pair of micrographs shows the same field viewed with fluorescence and phase

contrast optics. *A*, *B*: patches on collagen surface of cell. Note in particular the staining associated with some of the very fine processes at the cell edge. *C*, *D*: patches (see arrows) of stain on the free surface of the cell. Note that the lower patch consists of an aggregation of small fluorescent spots. Several autofluorescent granules are present in the perinuclear region. *E*, *F*: patch, on free surface of cell, consisting of a network of fine lines and spots.

PLATE 4

Colour micrographs of cultured muscle cells stained with rhodamine-labelled toxin. Three-day-old cultures fixed in ethanol, mounted in glycerol, and examined with transmitted dark-field illumination (see Methods). *A*: from a culture without neural tissue. The central patch of stain is on the free surface of the cell and consists of an aggregation of small fluorescent spots. Two other patches, not in the plane of focus, are on the collagen surface of the cell. *B*, from a mixed culture containing both nerve and muscle cells. The narrow bands of fluorescent stain are on the collagen surface of two muscle cells. Such bands of stain are typically associated with the path of nerve contact (not seen). Note the absence of stain elsewhere on these cells. The small bright refractile spots (*A*, *B*) were not seen with incident-light fluorescence.

PLATE 5

Patterns of fluorescent stain on identified, innervated muscle cells. Three-day-old cultures were stained with rhodamine-toxin and examined alive. All of the nerve-contacted muscle cells in this plate had twitched spontaneously before being stained. Each pair of micrographs shows the same field viewed with fluorescence and phase-contrast optics. Note that in all cases the stain is restricted to regions of nerve–muscle contact.

PLATE 6

Patterns of fluorescent stain on nerve-contacted muscle cells. Cultures were stained with rhodamine-labelled toxin. *A*, *B*: fluorescence and phase contrast views of the same field in a 4-day-old living culture. The stain follows the path of nerve–muscle contact and extends across the entire breadth of three successive cells. *C*, *D*: fluorescent staining in a 2-day-old culture, fixed in ethanol and mounted in glycerol. Note the long lengths of stain along edge of cell (*C*) and across breadth of cells (*D*). Paths of nerve–muscle contact were not visible in these examples. Scale as in *B*. *E*, *F*, *G*: a field in a 5-day-old living culture viewed with fluorescence (*E*) and phase-contrast (*G*) optics. In *F* both the fluorescence and phase-contrast images are superimposed to show that the fluorescent stain is entirely restricted to the site of nerve–muscle contact.

PLATE 7

Patterns of fluorescent stain on nerve-contacted muscle cells in cultures to which neural tube cells were added on day 3. Cultures were stained with rhodamine-toxin and examined alive. Each pair of micrographs shows the same field viewed with fluorescence and phase-contrast optics. *A*, *B*: a field in a culture stained 1 day after adding neural tube cells. Note the intense staining associated with one of the nerve contacts and the scattered small spots of fluorescent stain elsewhere on the cell. *C*, *D*: a field in a culture 2 days after adding neural tube cells. The stain is restricted to the path of nerve–muscle contact. *E*, *F*: from a culture stained 1 day after adding neural tube cells. The same nerve process has contacted several muscle cells and there is considerable variation in the amount of stain associated with the

