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NEURITE OUTGROWTH FROM EXPLANTED *XENOPUS* RETINA: AN EFFECT OF PRIOR OPTIC NERVE SECTION

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SUMMARY

Experimental conditions are described for the in vitro study of explanted eyes from embryos of *Xenopus laevis* and of retinas from older larvae. When eyes are explanted from embryos of stages 25–34, a rapid outgrowth of fibroblast-like and pigment cells is observed, upon which a neuritic outgrowth is eventually superimposed. Outgrowth from the retina of later stage tadpoles (50–54) is not seen until about a week following explantation and resembles the mixed cell outgrowth observed in whole eye explants from early stage embryos. If, however, the optic nerve of an older tadpole is cut 7 days prior to explantation, a purely neuritic outgrowth is seen from the previously denervated retina within 1–3 days.

INTRODUCTION

Most experimental investigations of the formation of visual connections have thus far been carried out in vivo. And while for many purposes this is necessary, a number of considerations have led us to investigate the possibilities of growing the relevant tissues in vitro. For example, advantages may follow from the ability to observe the behavior of growing fibers directly and to apply various treatments, chemical and otherwise, to the isolated tissue in culture. We have chosen to study initially the conditions for growth of explanted whole eyes and isolated retinas in embryos and larvae of *Xenopus laevis*.

MATERIALS AND METHODS

Xenopus embryos and larvae from a laboratory colony were decontaminated prior to operation by storage for a day in sterile 90 ml petri dishes containing 25 ml of Niu–Twitty solution[23] containing mycostatin (Nystatin, Sigma, London) 100 U/ml and neomycin sulfate (Sigma) 100 μg/ml[2]. In some instances, gentamycin sulfate (Flow

Laboratories) was also added, at a concentration of 70 μg of free base/ml. This decontamination mixture, always containing gentamycin, was used to rinse eyes and dissected retinas before they were set out in the culture chambers. For surgical procedures, the same mixture was supplemented with the anesthetic, tricaine methanesulfonate (MS-222, Sandoz, Basel), 0.67 ml of a 5% filter-sterilized solution/100 ml of decontamination mixture.

Eyes were excised from stage 25–34 embryos by means of sterile sharpened tungsten needles, under a dissecting microscope, and were transferred to a rinse solution by a suitable sized glass micropipet fitted with a plugged mouth tube. If the eyes did not rupture during transfer (and this did not often happen unless the tissue came in contact with the air-water interface), they were opened with needles to ensure exposure of the retinal cells to the substratum. After removal of the eyes from older tadpoles, the retina and lens were dissected free of the remainder of the eye in a sterile, paraffin-lined dish containing the salt–antibiotic mixture. In most cases the lens was then removed.

Plastic petri dishes for tissue culture, 30 mm $\times$ 10 mm (Nunclon N 1470 Vestric, London) proved to be suitable for the adhesion of collagen gels and were therefore routinely used as growth chambers throughout this study. For growth on glass, a 20 mm hole was made in the plastic bottom of a dish and a glass cover slip was cemented into place[5]. The top was replaced and the dish was sterilized by irradiation from a ^{90}Co source.

Cultures were stored either at room temperature or in a controlled temperature room at 20.5 °C, in humidified plastic boxes. They were examined, usually daily, in a Wild M40 inverted microscope through which 35 mm photographs or 16 mm time-lapse cine films were made. A metal adapter was designed to support the culture dishes on the microscope stage. A 10 $\times$ phase contrast objective was used routinely and a long working-distance 20 $\times$ phase contrast objective also proved satisfactory for examination of cultures through the plastic and the collagen gel substratum. A Leitz Diavert inverted microscope was also used.

Collagen, prepared according to Elsdale and Bard[6] was the generous gift of Dr. B. Hogan of the Imperial Cancer Research Fund Laboratories, Mill Hill. The collagen was stored as a solution at low ionic strength and pH 4 at 4 °C.

