

NERVE FIBERS IN CULTURE AND THEIR INTERACTIONS WITH NON-NEURAL CELLS VISUALIZED BY IMMUNOFLUORESCENCE

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ABSTRACT

Cultures of embryonic mouse spinal cord explants, alone or in combination with rat myotubes, were stained by indirect immunofluorescence using antibodies against three structural proteins to: (a) reveal the distribution of these proteins among different cell types, and (b) test the usefulness of antibody staining to reveal the gross morphology of the neurite network in complex cultures.

Affinity column purified antibodies were used against chicken gizzard actin, porcine brain tubulin, and skeletal muscle α -actinin. Neurites were stained intensely by anti-actin as was the stress fiber pattern of underlying fibroblasts. With anti-tubulin, the staining of neurites was an order of magnitude more intense than the staining of the microtubule pattern of background fibroblasts. Neurite cell bodies and astrocyte-like glia cells were stained with anti-tubulin and their nuclei remained unstained. Anti-tubulin could thus be used to trace even the finest extensions of nerve processes in spinal cord and spinal cord-muscle cultures. Furthermore, it could be combined with the histochemical reaction for acetylcholinesterase (AChE, EC 3.1.1.7) to demonstrate AChE-positive neurons and specialized nerve-muscle contact sites.

The staining of neural elements with anti- α -actinin was generally much weaker than with anti-actin and anti-tubulin. Neurites were stained only moderately in comparison to myotube Z lines in the same culture. However, a distinct staining of the periphery of dorsal root ganglion cells was observed. Thus, a protein immunologically related to muscle α -actinin is present in the nervous system. In myotubes, Z lines were stained intensely with anti- α -actinin while I bands were only faintly stained with anti-actin. In isolated myofibrils, both structures were stained intensely with the same antibody preparations.

KEY WORDS tubulin · muscle
proteins · immunofluorescence · nerve-muscle
culture · acetylcholinesterase

Cultured nerve cells are able to send out processes that interact in characteristic ways with other cells.

It is usually necessary, in gross-morphology studies of such cultures, to stain the nerve processes selectively, to follow their course on a background of non-neural cells. A variety of silver impregnation methods originally devised for tissue sections (cf. reference 27) are in use for that purpose (e.g.

reference 22); some can be combined with the histochemical enzyme reaction for acetylcholinesterase (AChE, EC 3.1.1.7, reference 19) and can be applied to coupled nerve-muscle cultures (2). However, fine structures, such as the spikes of growth cones, are usually not preserved after silver impregnation. Furthermore, AChE staining of neurons will be totally obscured and details of the enzyme staining of myotubes may be lost.

We have therefore tried indirect immunofluorescence using antibodies against actin, α -actinin, and tubulin as an alternative to silver impregnation of nerve processes. This approach was based on the knowledge that tubulin (34) and contractile proteins (10, 23, 35) are present in the nervous system, and that nerve processes are rich in microtubules (21) and rapidly accumulate newly synthesized actin and tubulin (5). Accordingly, anti-tubulin turned out to be the most useful stain for nerve processes in culture. Its use could be combined with the histochemical reaction for AChE in the same culture, thus allowing the identification of AChE-positive neurons, nerve processes, and contact sites in combined nerve-muscle cultures, while preserving fine details of cellular morphology. A preliminary report of this work has been presented (13).

MATERIALS AND METHODS

Tissue Culture

Slices of mouse embryonic spinal cord (12–14 d after conception, strain CBA, inbred), 1–1.5 segments in thickness, were placed on 15-mm diam round coverslips coated with collagen (22). Two–three of these coverslips were kept in a 35-mm Petri dish with 0.7 ml of medium B (10% heat-inactivated horse serum, 10% fetal calf serum, both from GIBCO [Grand Island Biological Co., Grand Island, N. Y.], Dulbecco's modified Eagle's medium with glutamine, 100 units of penicillin, and 100 μ g of streptomycin per ml). Attachment and outgrowth of some flat cells had usually occurred after 24 h. After 1.5–2 d the medium was replaced by 2 ml of medium A (10% horse serum, 1% 10-d-old chick embryo extract, 89% Dulbecco's modified Eagle's medium with antibiotics as in B) of which 1 ml was replaced every 4 d. Cultures were grown at 37°C in the presence of 8–10% CO₂. For combined nerve-muscle cultures, rat myoblasts were prepared from newborn to 2-d-old Sprague-Dawley or Wistar rats (outbred) by the method of Yaffe (35) and seeded at a density of 8×10^5 per 35-mm dish. Each dish contained two–three collagen-coated coverslips. The cells were cultured in 2 ml of medium A for 4 d; the medium was then removed, two disks of spinal cord

were placed on top of the myotube layer (cf. reference 6), and 0.7 ml of medium B was used for 2 d, as above, to allow attachment of the spinal cord explants. Further culture was done in 2 ml of medium A.

Purification of Antigens

Smooth muscle actin was prepared from chicken gizzard by using procedures described for the isolation of skeletal muscle actin (25, 29), with slight modifications. Chicken gizzards were cut into cubes and minced in a meat grinder at 0°C. The minced meat was immediately extracted in 3 vol of Straub's solution (22.36 g KCl, 21.8 g KH₂PO₄, and 8.0 g KOH per liter, pH 6.5) for 20 min at 0°C. The residue was spun down (2,700 g, 10 min) and washed once in 0.6 M KCl, three times in 0.1 M KCl, twice in distilled water, once in 0.01 M NaHCO₃, and finally again in distilled water. Acetone powder was prepared by extracting the residue four times in 3 vol of acetone and drying it over CaCl₂.

G-actin was extracted from the acetone powder by the procedure of Spudich and Watt (29) and was finally chromatographed on Biogel P 150 (Bio-Rad Laboratories, Richmond, Calif.) (33). The actin-containing peak (monitored at an optical density of 280) was pooled and analyzed on sodium dodecylsulphate (SDS)-containing polyacrylamide slab gels. To reveal impurities, 20–40 μ g of G-actin were applied to a gel suited to reveal 0.2 μ g of protein per band after staining with Coomassie Brilliant Blue. Fresh actin preparations showed only one band co-migrating with purified rabbit skeletal muscle actin.

α -actinin was isolated from porcine skeletal muscle as described (9, 30). After two passages over diethylaminoethyl (DEAE)-cellulose (elution with a KCl gradient from 0–0.5 M), some of our preparations contained >90% α -actinin and 4–7% actin (as judged from SDS-polyacrylamide gels). These preparations were used without further purification. Other preparations (also from porcine muscle) showed additional polypeptides larger than α -actinin (which has a mol wt of ~100,000) and some minor bands in the 60–90,000-dalton region of the gel. The larger contaminants were removed, and the amount of smaller ones was greatly reduced by an additional passage through hydroxyapatite, as described by Suzuki et al. (30).