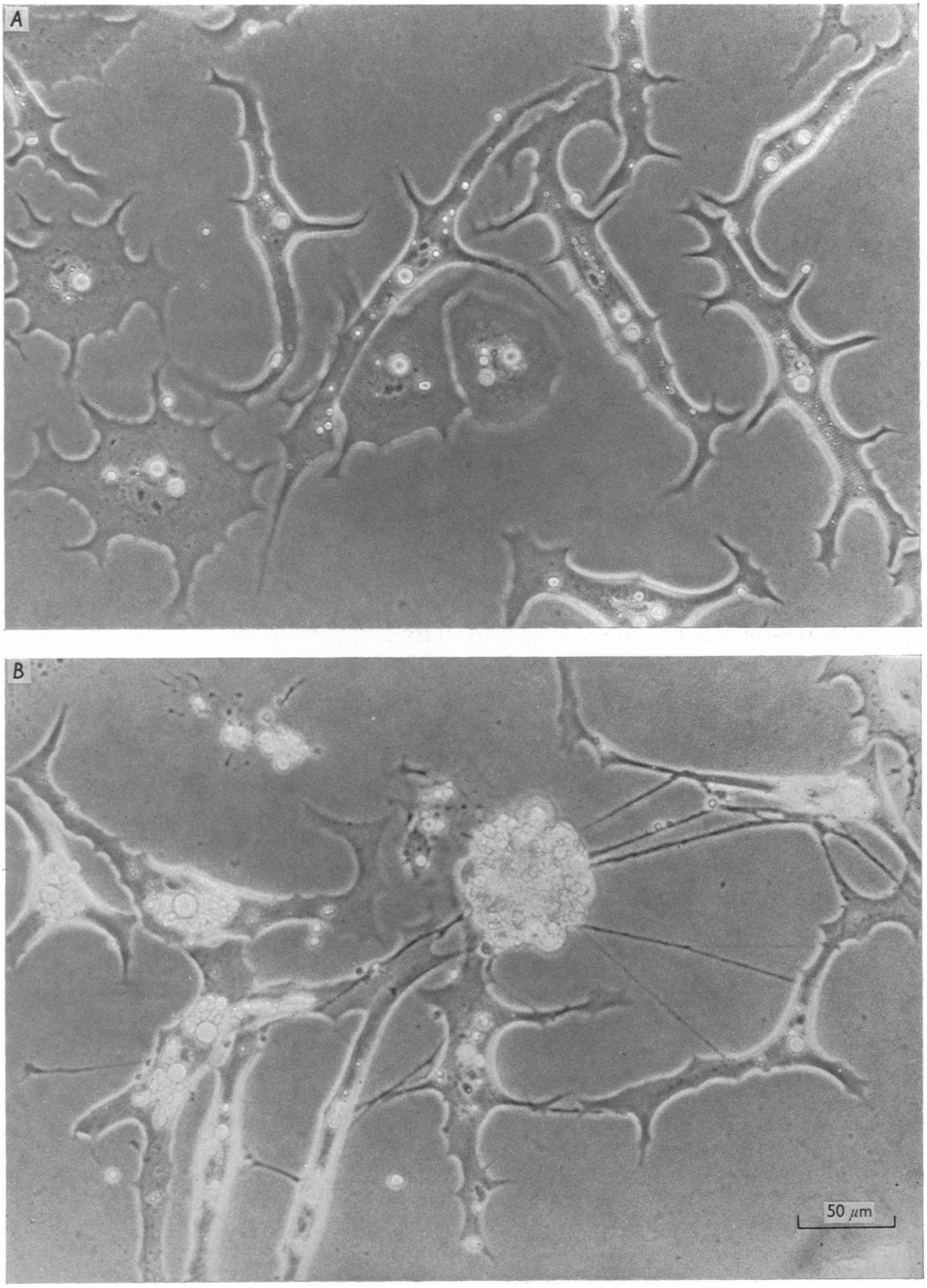
sites of contact. Note also the widespread scattering of spots of fluorescent stain on the cell in the upper left-hand quadrant. In *A* and *E* cells have been outlined and nerve processes are indicated by dotted lines.

PLATE 8

Reversible inhibition of fluorescent staining by cholinergic agents. *A, B, C:* a field in a 2-day-old mixed culture which was exposed to rhodamine-toxin in the presence of curare (10^{-4} g/ml.) and then to fluorescein-toxin after washing out the curare. The phase-contrast view (*A*) shows a nerve process contacting a muscle cell. When examined for fluorescence virtually no rhodamine staining (*B*) was observed but the fluorescein staining (C) was bright. *D, E, F*: a field in 3-day-old mixed culture grown in the presence of carbachol (10^{-5} g./ml.). The culture was exposed to fluorescein-toxin in the presence of carbachol and then to rhodamine-toxin after washing out the carbachol. The phase-contrast view (*D*) shows a nerve process in contact with two muscle cells. When examined for fluorescence virtually no fluorescein staining (*E*) was observed but the rhodamine staining (*F*) was bright. Cultures were fixed in ethanol and mounted in alkaline glycerol (see Methods). After such treatment it was usually impossible to visualize the path of nerve–muscle contacts (*A, D*).