Gel formation was initiated by the simultaneous addition of sufficient NaOH to bring the pH to neutrality and of a concentrated Steinberg's solution[12] to achieve the desired osmolarity. The components were mixed, and 0.4 ml was quickly pipeted into each culture dish, which was then rapidly agitated to ensure that the bottom was completely wetted. The dishes were not moved again for at least 1 h after the collagen had been added, to permit uniform gelation. They were then stored at room temperature in humidified containers for up to 2 weeks. Before use, 2 ml of culture medium was carefully laid over the gel and the excess removed 2 h later with a Pasteur pipet. Sufficient liquid was left over the collagen surface to allow the explants to be moist but not submerged in the medium. The gels contain only about 0.1% collagen[6] and care was taken to avoid mechanical jarring, since this could cause retraction of the gel from the side of the dish.

Fetal calf serum was obtained from Gibco Bio-Cult (Renfrewshire). Eagle's medium (Dulbecco's modification) was prepared from components obtained from BDH chemicals. L-15 mixture[14] was prepared without salts. Steinberg's solution was made 20 m*M* with respect to HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethane-sulfonic acid) and was brought to pH 7.4 with 1 *N* NaOH. It was prepared and stored as a 10-fold concentrate. Glutamine, 0.24 *M*, was stored at —20 °C, as were all the other media components except for the L-15 and Steinberg's solutions, which were kept at 4 °C.

For electron microscopy the cultures were fixed in 1% formaldehyde and 1% glutaraldehyde in 0.1 *M* phosphate buffer. The fixative was introduced slowly onto the floor of the culture dish without removing the culture medium and left at 4 °C overnight. The cultures were then washed with buffer and post-fixed for 1–2 h in 2% osmium in 0.1 *M* phosphate buffer, dehydrated in a series of graded alcohols to absolute alcohol, placed in a solution of equal parts absolute alcohol and Taab embedding resin for 2 h, and then left in resin at room temperature overnight. An open ended embedding capsule was then placed over the culture in the petri dish filled with resin and cured in a 60 °C oven for 2 days. The plastic dish was then cut away with a razor blade and ultrathin sections cut on a LKB ultramicrotome. Sections stained with 3% uranyl acetate in methanol and Reynolds lead citrate[24] were examined in a Philips 201 electron microscope.

RESULTS AND DISCUSSION

Growth surface

Explants of stage 25–34 eyes in various media indicated the superiority of collagen as a substrate for outgrowth of neurites. Gels of the denatured form of collagen, gelatin, (2%) or agar (1–2%, Difco Noble) showed no outgrowth although the explanted tissue appeared to adhere well. Excised eyes, maintained in a liquid medium for 10 days and then pipeted onto a collagen gel, showed good outgrowth within 2 days. Native collagen may have proved successful because it provided a substrate which most closely resembled the in vivo state of the polymer or alternatively, its apparent suitability for neurite outgrowth may perhaps be attributed to the fact that it produces a mechanically stable gel at a low concentration, resulting in a substratum with a high degree of porosity. While some purely neuritic outgrowth was seen, more often the neuritic outgrowth remained confined to a 'lawn' of non-neural cells. This was quite apparent even 1 week after explantation. Attachment and outgrowth were also seen, somewhat less reliably, from explanted eyes placed directly on the surface of the culture dish. Outgrowth on plastic was best when the dishes were not moved for 2–4 days after explantation; even then, detachment was sometimes seen after initial indications of outgrowth.

The appearance of pigment cells and fibroblast-like cells that had migrated away from the explant was much more frequent on a glass or plastic base than on a collagen growth surface. Pretreatment of the plastic or glass surface with dilute gelatin[29] facilitated initial positioning and adherence of explants but did not appear to in-

fluence the proportion of explants that ultimately showed good outgrowth. The usefulness of coating the growth surface with a polycation has recently been shown[15]. The availability of a growth surface that has the adherence and outgrowth promoting properties of collagen, but that is 2- rather than 3-dimensional, is potentially useful for the investigation of possible trophic influences between neighboring explants. It has previously been noted[28] that apparent increased growth and orientation of fibers between explants may result from stresses within the substrate, induced by the adjacent centers of biological activity, or to diffusion-limited gradients of metabolites. These possibilities would be much reduced or absent when growth is on a rigid, nonporous surface.