Tubulin was prepared from bovine brain as described (8, 28). Brain tissue was homogenized in reassembly buffer, and microtubules were allowed to reform in three polymerization cycles. Most of the high molecular weight components were then removed by a passage over Sepharose 4 B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Sweden). Polyacrylamide gel analysis in the presence of SDS revealed the two tubulin polypeptides at 55,000 and 53,000 daltons, and <3% high molecular weight components.

All proteins purified as described above were used as antigens and also as ligands for affinity chromatography.

Immunization

Female rabbits (Swiss Hare or Chinchilla, outbred stock, specific pathogen free, obtained from Institut für Biol. Med. Forschung AG, Füllinsdorf, Switzerland; or WIGA, Sulzfeld, Germany) were bled through the ear vein to obtain preimmune sera and subsequently immunized essentially as described by Lazarides (16). Briefly, two intramuscular injections (native antigens emulsified with Complete Freund's adjuvant, 1:1) were followed by a series of intravenous injections (into the ear vein) with increasing amounts of the antigens adsorbed to alum, up to a total amount of 2–2.5 mg of protein per rabbit. All the experiments described under Results were carried out with antibodies against native proteins, although we also obtained antibodies against SDS-denatured actin and tubulin with the same immunization scheme.

Testing of Antisera and Preparation of Specific Antibody

At the end of the immunization period (day 38–40), the rabbits were bled through the ear vein. After removal of the blood clot, a crude IgG fraction was prepared from the serum at 50% ammonium sulphate. The precipitate was centrifuged, dissolved in phosphate-buffered saline (PBS), and dialyzed against PBS. Double diffusion tests were essentially carried out as already shown for our anti-actin antibody (14). All rabbits immunized with actin, α -actinin, or tubulin gave positive precipitin reactions. 10 μ l of crude γ -globulin (10 mg/ml) was diffused against 10 μ l of the purified antigen (1 mg/ml), crude acetone powder extract (to test for anti-actin activity), crude muscle extract (anti- α -actinin), or brain homogenate (anti-tubulin). In each case, a single precipitin line was observed.

Affinity columns were prepared by linking the antigens to activated Sepharose 4 B beads, and specific antibodies were purified from the crude IgG fractions on such columns essentially as described before for anti-tubulin (7), anti- α -actinin (12) and anti-actin (14). The bound molecules were eluted with 4 M MgCl₂. The pooled peak fractions of the elution profile (monitored at an optical density of 280) were immediately dialyzed against 0.1 M borate, pH 8.0. The yield of this immuno-adsorbent purification (5–8% of the IgG molecules were bound) indicated that the majority of the antibody molecules evoked by the immune response must have been directed against the antigens (actin, α -actinin, or tubulin, respectively) and not against a minor impurity.

Specificity Analyses of the Antibodies

Affinity columns, indirect immunofluorescence with fluoresceine-isothiocyanate-conjugated goat anti-rabbit antibody (FITC-GAR, Miles, Seravac, Lausanne, Switzerland), and electron microscopy with ferritin-coupled goat anti-rabbit antibody (Fer-GAR, Nordic, Tilburg/

London) were employed to test the quality of the antibody preparations.

The α -actinin-Sepharose column did not retain protein from anti-actin serum, and, likewise, the anti- α -actinin antibody did not contain any anti-actin activity. Neither the actin-Sepharose nor the α -actinin-Sepharose columns bound protein from the anti-tubulin preparation.

In immunofluorescence tests, anti-actin bound specifically to the I region of isolated relaxed myofibrils (14), whereas anti- α -actinin was restricted to the Z lines (12). Anti-actin stained stress fibers of mouse 3T3 cells homogeneously (cf. references 14 and 18), whereas anti- α -actinin stained only portions within such stress fibers and also their presumptive attachment sites at the cell membranes (cf. reference 17). Anti-tubulin stained mouse sperm tails brilliantly as well as the cytoplasmic microtubule network and cytocenters of interphase and the spindle apparatus of mitotic 3T3 cells (cf. references 7, 31, and 32). After incubation with univalent Fab fragments prepared from the specific anti- α -actinin and subsequent incubation with Fer-GAR, thin sections of rabbit skeletal muscle revealed the anti- α -actinin molecules exclusively restricted to the Z line. In particular, there were virtually no ferritin molecules seen in the M line region (A. C. Allison and B. M. Jockusch, manuscript in preparation).

Preimmune sera from all rabbits were tested for their anti-actin, anti- α -actinin, and anti-tubulin activity. No measurable amount of protein was retained on the immuno-adsorbent columns, and no staining of cellular structures (including neural cultures) was observed in immunofluorescence experiments with appropriate dilutions of the preimmune sera.

Staining Procedures

Silver impregnation of nerve fibers was done by the method of Namba et al. (19) with or without AChE histochemistry, or by procedures given by Bodian (1).

Acetylcholinesterase histochemistry was carried out by the technique of Karnovsky and Roots (15). Samples were preincubated for 30 min at room temperature in 10⁻⁵ M tetraisopropylpyrophosphoramidate (isoOMPA), to inhibit unspecific esterases. Incubation was carried out for 1–2 h at 30°C; the substrate solution at pH 6.0 contained 10⁻⁵ M isoOMPA. In experiments combining the AChE test with indirect immunofluorescence, fixation of the sample was done for 1 h at 0°C in calcium formalin (1% wt/vol CaCl₂, 3.7 wt/vol formaldehyde in water) or in 3.7% wt/vol formalin in Dulbecco's calcium- and magnesium-free phosphate-buffered saline (CMF-PBS), with similar results. After AChE staining, the cultures were washed with water and kept (up to 3 d) at 4°C under water or in CMF-PBS. The immunofluorescence procedure was continued, starting with the Triton X-100 treatment.

Indirect immunofluorescence was done by a scheme

modified from several published procedures (3, 17, 18): (a) Rinse with CMF-PBS; (b) Fix for 20 min in 3.7% formaldehyde in CMF-PBS at room temperature (a stock solution of commercial "stabilized" 37% formaldehyde was used); (c) Transfer to 3.7% formaldehyde, 0.2% (vol/vol) Triton-X 100 in CMF-PBS for 10 min at room temperature; (d) Wash for 3 min in an acetone-water solution (1:1), 5 min in acetone, and 3 min in a fresh acetone-water solution (1:1), at 4°C; (e) Wash in several changes of CMF-PBS at room temperature; (f) Drain excess liquid, apply 20 μ l of γ -globulin solution (~1 mg/ml) in CMF-PBS, or monospecific antibody (50 μ g/ml, in some cases dilutions of 5 μ g/ml), incubate in a moist chamber for 1 h at 37°C; (g) Drain, wash in three changes of CMF-PBS, apply 20 μ l of fluorescein conjugated goat anti-rabbit γ -globulin (FITC-GAR, Miles), incubate as in (f); (h) Drain, wash extensively in CMF-PBS, dip in water, mount in glycerol-CMF-PBS solution (7:3). Observe and photograph within 24 h.

