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SPECIFIC CHANGES IN AXONALLY TRANSPORTED PROTEINS DURING REGENERATION OF THE FROG (*XENOPUS LAEVIS*) OPTIC NERVE[1]

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Abstract

Labeled proteins in intact and regenerating optic nerves of juvenile *Xenopus* clawed frogs were examined at three different time points (2 to 4 hr, 18 hr, and 5 to 9 days) following [^{35}S]methionine injection into the eye. The distal axon tips of optic nerves were transected at the margin of the tectal lobe and regeneration of the nerve was followed by three methods: autoradiography, tissue section counting following [^{3}H]proline injection into the eye, and electrophysiological mapping of the visual field projection. By these methods, regrowth was found to occur 2 weeks after transection, but the fibers had not yet sorted their retinotopic pattern. Two-dimensional gel separation of labeled nerve proteins revealed 250 to 300 identifiable proteins, 89 of which (including all spots which differed consistently upon direct comparison of regenerating versus normal nerves) were selected for quantitative treatment. Nine of these spots (240, 135, 65, 64, 58, 54, 56, 31, and 26 kilodaltons) were shown to increase and six (56, 49, 42, 29, 17, and 15 kilodaltons) were shown to decrease significantly in regenerating nerves. By using a crush control and tracking the labeled proteins into the tectum over time, these proteins were shown to be axonally transported proteins. In addition, four other nonaxonally transported proteins also changed during regeneration.

Unlike their mammalian counterparts, the neurons in the central nervous systems of lower vertebrates readily regenerate axonal pathways and connections even in mature organisms (Matthey, 1925; Stone and Chace, 1941; Sperry, 1944; Sperry, 1948; Attardi and Sperry, 1963; Gaze, 1959, 1960). By present anatomical and electrophysiological crtieria in the best cases, regenerated optic nerve fibers of frogs and fishes can reestablish connections with the correct class of postsynaptic cell, organize their arbors in the proper laminar order, and recreate the topographic order of the original retinotectal projection (Maturana et al., 1959; Gaze and Jacobson, 1963; Gaze and Keating, 1969; Freeman, 1977; Schmidt and Edwards, 1983).

Optic nerve injury stimulates reactions in both the neuronal elements and their support cells which can be recognized acutely in histological material (Sjostrand, 1965; Murray and Grafstein, 1969; Murray and Forman, 1971; Ostberg and Norden, 1979; Wolburg, 1981). More chronic responses to axonal injury include the reinitiation of axon growth, elongation of the axon, reinnervation of the proper "target" neural center, recognition of correct synaptic sites within the target center, and reestablishment of functional connections with the appropriate postsynaptic cells (Cowan and Hunt, 1985; Stuener and Easter, 1984). While these events have been vigorously investigated using anatomical and electrophysiological methods (Maturana et al., 1959; Jacobson, 1961; Attardi and Sperry, 1963; Gaze, 1970; Chung, 1974; Hunt and Jacobson, 1974; Edds et al., 1979; Fraser and Hunt, 1980; Meyer, 1982), the literature on molecular correlates of these processes consists of but a few pioneering papers.

Because neuronal protein synthesis occurs in cell bodies and not axons (reviewed in Grafstein, 1969), axonal proteins relevant for regeneration must pass through the nerve to its growing terminals by axonal transport. Axonal transport, moreover, can geographically separate the relevant retinal ganglion cell proteins from other proteins in the eye (Lorenz and Willard, 1978; Droz et al., 1975). For these reasons, studies on the axonal transport of proteins in regenerating nerves (Hoffman and Lasek, 1975; Bisby, 1980; Perry and Wilson, 1981), including the optic nerves of goldfish and toads (Benowitz et al., 1981;

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Giuliane et al., 1980; Skene and Willard, 1981a, 1981b) have become increasingly valuable, especially with the improved resolution of two-dimensional gel electrophoresis (O'Farrell, 1975; Wilson et al., 1977).