The growth medium

Amphibian tissue culture is noted for its lack of stringent growth requirements[18,19]. A wide range of temperature and pH is tolerated by a number of explanted tissues. In initial studies, we used an available culture medium, Dulbecco's modification of Eagle's medium, diluted 7:3 with sterile distilled water, to approximate amphibian osmolarity. Good outgrowth was seen from explanted eyes, and there was no obvious difference in the amount or degree of outgrowth when the dishes were stored in air instead of under 5% CO_2, even though in the former case, the pH rose to 8.2. Media specifically designed for amphibian tissue culture, however, gave better outgrowth.

The medium used in the present experiments consisted of Steinberg's solution × 10 buffered with Hepes (pH 7.4), 0.5 ml; L-15 mixture without salts, 5.7 ml; fetal calf serum, 2.5 ml; gentamycin 7 mg/ml, 0.1 ml; and L-glutamine 0.24 *M*, 0.1 ml.

Antibiotics

The practical problem of which antibiotics to use in *Xenopus* tissue culture has been discussed by Laskey[13]. In agreement with his findings, gentamycin served adequately in preventing bacterial contamination in the present experiments. Using minimal sterile precautions, we did not encounter serious problems of bacterial contamination in the presence of this antibiotic. Penicillin and streptomycin, the antibiotics usually employed in mammalian tissue culture, were ineffective, presumably because of the gram-negative nature of the organisms present in the maternal cloaca of *Xenopus* and which contaminate the eggs, although this antibiotic mixture has been used in the maintenance of whole excised *Xenopus* eyes in a liquid medium[10].

In a few instances in which retinas from postmetamorphic eyes were explanted, it was not practicable to decontaminate the entire frog. Instead, the eye was removed from the anesthetized animal and was immersed for 30 sec in 70% ethanol, followed by rinses in sterile decontamination mixture.

The only fungicide used in these experiments was the mycostatin for preliminary washes. While amphotericin B (Fungizone, Squibb) is often used for tissue culture purposes, it was not added because of its known toxicity for *Xenopus* embryos[13]. Fungal infection was nevertheless rarely seen for about 2 weeks following explantation, and occurred in about 20% of cultures maintained for longer periods.

Retinal explants

Using embryonic eyes, we had established satisfactory conditions for outgrowth of neurites on a collagen substratum. It was then of interest to see whether this response could be elicited from older retinas dissected free of other eye tissue. Young eyes of stage 25–34 have not yet formed tectal connections[22], although the first ganglion cells to appear in the developing retina do so before stage 32, by which time the retina is also polarized[11]. Eyes from larvae at stages 50–54, however, have already formed synaptic contacts in the tectum[3,25] and have generated an orderly retinotectal projection[8].

In initial experiments, the larval eye was cut open and retinal fragments were dissected out and were positioned on the collagen surface by means of a pipet. It eventually became possible to 'peel' the eye and thus remove the intact retina, together with the lens, from the eye. After removal of the lens, 1–5 retinas or retinal fragments were placed in each culture dish and, for purposes of sequential observation, each fragment was identified in relation to a mark on the outside of the dish. No neuritic outgrowth was seen for 4–7 days, at which time a mixed outgrowth of neurites and other cell types was observed. In general, the neurites appeared to be contained, not growing beyond a base-layer or lawn, of non-neuronal cells which included pigment cells and fibroblast-like cells, as was often observed with explanted eyes from earlier stage larvae.