Observations and microphotography were with a Zeiss "Universal" microscope equipped with a MBO 100-W mercury lamp and a III R S epifluorescence condenser, using the excitation filter B G 12, Zeiss barrier filter 50, and phase-contrast objectives 25 \times , 40 \times , and 63 \times (oil immersion). Photomicrographs were usually taken on DIN 27 black and white film (Kodak Tri-X-Pan [fluorescence]) and DIN 15 Agfa-pan 25 (phase contrast).

RESULTS

Morphology of Cultures and Identification of Cell Types

The development and morphology of cultured mouse spinal cord explants (Peterson and Crain, 22) and rat myotubes (35) have been the subject of detailed publications. In the following brief description, only the features pertinent to our immunofluorescence experiments will be mentioned.

Spinal cord explants attached and showed cellular as well as fiber outgrowth after 20 h. Dorsal root ganglia flattened more rapidly than the spinal cord proper. In the course of 1 wk, a lawn of fibroblastlike cells spread around the explants, on which the network of neurites extended. In later stages, neurites showed a strong tendency to form parallel bundles. Some larger neurons of the spinal cord proper were usually found outside the explant, and flat cells were sparse or absent in their immediate neighborhood. Despite these later changes, the gross morphology of a spinal cord cross-section plus attached dorsal root ganglia was recognizable for 10–15 d, thus allowing

the identification of ventral-horn and dorsal-root-ganglion neurons. Neurons were generally identified by their strongly refractile cell bodies, long processes (24), and by the black staining of their processes and the fibrillar network in their perikarya after silver impregnation. Astrocytes were tentatively identified by their morphology. Their processes were not stained black by silver impregnation as described by Namba et al. (19). Rat myoblasts started fusing on day 2 after seeding, and most nuclei were in myotubes on day 4, at which time spinal cord explants were usually added. Contractility was seen 1–2 d later, and at the same time cross striations started to develop.

In unstained combined nerve-muscle cultures, it was difficult to follow the fiber outgrowth from the spinal cord explants. Muscle fibers seen in phase contrast showed no obvious differences whether spinal cord explants were present or absent in the culture.

Comparison of Different Antibodies

Affinity column purified antibodies against tubulin, actin, and α -actinin applied to spinal cord cultures yielded characteristic and clearly distinguishable staining patterns.

In all three cases, corresponding crude γ -globulin fractions from pre-immune sera gave only weak and diffuse background staining, even when their protein concentration was one or two orders of magnitude higher than that of the purified immune antibodies. As an example, Fig. 1 shows the lack of staining of nerve fibers with pre-anti-tubulin. Though tubulin is a ubiquitous protein of the animal cell, staining with anti-tubulin can be used to demonstrate the network of nerve fibers on a background layer of flat cells (Figs. 2 and 3). The reason for this is probably the dense packing of microtubules as they converge into a cell process as opposed to the loose network in spread flat cells. This can be seen in an anti-tubulin-stained fibroblast with process-like extensions (Fig. 4). Anti-tubulin stained the perikarya but not the nuclei of neurons and glia cells (Fig. 5).

With anti-actin, nerve fibers were intensely stained, but so was the stress fiber pattern of background fibroblasts (Fig. 6). Therefore, anti-actin cannot be used as a differential stain for nerve fibers.

Nerve fibers were only slightly stained by anti- α -actinin, also in combined cultures in which the