We have chosen *Xenopus laevis* for our studies on axonally transported proteins during optic nerve regeneration, because histological and electrophysiological data exist on its time course of optic nerve regeneration (Gaze and Jacobson, 1963; Gaze and Grant, 1978; Glastonbury and Straznicky, 1978; Straznicky et al., 1979; Ostberg and Norden, 1979; Gaze and Fawcett, 1983) and on the altered retinotectal patterns which ensue when surgical rearrangement of the eye or tectum is coupled with an optic nerve crush or transection (Jacobson and Levine, 1975; Gaze and Straznicky, 1979; Rho and Hunt, 1980; Straznicky and Tay, 1981). Abundant data on visual ontogeny in embryos and larvae (Jacobson, 1968, 1976; Hollyfield, 1971; Straznicky and Gaze, 1971, 1972; Gaze et al., 1974; Beach and Jacobson, 1979; Grant and Rubin, 1980; Conway et al., 1980) give the clawed frog a further advantage over goldfish and toads: the longer term prospect that certain molecular correlates of optic nerve regeneration might point the way to molecular events in the normal ontogeny of the animal.

In a previous paper, we described the phases of transport in the intact optic nerve of normal juvenile *Xenopus* froglets. In the course of that study, it was necessary to distinguish transported ganglion cell proteins from contaminant proteins, which nerve sheath cells distal to a nerve crush can contribute to the SDS gel profiles of the optic nerve proteins, after intraocular injections of [^{35}S]methionine (Szaro and Faulkner, 1982; Szaro et al., 1984). In the present paper, we have examined changes in axonally transported proteins in the regenerating optic nerve, 2 weeks after severing the distal tips of the optic axons at the rostral and lateral margins of the tectum (Giuliane et al., 1980; Szaro et al., 1982). The surgery used minimizes the contributions that injured support cells make to the profiles of labeled optic nerve proteins and ensures the role of the optic nerve as a relatively passive conduit from which transported proteins can be harvested en route to the growing axon tips. Scintillation counting of tectal sections, [^{3}H]proline autoradiography, and electrophysiological visual field mapping were all used to examine the time course of optic nerve regeneration in *Xenopus* following the transection of the optic axons at the edge of the tectum; they suggested a time point (of 2 weeks) during regeneration when we could expect to see maximal changes in the profiles of axonally transported proteins. We describe specific changes in these protein profiles as detected by O'Farrell two-dimensional gel electrophoresis (O'Farrell, 1975; Wilson et al., 1977).

Materials and Methods

Charting the time course of regenerating optic fibers

Optic nerve lesions. Juvenile (1 to 6 months postmetamorphosis, 2.5 to 3.3 cm in length) *X. laevis* frogs (Nasco Biological Supply, Ft. Atkinson, WI) were anesthesized by immersion in 0.1 to 0.2% tricaine methane sulfonate (Finquel, Ayerst, NY). The diencephalon and tectal lobes were exposed dorsally by surgical retraction of cranial skin and bone, and the optic fibers innervating the left tectum were severed with a fine wire electrode. The wire was inserted through the full thickness of tectum to the ventricle, at the rostromedial corner of the tectum and then drawn laterally across the rostral border of the tectum, and finally drawn caudally along its lateral edge (see Fig. 1). The skull and overlying skin were replaced; and the animals were allowed to recover.

In *Xenopus*, the optic tectum is directly innervated by fibers emanating from the contralateral eye. Hereafter, the right eye, whose optic nerve innervates the left tectum, will be referred to as the experimental eye, and its (right) optic nerve designated as the experimental optic nerve. The left eye and nerve which innervates the intact right tectum were used as controls within the same animal.

Isotope injections. L-[2,3,4,5-^{3}H]Proline (Amersham; Arlington Heights, IL; 80 to 100 Ci/mmol, 1 μCi/μl) was allowed to sit briefly at room temperature and then was pressure injected (0.7 to 1 μCi in 0.7 to 1 μl) through a micropipette into the eyes of anesthetized frogs. To avoid damaging the retina, we inserted the micropipette into the eye's posterior chamber by passing it through the cornea and iris near the margin of the retina.