Since neurites grew out from the retinal elements of the younger, embryonic eyes with a much shorter delay, it was of interest to see whether there was any interaction between the two kinds of explant, such as acceleration of outgrowth from the older retinas, or even retardation of outgrowth from the younger eyes, when they were grown in the same dish. The results of such an experiment was that the two pieces of tissue behaved independently. Neuritic outgrowth from the embryonic eye was seen within 24 h but there was not outgrowth from the stage 54 explanted retina in the same dish, until 5 days later. Thus, the differential time of outgrowth was not mediated by readily diffusible factors that cause cell outgrowth, such as have been reported in cultures of neuroblastomas[17] and embryonic brain cells[16]. Neuroblastomal neurite extension seems also to result from a number of non-specific conditions, including the depletion of metabolites necessary for replication[27]. It seemed possible, by analogy, that the rapid response of a younger eye might reflect a greater metabolic rate and the more rapid depletion of some critical nutrient, in comparison with an older retina. The double explant experiment militates against this possibility as well.

A further possibility is that the different times of initial outgrowth of embryonic and larval eyes may merely reflect anatomical differences. For example, the embryonic eye is much smaller than the larval retina and perhaps growing fibers have a shorter distance to go before appearing at the edge of the explant. While such factors presumably have some relevance for the results, it is unlikely to be the only, or even the main explanation since different times of initial outgrowth can be caused in larval eyes by different treatments of the eyes before explantation, as described in the next section. The rate of neurite outgrowth appears to be an intrinsic property of the retina and to reflect its developmental stage or its physiological state.

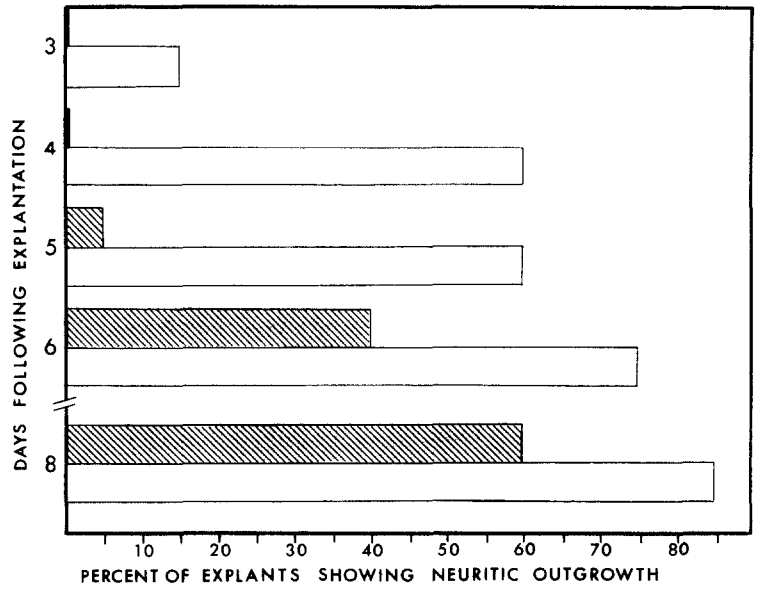

Fig. 1 Prior cutting of the optic nerve greatly accelerates the appearance of neurites from explanted retinas. The left optic nerve was cut in 20 tadpoles at about stage 54 one week prior to explantation. Five right or left retinas were placed on a collagen substratum in culture medium in each of 8 petri dishes and maintained at 20.5 °C Open bars, left eye (cut nerve); shaded bars, right eyes (uncut optic nerve).

Regeneration experiments

Although the neuronal cell type or origin of the neuritic outgrowth is unknown, the age-dependent effect suggested that the early outgrowth of embryonic eyes could be a consequence of the fact that the ganglion cell axons in the optic nerve had not yet reached the tectum and might thus be expected to be in a state of maximal production of cellular materials. If this were so, we should mimic this state in the stage 50+ retina by cutting the optic nerve some time before explantation of the eye.