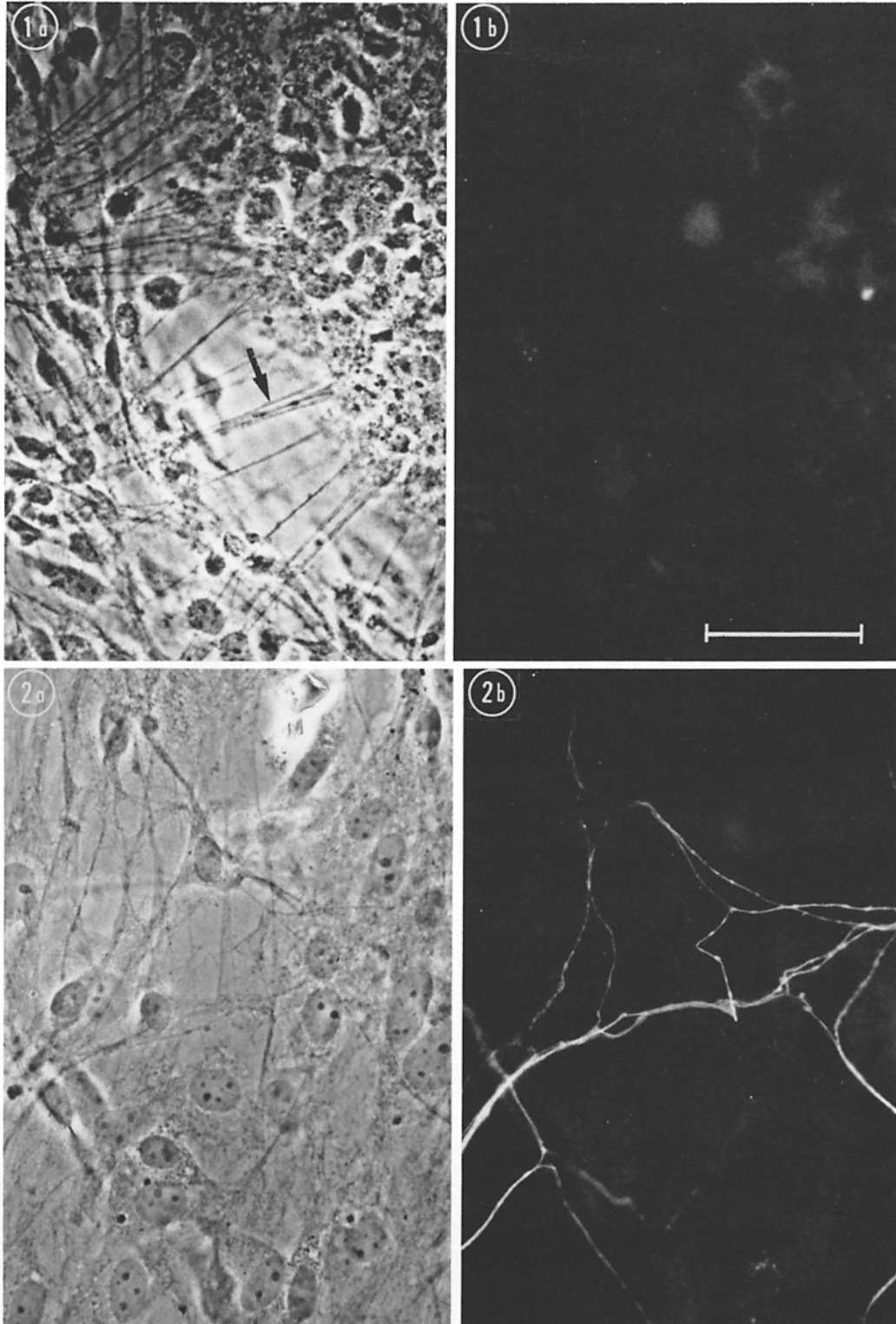
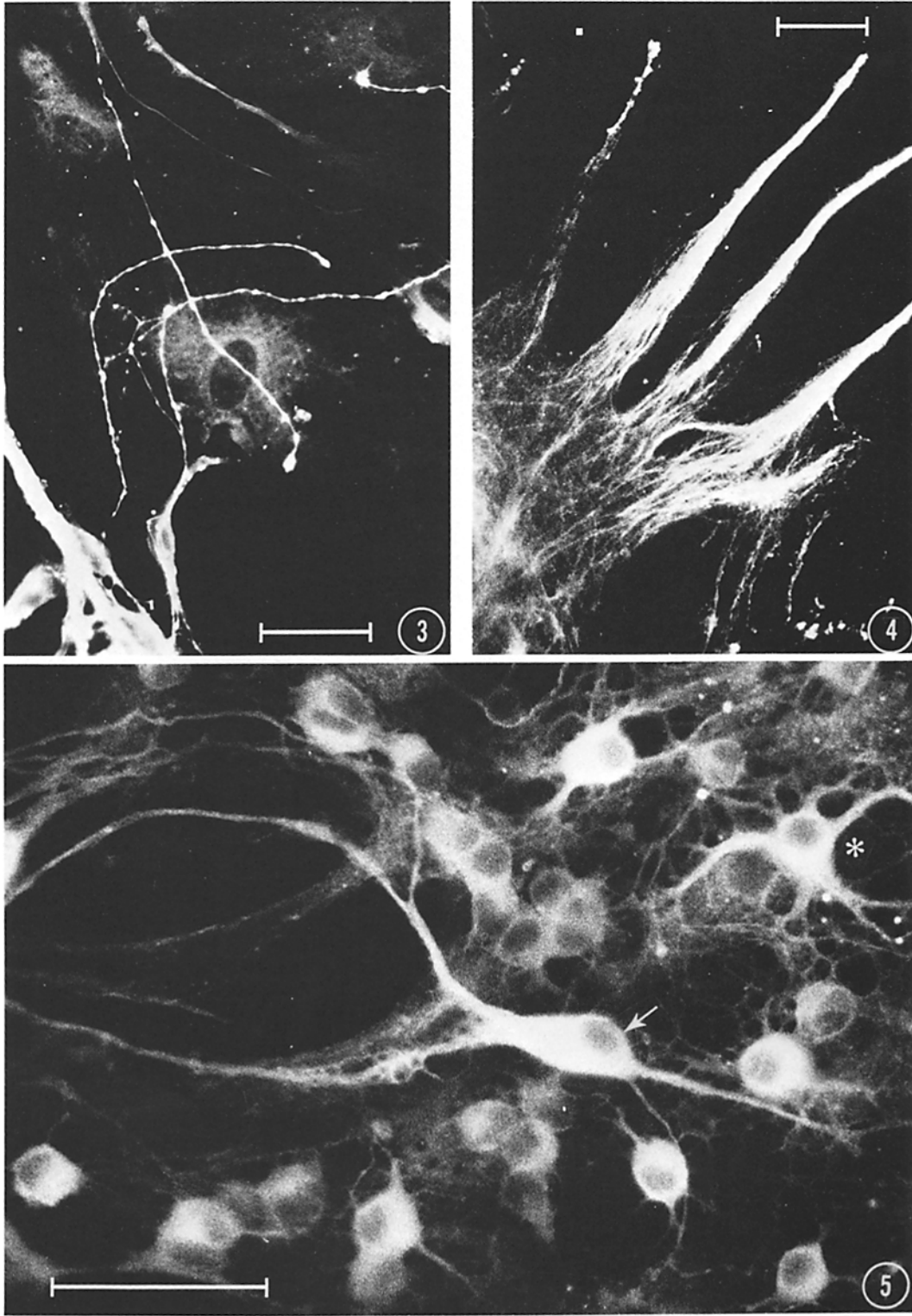


FIGURE 1 Control experiment with preimmune serum: Lack of nerve fiber staining with pre-anti-tubulin (γ -globulin fraction, concn. 1 mg/ml). Spinal cord explant 7 d in culture. (a) Phase-contrast: margin of explant (to the right) with outgrowing fibers (arrow). (b) Same field, fluorescence (exposure and printing as in Fig. 2 b). Bar, 50 μ m. \times 480.

FIGURE 2 Staining of fine nerve fibers with anti-tubulin (affinity purified antibody, diluted to 5 μ g/ml). Outgrowth zone of spinal cord explant 7 d in culture. (a) Phase-contrast, showing lawn of flat cells. (b) Same field, fluorescence, showing neurites. \times 480.



Z lines of myotubes were brightly fluorescent with the same antibody (Fig. 7). The peripheries of the cell bodies of dorsal root ganglion cells were reproducibly fluorescent with anti- α -actinin, thus indicating the presence in nervous tissue of a protein immunologically related to skeletal muscle α -actinin (Fig. 8).

The antibodies against the two muscle proteins were tested on isolated myofibrils and on cross-striated myotubes. In these preparations, the binding of the antibody strongly influenced the appearance of the cross-striation pattern in phase contrast. The visibility of the I bands was enhanced by anti-actin (Fig. 9*a*) while the visibility of Z band was strongly enhanced by anti- α -actinin (Fig. 10*a*). In a rather contracted myofibril, anti-actin fluorescence was seen in narrow bands corresponding to the I band (Fig. 9*b*). Thus, the actin contained along with myosin in the H band is probably not available to the antibody. In intact myotubes, even the staining of I bands was faint (Fig. 9*b*) or absent. Anti- α -actinin stained the Z lines intensely in both myofibrils and intact myotubes. In addition, the periphery of the whole myotube was stained, though more weakly than the Z lines (Fig. 10*b*). In immature myotubes, staining with anti- α -actinin was much less intense and lacked the cross-striation pattern (Fig. 10).