PLATE 9

A, B: fluorescence and phase-contrast views of a field in a muscle culture which was fixed for 1 hr in 3% paraformaldehyde before being stained with rhodamine-toxin. Note the typical patches of stain. *C, D, E*: radioautographs from a 2-day-old muscle culture exposed to ^{125}I-labelled toxin. Bright field (*C*) and phase-contrast (*D*) views of the same field show a scattering of grains over the muscle cell as well as a patch of high grain density. Another field (*E*) viewed with phase contrast optics.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B
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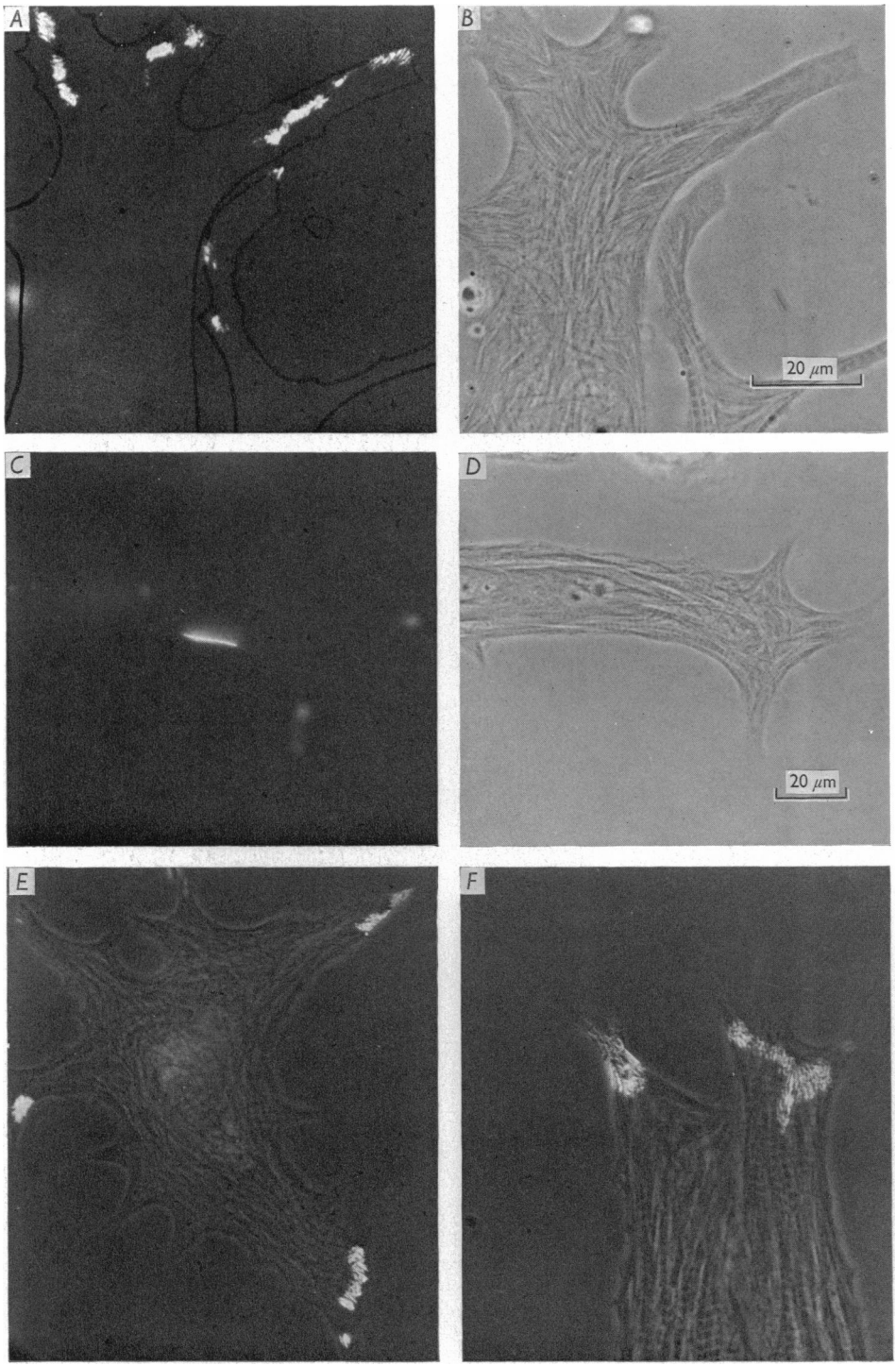
A
B
20 μm
C
D
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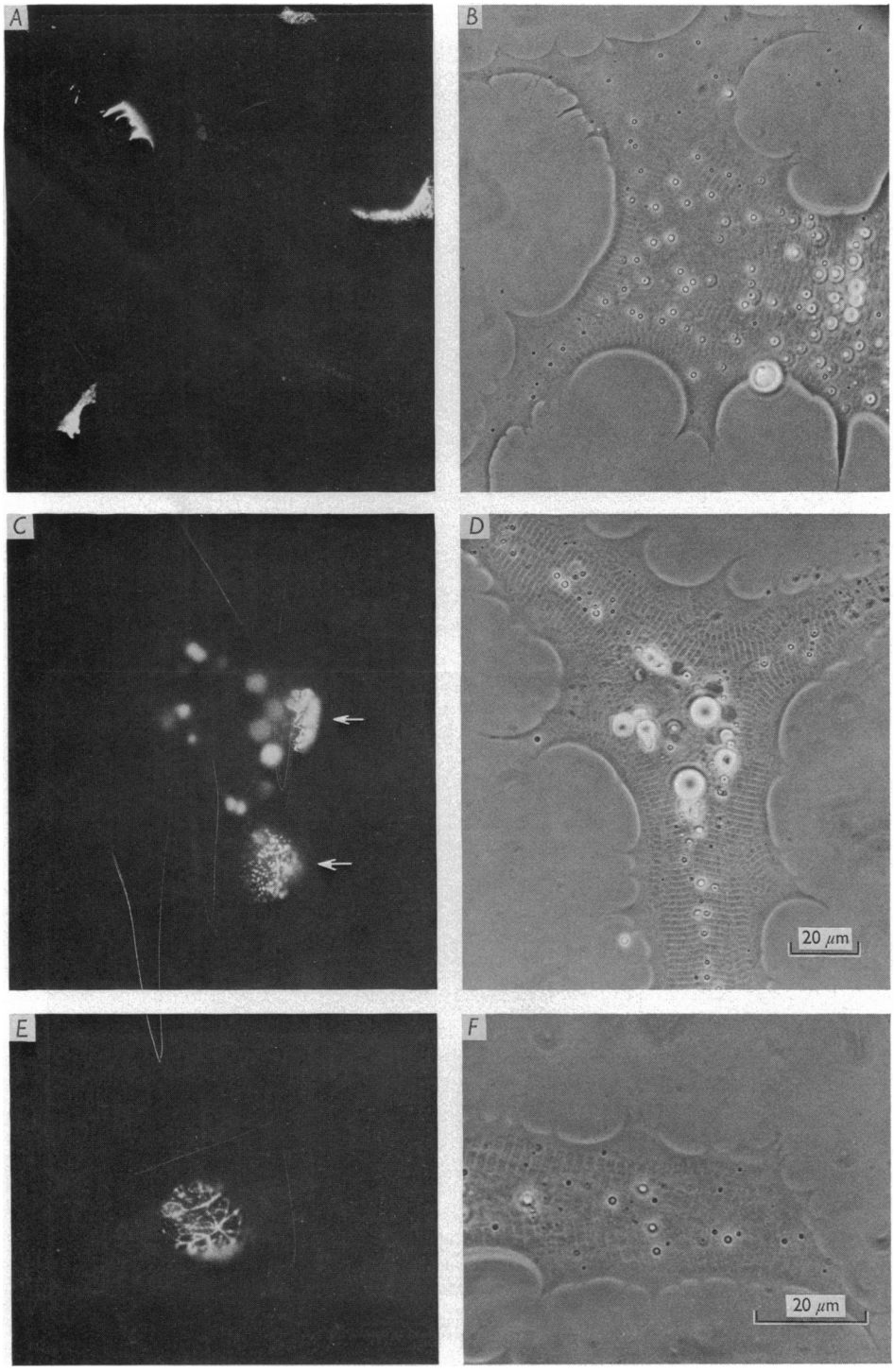
E
F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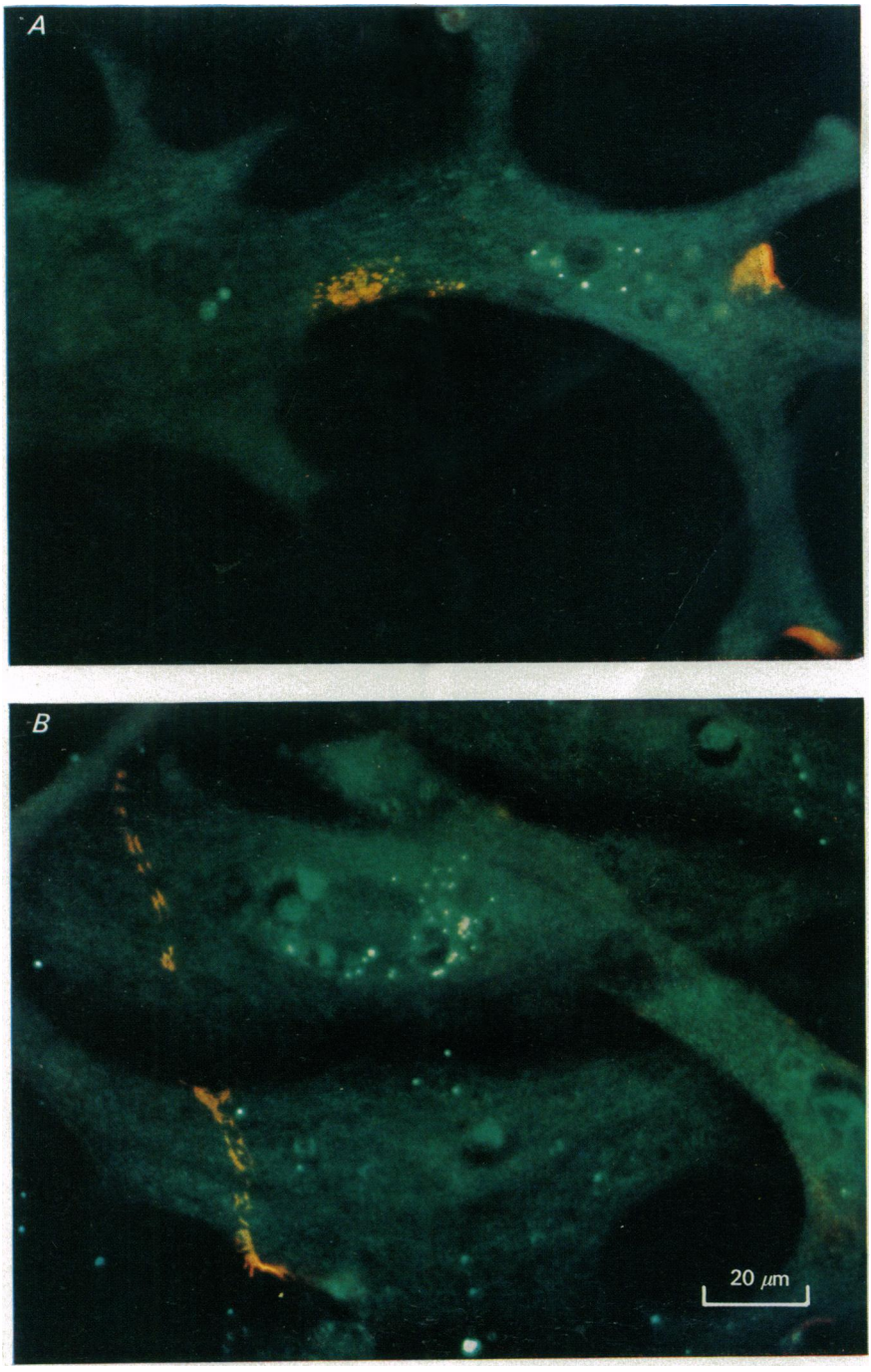