Since regeneration of the optic nerve in the *Xenopus* larva takes 10–18 days[7], we selected 7–8 days as a convenient interval to permit the development of a retinal response. Various cytological and autoradiographic studies in the goldfish indicate that the retina shows an increase in RNA and protein synthesis within about 2 days of disruption of the optic nerve[20,21].

In larvae of about stage 54, the left optic nerve was cut by means of a piece of razor-blade, close to its entry into the brain in order to avoid significant damage to the retinal blood supply. A week later the right and left eyes were excised and the retinas explanted into separate dishes, each containing 1–5 retinas in culture medium over collagen. In all, 3 separate experiments were performed with a total of 56 retinas. A typical experiment is summarized in Fig. 1. Prior section of the optic nerve resulted in an early outgrowth of a purely neuritic nature. Retinas from the intact side showed a delayed outgrowth of combined cell types often in a 'lawn'. In general, 6 out of 7 retinas eventually showed neuritic outgrowth. An example of outgrowth is shown in Fig. 2. In every case where sprouting occurred it was seen in 2–4 days if the optic nerve had

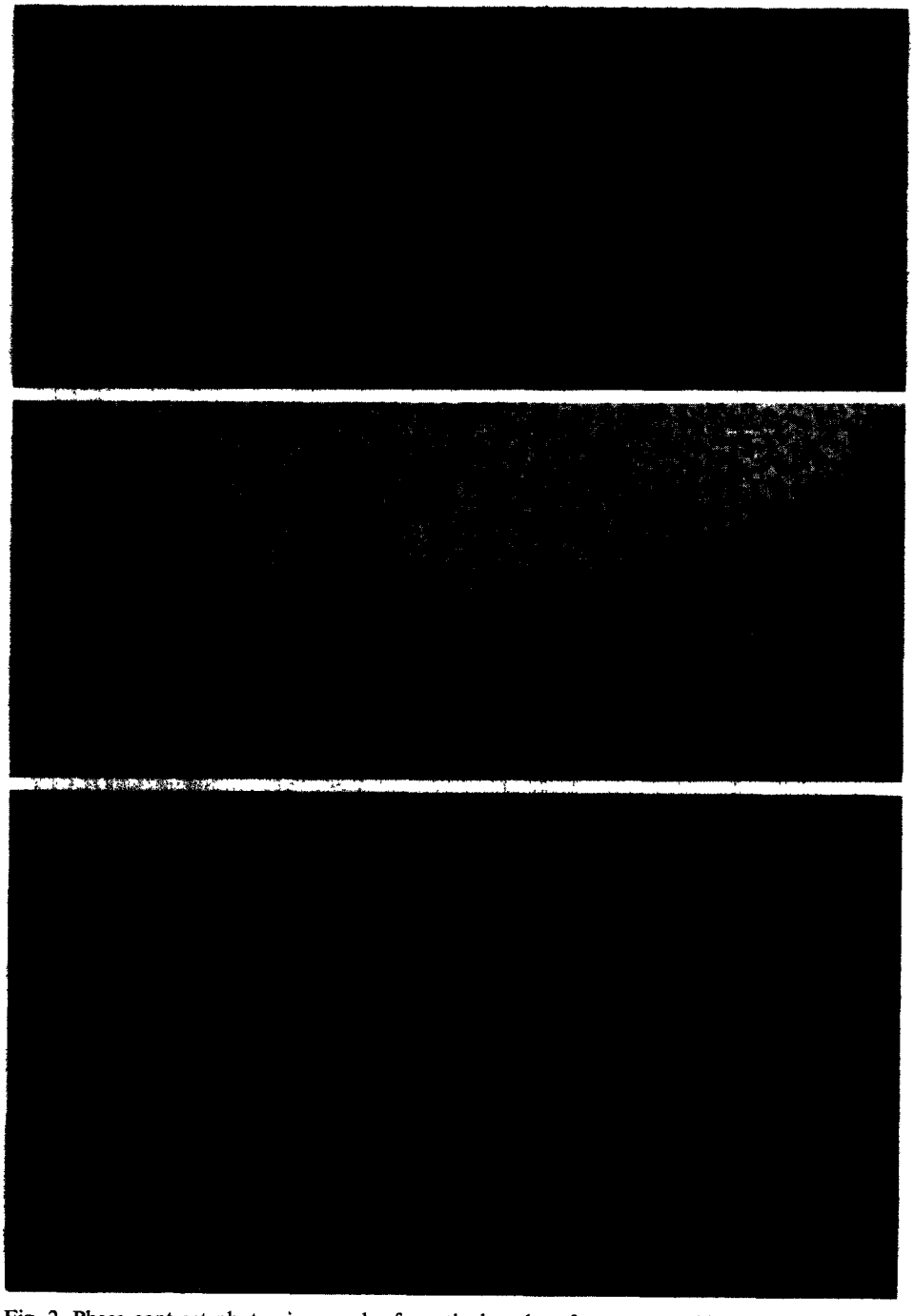

Fig. 2. Phase contrast photomicrograph of a retinal explant from a stage 50+ tadpole. The optic nerve had been cut 7 days prior to explantation. A: after 2 days in culture. B: after 4 days in culture. C: after 10 days in culture. Bar = 100 μm.