Tissue section scintillation counting. To track the early phases of optic nerve fiber regrowth, [^{3}H]proline was injected into the right eyes of unoperated animals and animals whose optic nerves had been severed 30 min, 3 days, 6 days, 10 days, and 13 days earlier. Eighteen to 24 hr after isotope injections, the animals were anesthetized and perfused first with isotonic frog saline, and then with Karnovsky's fixative. The brains were removed, postfixed in Karnovsky's, and washed overnight in a 30% sucrose solution in 0.1 M phosphate buffer, pH 7.4. The brains were then embedded in gelatin, frozen, and sectioned transversely at 75 μm with a sliding microtome. Each section was placed in a scintillation vial with an appropriate cocktail containing NCS tissue solubilizer (Amersham) (Loh and Gainer, 1975). Vials containing sections marking the anterior and posterior tectal margins were marked. The sections were then allowed to dissolve at 55°C overnight and subsequently were cooled and counted in a Packard scintillation counter to an average error of 5%.

Light microscope autoradiography. The right eyes of unoperated animals and those of animals whose nerves had been severed 30 min and 2 weeks earlier were intraocularly injected with [^{3}H]proline and fixed in Bouins' fixative 18 to 22 hr later. After several days in fixative, their heads were dehydrated, embedded in paraffin, and subsequently cut parasagittally at 10 μm. Slides were dipped in a 1:1 dilution of Kodak NTB2 emulsion (Rochester, NY), air-dried, and allowed to expose the emulsion for 5 to 8 weeks at 4°C. The slides were then developed in Dektol, stained in hematoxylin, and counterstained with phloxine B or eosin.

Electrophysiological mapping of the visual field projection. The visual field projection to the contralateral optic tectum was also used to assess the time course of optic fiber regrowth. The frogs were first anesthetized and their tecta were then exposed dorsally. The animals were then immobilized with an intramuscular injection of 0.05 to 0.15 ml of *d*-tubocurarine (0.1 mg/ml, Lilly, Indianapolis, IN) and then mounted at the center of a visual perimeter with the (right) experimental eye aimed at the center of the perimeter arc. The perimeter was capable of presenting a spot of light (0.4 to 2° in diameter) anywhere within a 200° solid angle. The (left) control eye was covered with a black contact occluder. A platinum-tipped, platinum iridium electrode (F. Haer, Brunswick, MI), with a characteristic untipped resistance of 10 megaohms, was lowered into the tectum to detect optic evoked responses. These responses, consisting of action potentials from several units firing together in the superficial neuropil of the tectum, are believed to arise from terminal arborizations of optic nerve fibers (George and Marks, 1974; Hunt and Jacobson, 1974; Chung et al., 1974). The evoked responses were passed through a 60-Hz rejection filter, amplified by a band pass amplifier (100 Hz to 3 kHz), and then simultaneously displayed on a Tektronics oscilloscope and monitored over an audio speaker. When a focal response to a spot of light was detected, the entire multiunit receptive field at which the spot (stationary, moving, or flashing on and off) evoked a response, was mapped out and recorded on the perimetry card. The electrode was then moved systematically and responses were sampled at recording sites throughout the dorsal surface of the tectum. Further details of the recording methods have been reviewed by Hunt and Jacobson (1974) and Ide et al. (1979).

Gel electrophoretic fractionation of labeled optic nerve proteins during regeneration

Isotope handling and injections. For gel electrophoresis studies, L-[^{35}S]methionine, stored in 0.2 M potassium acetate and 0.1% 2-mercaptoethanol at a concentration of 5 to 11 mCi/ml, was obtained from Amersham (specific activity, 600 to 1400 Ci/mmol). Approximately 6 to 10 μCi of [^{35}S]methionine in a volume of 0.7 to 1.0 μl were intraocularly injected into both the experimental and control eyes in the manner previously described (Szaro et al., 1984).

Two weeks after optic fiber transection at the tectal margins, both the experimental and control eyes were injected with isotope. At varying intervals after the injections (2 hr, 4 hr, 18 hr, and 7 days), the frogs were reanesthetized and then exsanguinated by slicing the aorta. The two eyes, tectal lobes, and optic nerves from the eye to the chiasm were removed individually and frozen immediately on dry ice. The experi-