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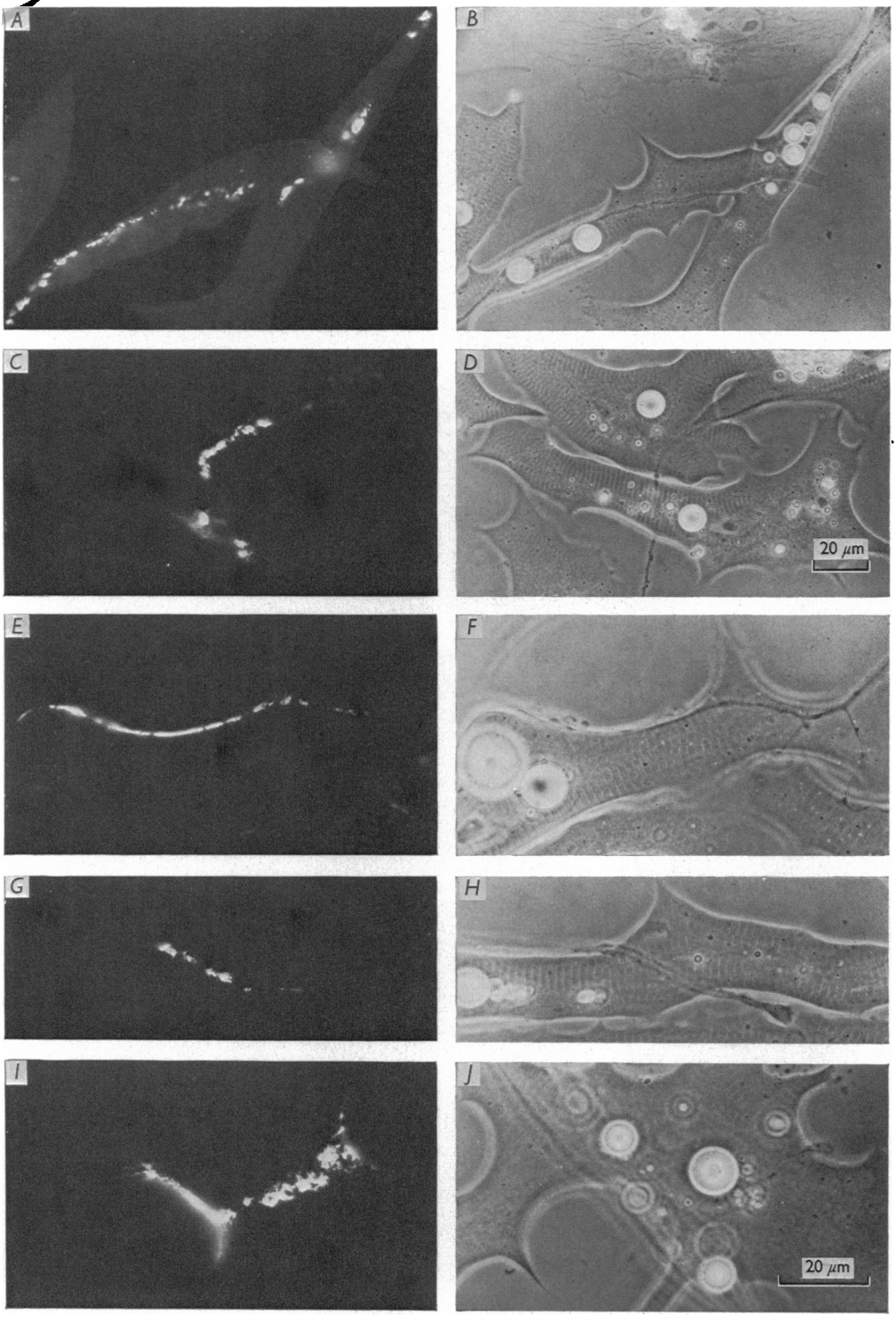