Nerve Fiber-Myotube Interaction

In the following experiments, anti-tubulin as a stain for nerve fibers was applied to mouse-nerve/rat-muscle cultures. The network of nerve fibers on a myotube lawn could be visualized, because the myotubes themselves showed only weak and diffuse staining with anti-tubulin (Fig. 11).

Most nerve fibers passed over muscle fibers with no obvious sign of interaction or contact formation (Fig. 11). At some places, however, a finer branching pattern of the nerve fibers developed in contact with a myotube (Fig. 12). At later stages of the culture, nerve fiber-myotube contacts had further developed in that (*a*) there was extensive local branching of the nerve fiber in the contact zone, and (*b*) the myotube surface in the same region showed AChE activity as could be shown with combined AChE and anti-tubulin staining (Fig. 13). Nerve fibers with high AChE activity were already conspicuous in phase contrast because of the refractility of the AChE reaction product, whereas fluorescence was quenched in AChE-positive regions. In contrast to the nerve terminal in a mature "endplate", in our cultures the main fiber continued beyond the contact zone. From the nerve branching pattern and AChE activity, it is clear at light-microscopic levels that these contacts are the results of a localized interaction of nerve fiber and myotube rather than mere accidental contacts. Specializations at the electron-microscopic level, like sub-neural folds, have not been investigated in these preparations nor have we made electrophysiological tests before fixation. Therefore, the neuromuscular contact sites described are not referred to as synapses.

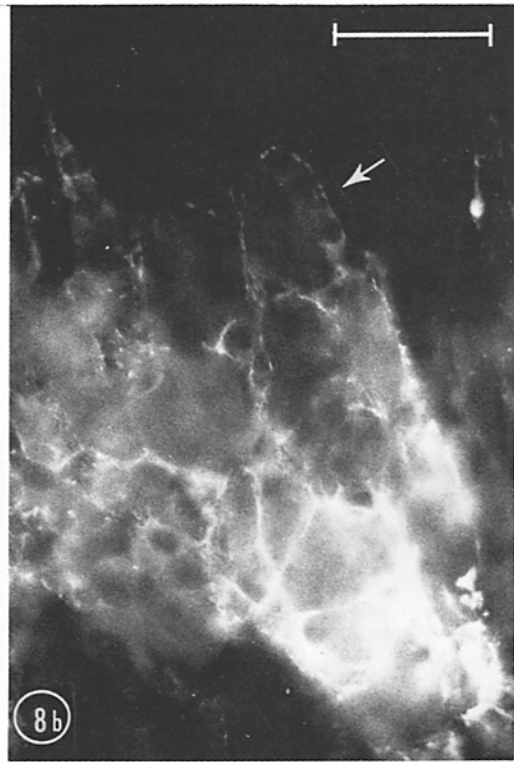
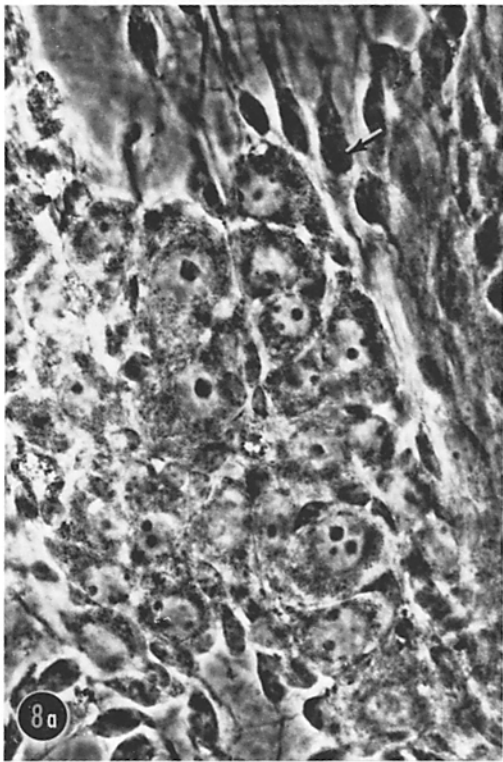
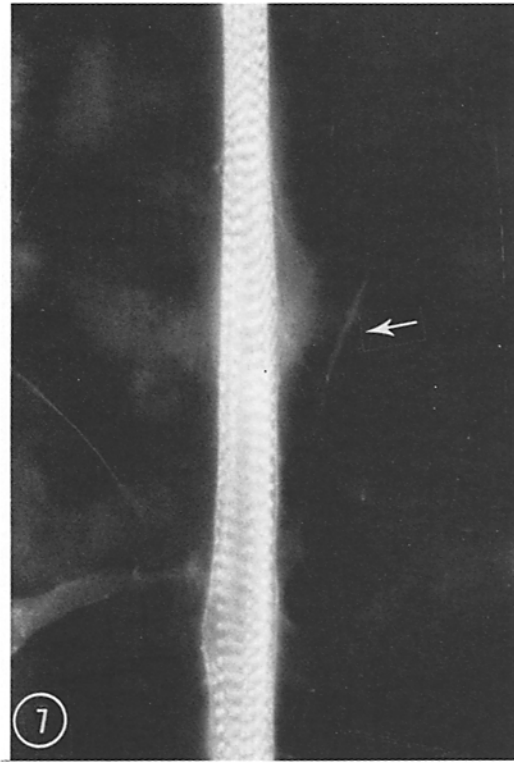
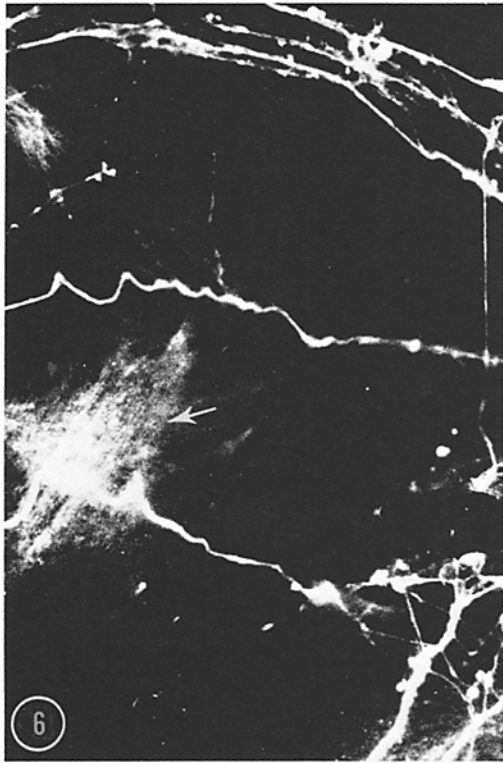
DISCUSSION