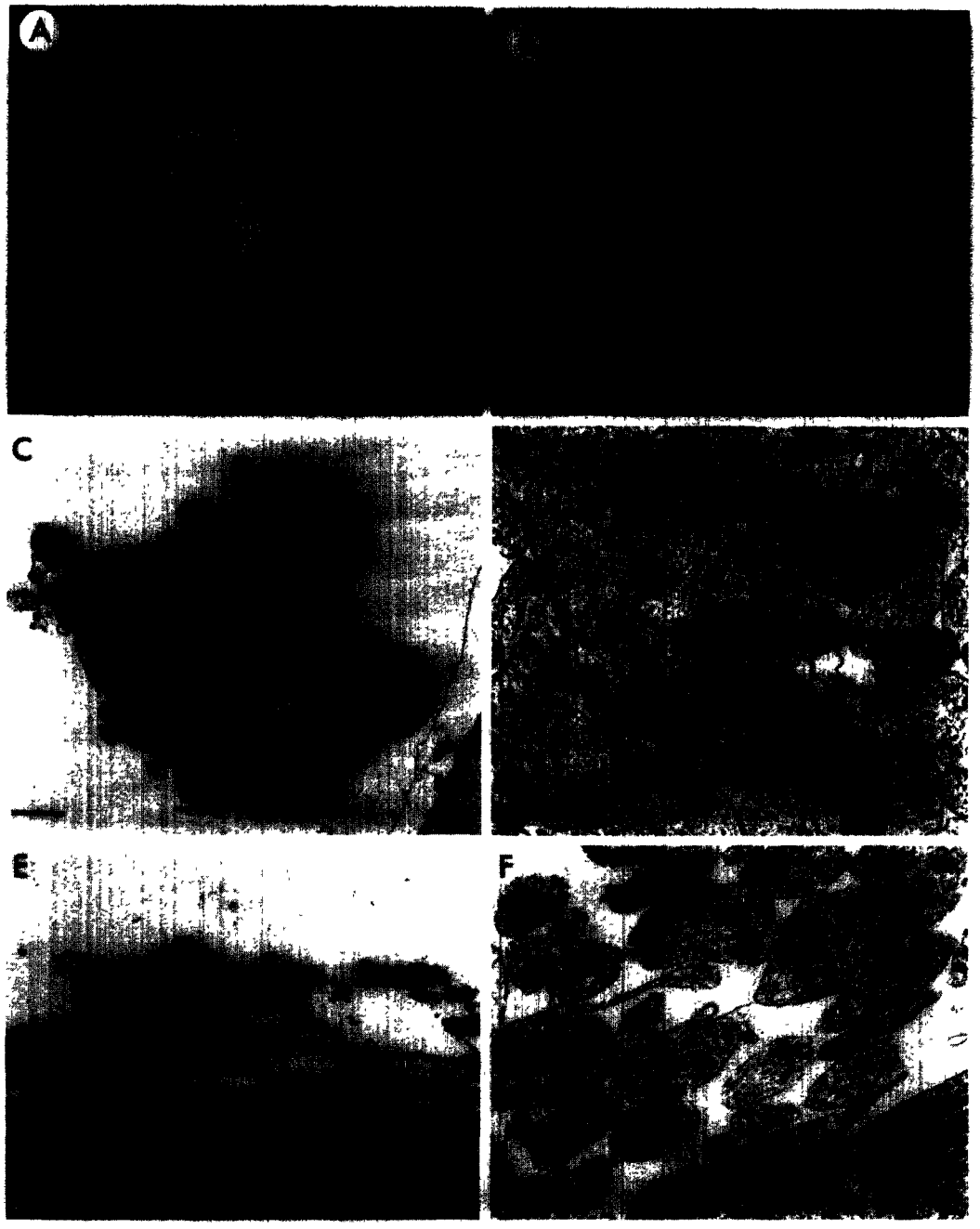

Fig 3. A. explanted retinal fragment from an eye, the optic nerve of which had been cut 7 days prior to explantation, photographed after 15 days in culture on a collagen substratum Bar = 1000 μm B: phase contrast enlargement of region of the explant shown in A. Bar = 200 μm. C. electron micrograph of transverse section of neuritic process shown in B. Bar = 2 μm. D: magnification of electron micrograph C. Bar = 0.5 μm E: electron micrograph of transverse section of neuritic process from an explanted retina, the optic nerve of which had not previously been cut Bar = 2 μm In such cases, neuritic outgrowth appears only after several days and is accompanied by an underlying cellular lawn (see text) F: higher magnification of E Bar = 0 5 μm.

previously been cut (19 out of 28 operated retinas; none out of 28 unoperated), and not until 3–4 days later if the retina was from the unoperated side (17 out of 28 unoperated; 5 out of 28 operated). Outgrowth was more rapid in each case if the cultures were maintained at room temperature (20–25 °C) than if they were kept in a constant temperature room at 20.5 °C. Early outgrowth (within 3 days of explantation) was seen only in retinas whose optic nerve had been cut a week earlier.

The cells of origin of the neuritic outgrowth are not known, but it is tempting to suggest that they are the ganglion cells, since section of the optic nerve is known to stimulate selectively macromolecular synthesis in this cell layer in the goldfish[20,21]. The thickly aligned axonal outgrowths, seen after several days of growth of explants (Fig. 3), further support this idea. Time lapse films showed continued extension and retraction of neurites. Active growth cones were seen for weeks following explantation.

The mechanism of fiber progression in neurite outgrowth differs from the mechanisms of cell outwandering. In the former case the cell body remains more or less constant in position and only the axon or process extends. It has been suggested that growth cones, which are not necessary for growth per se (at least in certain in vivo situations), are associated with an exploring mode of behavior, and with the processes of intercellular recognition. If so, the explanted amphibian retina may be suitable for studies of neuronal connectivity.

The most widely accepted hypothesis to account for the pattern of neuronal connection in many parts of the nervous system is that of neuronal specificity[26], which suggests that specific conditions are formed on the basis of intercellular recognition phenomena between cells or processes bearing corresponding cytochemical labels. There is evidence that, if this is the mechanism involved, it functions not only between, for instance, the optic nerve fibers and the tectal cells, but also between the optic nerve fibers themselves[1,4,9]. If interfiber recognition of this sort occurs, and if the cellular and axonal characteristics involved survive explantation into a culture situation, there is hope that selective fasciculation, selective synaptic formation or selective synaptic maintenance may eventually be demonstrable in culture.

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