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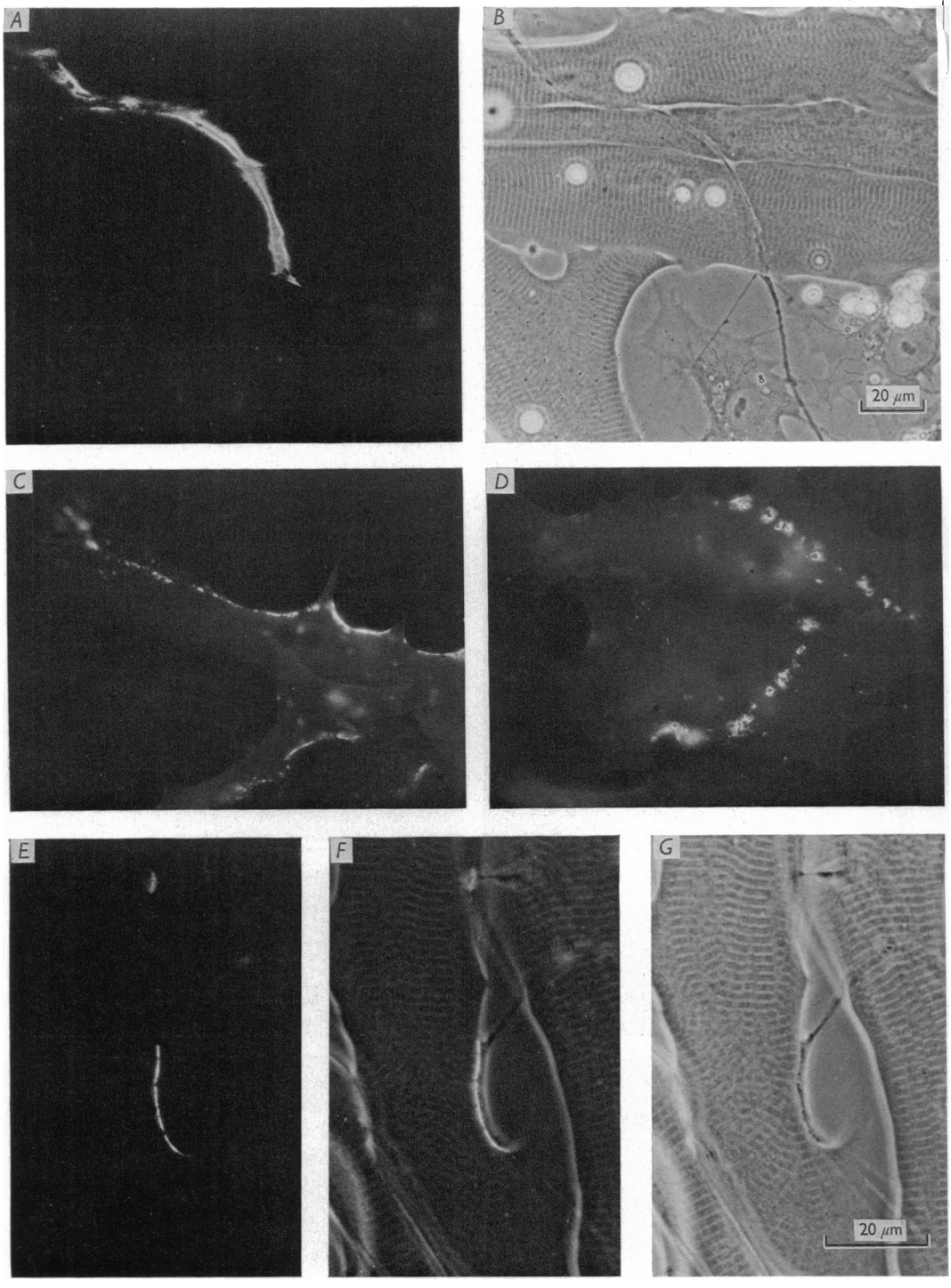