The presence of actin and tubulin (5) as well as of cytochalasin B-sensitive filaments and colchicine-sensitive microtubules (36) has been reported for nerve cells grown in culture. Much, but not all, of the tubulin in neural cells is assembled in microtubules (20). The presence of tubulin subunits on the axonal surface has been recently claimed (4). No data are available on

FIGURE 3 Neurite bundle (lower left) and exploratory fibers stained with anti-tubulin (purified antibody at 50 $\mu\text{g/ml}$ after 1-h reaction for AChE). In the middle, a large fibroblast with the nucleus unstained is seen slightly out of focus. 10-d-old spinal cord explant. Bar, 50 μm . $\times 335$.

FIGURE 4 Cytoplasmic microtubule pattern in a mouse fibroblast, line A9, stained with anti-tubulin (purified antibody, 50 $\mu\text{g/ml}$). Cells were grown as a monolayer on glass coverslips (without collagen). Under these conditions, $\sim 30\%$ of the A9 cells assume a starlike shape. Microtubules extend into the cytoplasmic projections and form densely packed bundles. (Culture was a gift of Dr. E. Jost, Heidelberg). Bar, 20 μm . $\times 750$.

FIGURE 5 Neuron and glia cells stained with anti-tubulin (purified antibody, 50 $\mu\text{g/ml}$). Spinal cord explant 11 d in culture. Note fluorescence-negative nuclei in the neuron (arrow), and the astrocyte-like glia cell (asterisk). Fluorescence. Bar, 50 μm . $\times 670$.



the quantity and distribution of α -actinin in nervous tissue.

Immunofluorescence experiments should provide information about the concentration of antigens which is independent of the biochemical approach. However, the results are complicated by the unknown degree of cross reactivity in those cases where the source of the antigen is not identical to the cell type tested. This would limit our conclusion on the relatively low concentration of α -actinin in nerve processes. On the other hand, with highly conserved proteins like actin and tubulin, the source of the antigen should be less important.

Other complicating factors are the state of assembly of the antigen and the preservation of the antigen after fixation and extraction. Thus, the antigenic sites visualized by anti-tubulin are not necessarily intact microtubules, because no special care has been taken to preserve these labile structures (26) during fixation.

Finally, well-preserved and highly cross-reacting antigens may not necessarily be available to the antibody. This is illustrated by the failure of anti-actin to stain the I bands of cross-striated rat myotubes, even though the same antibody stained stress fibers of fibroblasts in these cultures, and I bands of isolated rat myofibrils. On the other hand, Z lines of the cultured myotubes were stained by anti- α -actinin as they were in isolated myofibrils. The reason for the different availability of these two antigens is not clear.

In accordance with biochemical and ultrastructural evidence, we found intense staining of neurites with anti-tubulin and anti-actin. Isenberg et al. (11) have stained differentiating neuroblastoma cells with anti-tubulin and anti-actin. While their results with anti-tubulin are similar to ours, they find actin only at the tips of nerve

processes. It is likely that nerve processes extending from organotypic explants over several millimeters have achieved a higher degree of maturation and therefore differ from the relatively short processes sent out by neuroblastoma cells.

Anti-tubulin turned out to be a useful histological stain for studying the network of nerve processes in cultures; it is comparable to the Bodian silver impregnation (1) and is superior to the method of Namba et al. (19). Immunofluorescence staining is fast compared to silver impregnation, and can easily be combined with acetylcholinesterase histochemistry. It can therefore be used to identify AChE-positive neurons, to judge neurite outgrowth in mixed nerve-muscle cultures, and to facilitate the search for contact sites between nerve and muscle.

We thank Rudolph K. Meyer for help with the preparation of tubulin, and Kristin Kelley, Carlo Marchetti, and Beate Segnitz for skillful assistance.

This work was supported by grants 3.720-76 and 3.721-76 from Schweizerischer Nationalfonds, and grants Jo 55/6-8 and Jo 84/5 from Deutsche Forschungsgemeinschaft.

Received for publication 9 November 1977, and in revised form 10 July 1978.

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FIGURE 6 Staining of nerve fibers with anti-actin (γ -globulin fraction, 1 mg/ml). Outgrowth zone of a 7-d spinal cord culture. Stress fiber pattern of large fibroblast is visible (arrow) but not in focus. Identical results were obtained with purified antibody. Fluorescence. $\times 480$.

FIGURE 7 Nerve fiber (arrow) and a cross-striated myotube stained with anti- α -actinin (purified antibody, 50 μ g/ml). Combined mouse spinal cord/rat myotube culture, 8 d old, 4 d after addition of spinal cord. $\times 480$.

FIGURE 8 Dorsal-root ganglion cells stained with anti- α -actinin (purified antibody, 50 μ g/ml). (a) Phase-contrast, showing a group of about 10 neurons (arrow) in the center. (b) Same field, fluorescence, showing labeling of the cell periphery (arrow). In other cases, the staining was continuous, not patchy. No surface staining was seen with corresponding preimmune γ -globulins at 1 mg/ml. Spinal cord explant with attached dorsal root ganglia 7 d in culture. Bar, 50 μ m. $\times 480$.

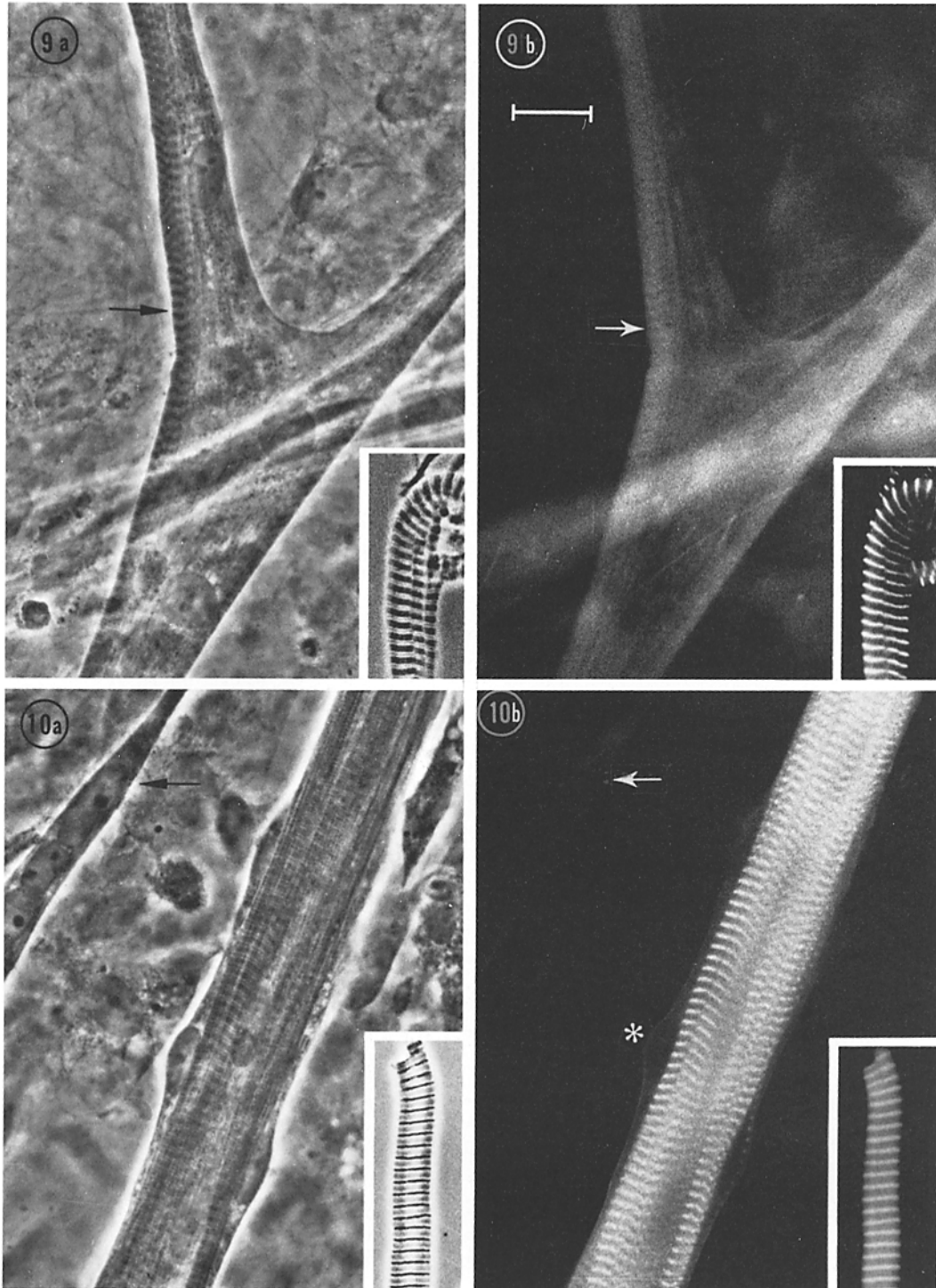


FIGURE 9 Rat myotubes in culture and isolated porcine myofibrils (*inset*) stained with anti-actin (purified antibody, 50 $\mu\text{g/ml}$). (a) Phase-contrast, showing enhancement of I-band pattern (arrow) by bound antibody. (b) Fluorescence, showing intense I-band staining in the myofibril and only slight staining (arrow) in the myotube. Combined rat myotube/mouse spinal cord culture, 8 d old, 4 d after addition of spinal cord. Bar, 20 μm . $\times 640$.

FIGURE 10 Rat myotube in culture and isolated porcine myofibrils (*inset*) stained with anti- α -actinin (purified antibody, 50 $\mu\text{g/ml}$). Arrow, an immature myotube. (a) Phase-contrast, enhancement of Z-line pattern by antibody binding. (b) Fluorescence, showing brightly fluorescent Z lines in the mature myotube and in the myofibrils, and weaker staining of the periphery of the myotube (asterisk). There is no Z-line pattern in the immature myotube. Rat myotubes were 15 d in culture. $\times 640$.