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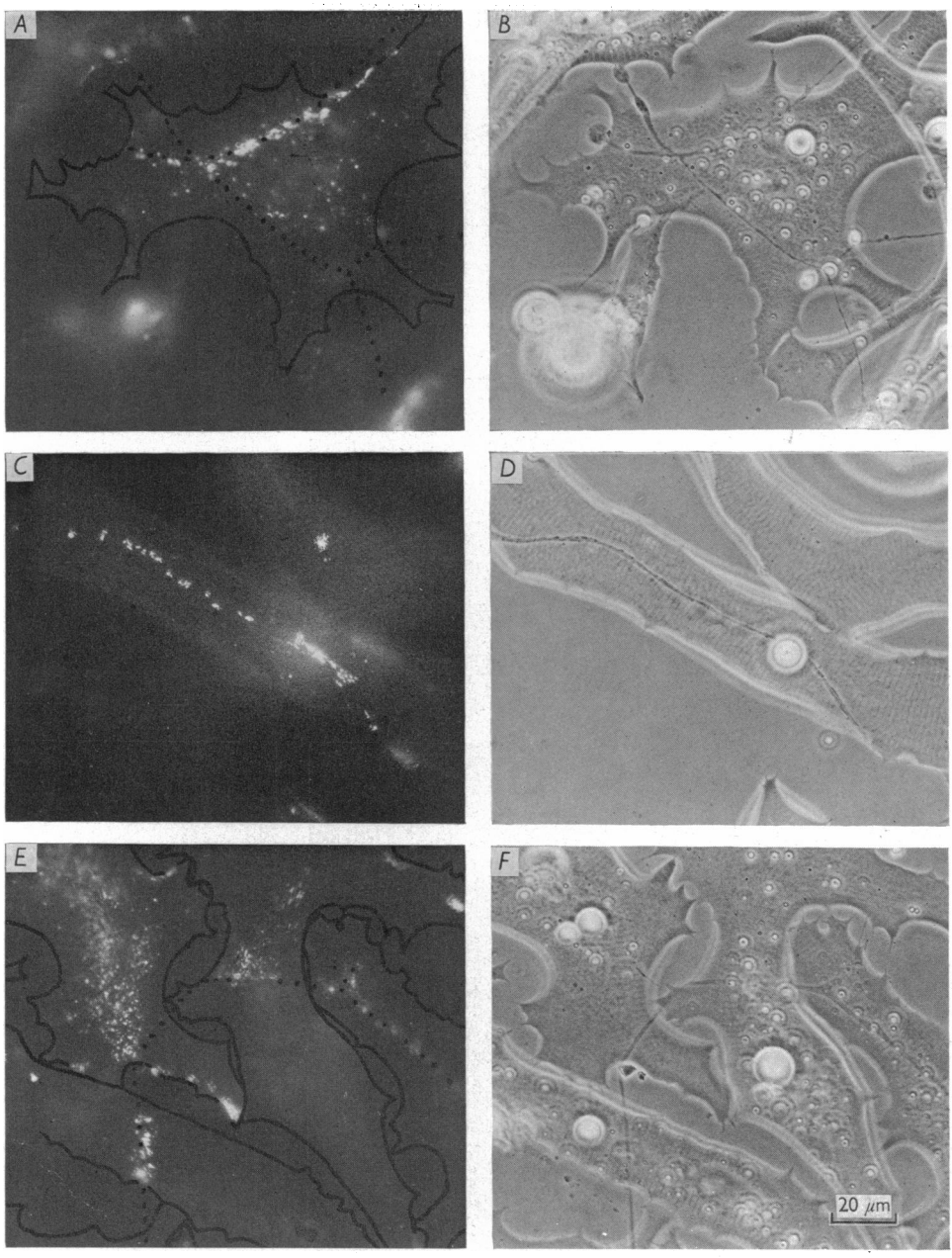
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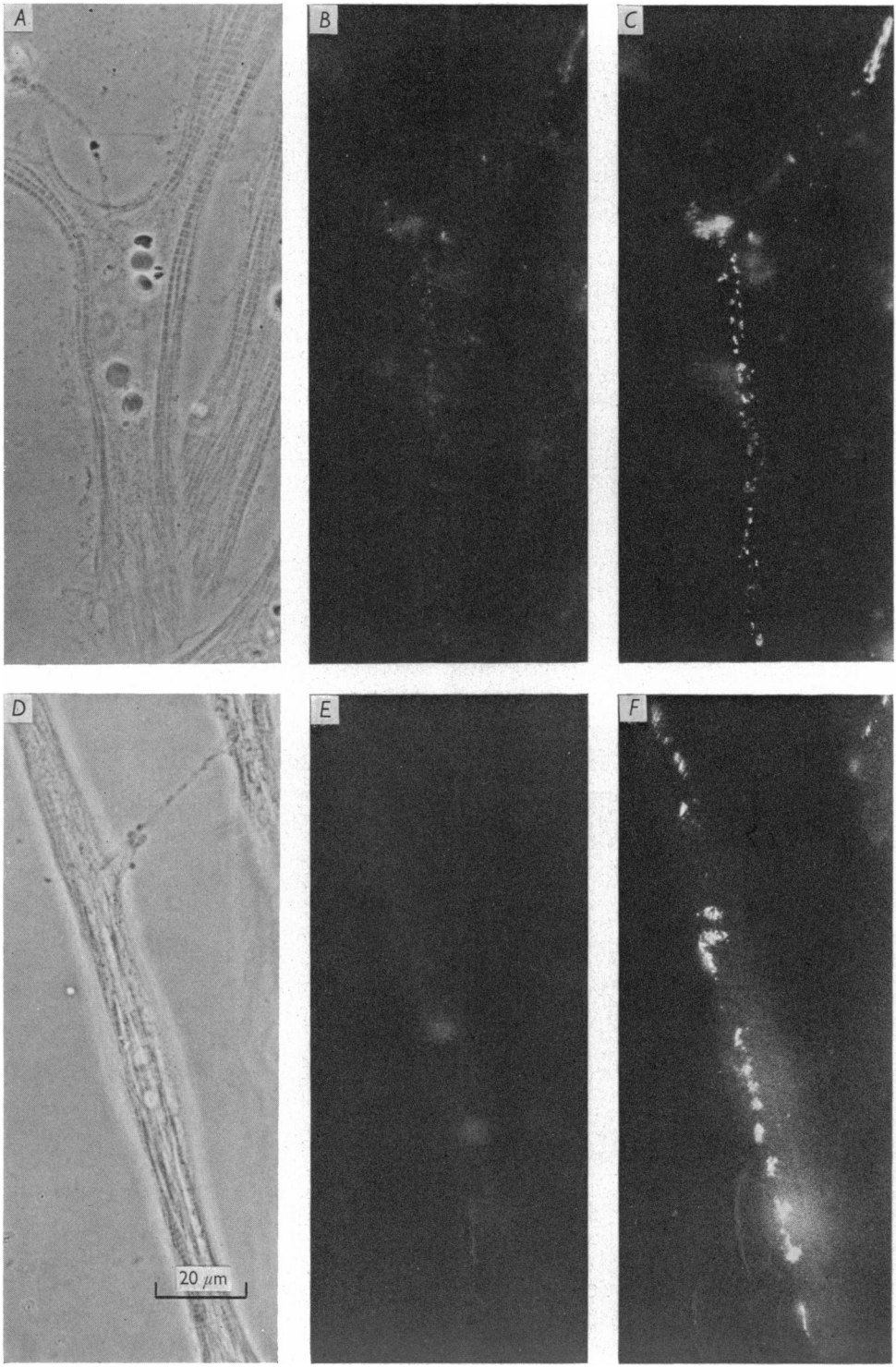
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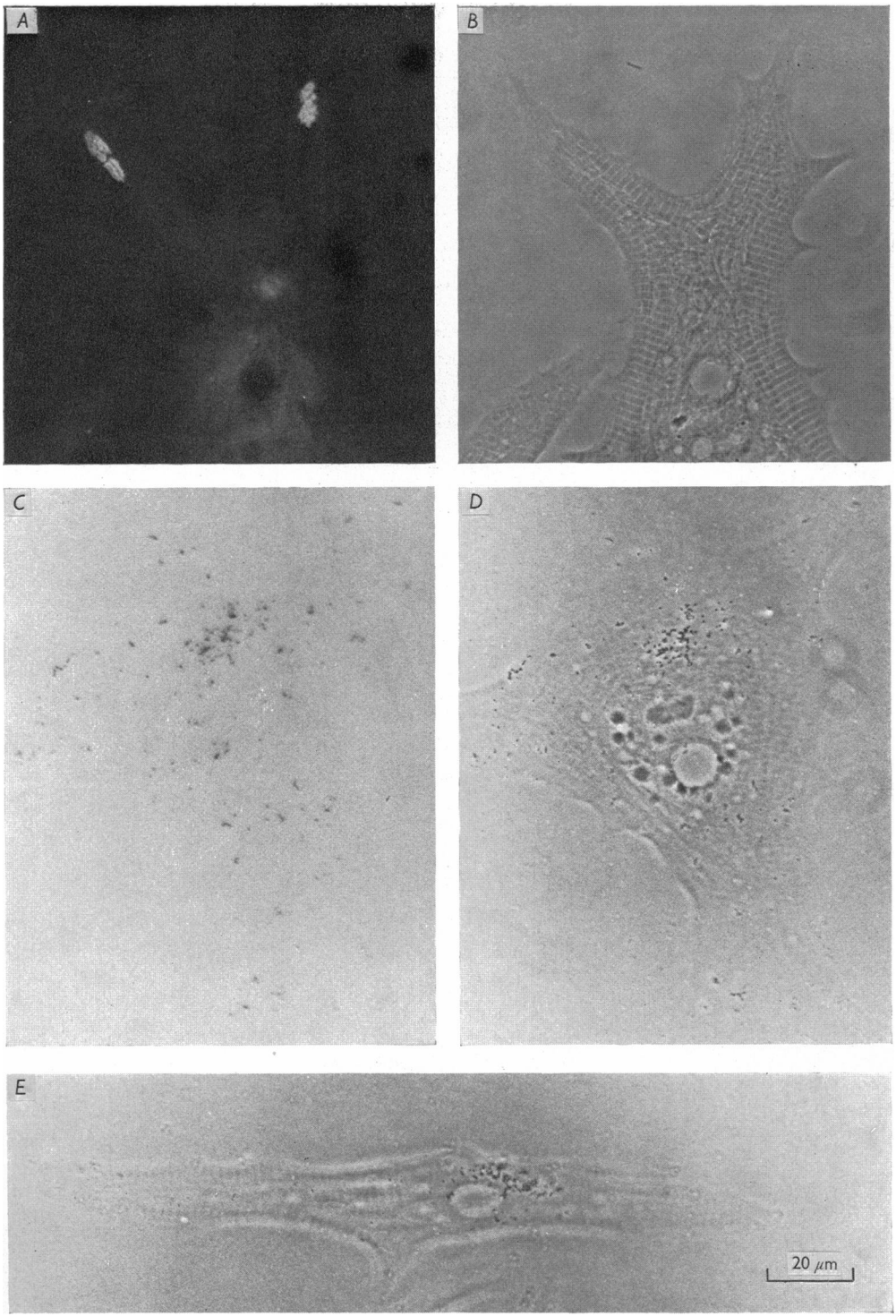
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