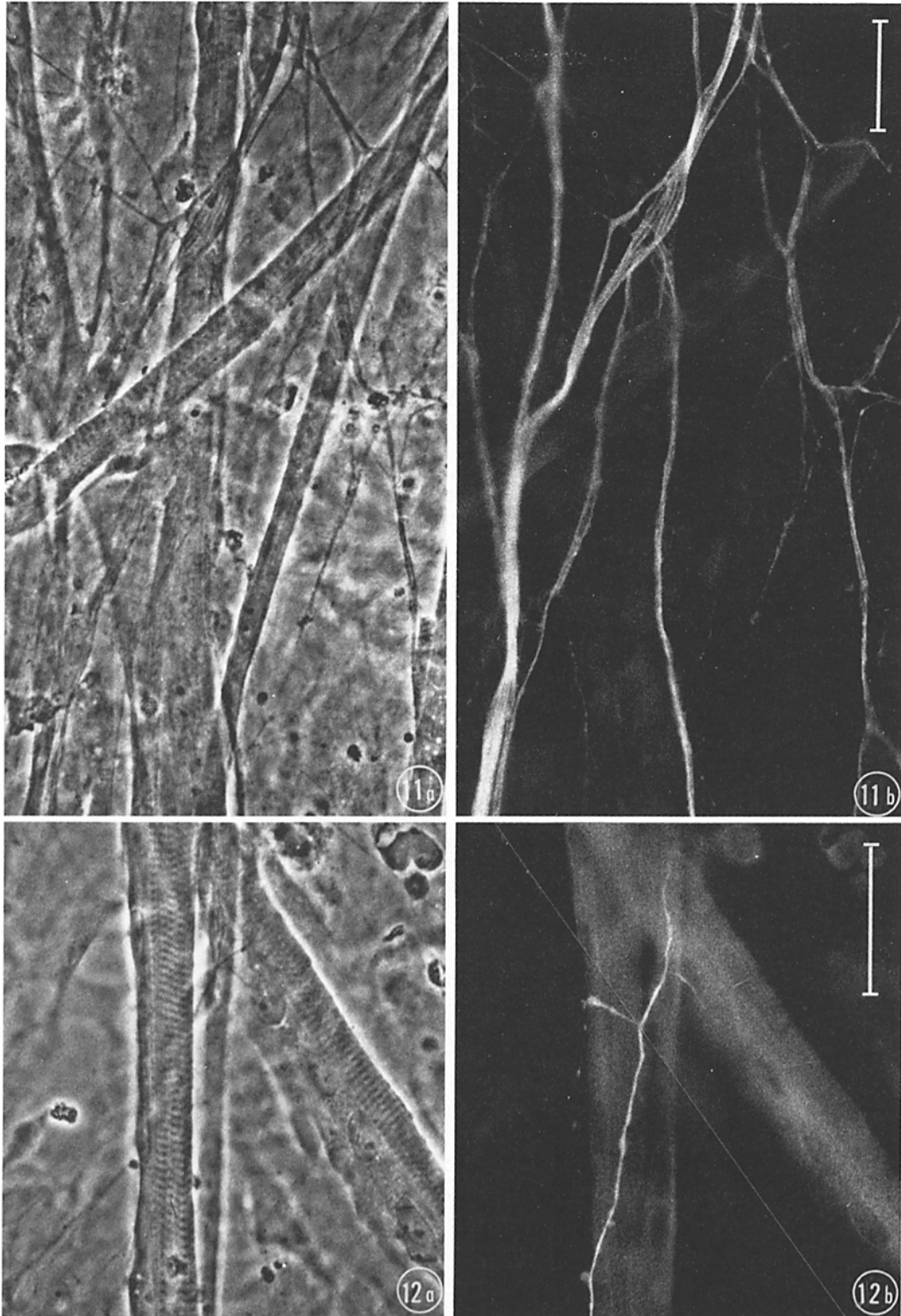


FIGURE 11 Mouse nerve fibers on rat myotubes, stained with anti-tubulin (purified antibody, $50 \mu\text{g}/\text{ml}$). Culture was fixed and stained 7 d after the mouse spinal cord had been placed on the myotube monolayer. (a) Phase-contrast; (b) Same field, fluorescence. Bar, $50 \mu\text{m}$. $\times 300$.

FIGURE 12 Contact of mouse nerve fibers with a rat myotube, anti-tubulin staining. Same culture as in Fig. 9. (a) Phase-contrast. (b) Same field, fluorescence, showing nerve ending on myotube (composite photograph of two different planes). Bar, $50 \mu\text{m}$. $\times 480$.

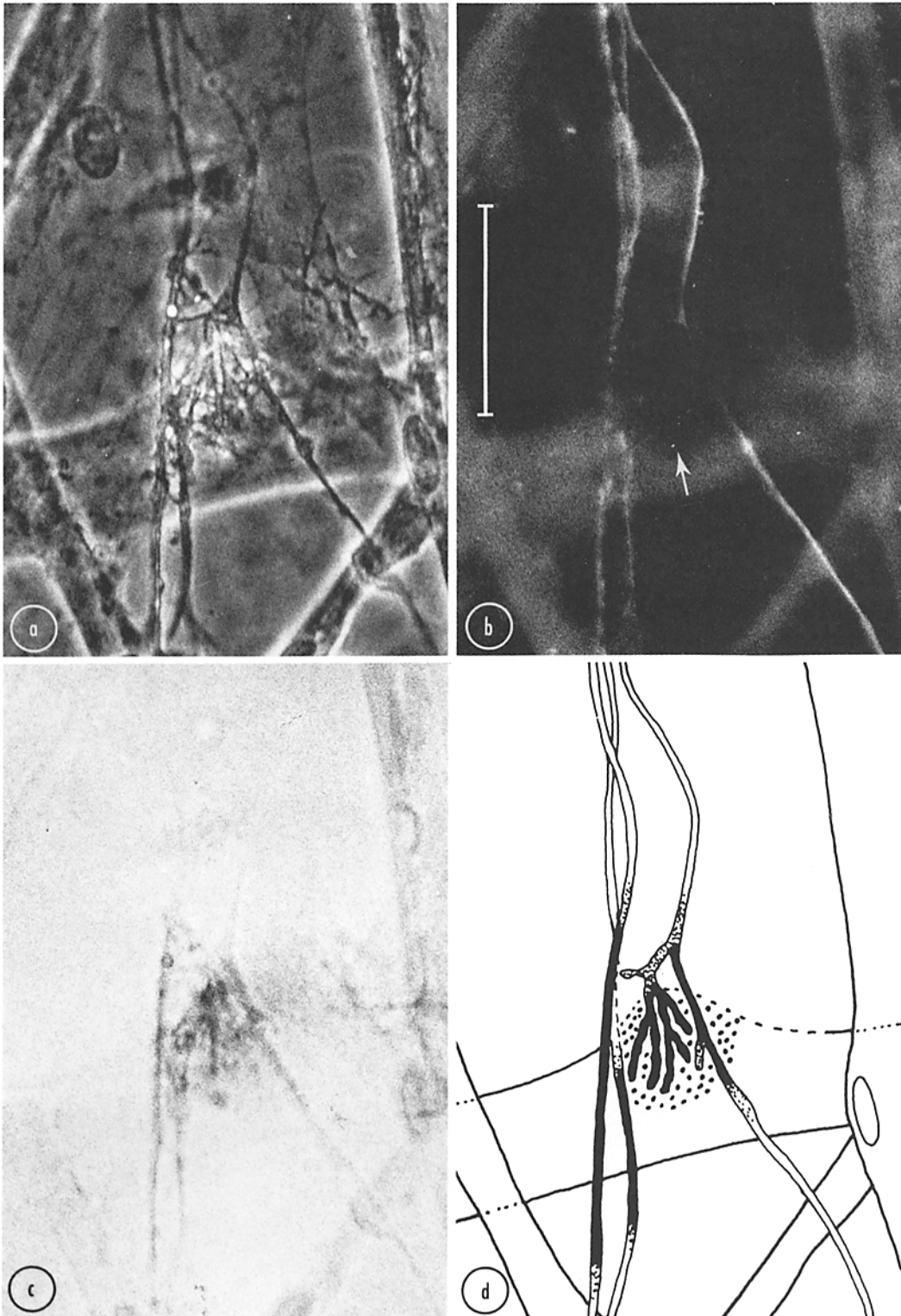


FIGURE 13 Neuromuscular contact stained for AChE (1 h at pH 6.0) and with anti-tubulin (purified antibody, 50 $\mu\text{g/ml}$). Culture as in Fig. 11. The same field is shown in four different ways: (a) Phase-contrast; AChE-positive regions of the nerve fibers are highly refractile. (b) Fluorescence, showing nerve fibers except terminal branches. In the region of AChE staining, fluorescence is abolished (arrow). (c) Bright field, to show AChE product. Nerve fibers and the surface of the myotube in the contact region are AChE positive; nuclei of myotubes are weakly stained. (d) Schematic drawing which combines information from (a), (b), and (c). The outlines of some myotubes and three nerve fibers are shown. One of the nerve fibers has sprouted at the contact zone, but continues beyond it. Black and stippled areas indicate AChE-positive regions. Bar, 50 μm . $\times 670$.

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