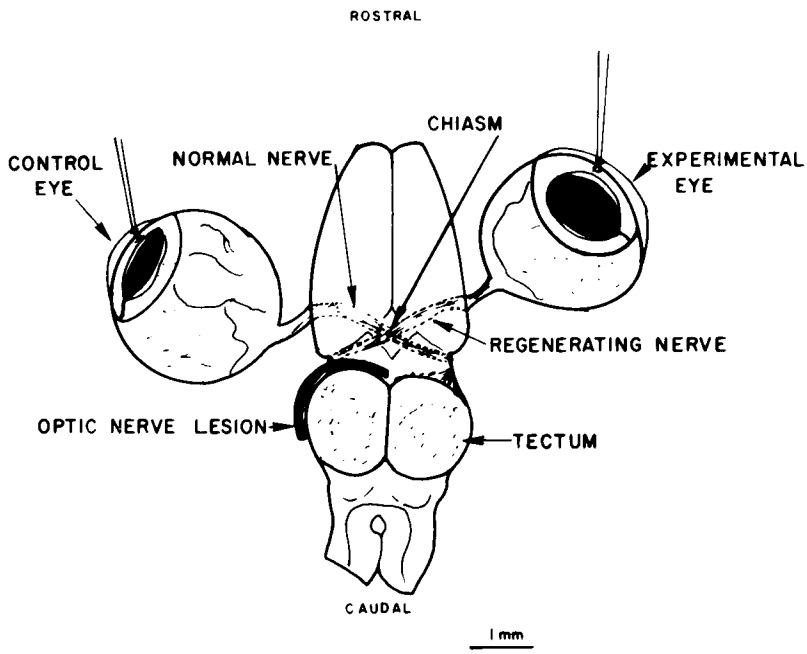

Figure 1. Dorsal view of the brain of *X. laevis*. This diagram illustrates the primary visual pathway in *Xenopus* and shows the site of fiber transsection at the rostral and lateral margins of the midbrain optic tectum. Note that the optic fibers of the right eye form the right optic nerve and after crossing at the optic chiasm innervate the left tectum. This drawing was traced from a photograph provided courteously by J. H. Rho, Children's Hospital, Boston, MA.

mental and control tissues from 10 animals were pooled to make each sample. The samples were then stored at −70°C until further needed.

Preparation of labeled tissue for two-dimensional electrophoresis

The frozen samples were thawed, solubilized, and centrifuged according to previously published methods (see Wilson et al., 1977; and Szaro et al., 1984). However, in place of Wilson's isoelectric focusing (IEF) buffer, we used a modified IEF buffer consisting of 0.5 ml of glycerol, 0.5 ml of Nonidet P-40 (Gallard Schlessinger, Carle Place, NY), 0.5 ml of pH 3 to 10 ampholytes (Bio-Rad, Richmond, CA) in 25 ml of 10 M urea (Schwarz/Mann, ultrapure). The final supernatant volumes were 55 μl for the nerve and tecta samples, and 110 μl for the eye samples.

Five per cent of the total volume was saved to assay the amount of label incorporated into protein by conventional trichloroacetic acid (TCA) precipitation on filter papers. The remainder was stored at −70°C until electrophoresis. After solubilization, in order to reduce differences caused by freezing artifacts, all experimental and control samples which were to be compared with each other were handled in parallel.

Two-dimensional gel electrophoresis

Isoelectric focusing was carried out according to the methods of O'Farrell (1975) and Wilson et al. (1977) on 5% polyacrylamide gels containing 6.25% of a commercially available stock solution of pH 3 to 10 ampholytes (Bio-Rad). The upper reservoir buffer was 0.2 M NaOH: the lower buffer was 0.1 M H_3PO_4. The samples were thawed at room temperature for 1 hr, sonicated for 5 sec, and then loaded onto the gels. The IEF gels were run first for 45 min at 150 V, and then at 300 V for a total of 5600 V hr. After the run was concluded, the gels were extruded and stored at −70°C. All experimental gels and their associated controls were run on the same day in the same gel apparatus.

Second dimension gels (SDS gel electrophoresis) were run on linear gradient gels poured from 4 and 17% monomeric acrylamide solutions through a linear gradient maker as described elsewhere (Neville, 1971; Loh and Gainer, 1975; Szaro et al., 1984). Next, the gels were fixed and stained overnight in 0.1% Coomassie blue. Later, destained gels were photographed and processed for fluorography (Bonner and Laskey, 1974).

X-ray film (SB-5 or X-AR, Eastman Kodak) was preflashed (Laskey and Mills, 1975; Szaro et al., 1984) and exposed to the processed gel at −70°C. The exposure times were adjusted so that the TCA-precipitable counts loaded on each gel multiplied by the length of the exposure in days (corrected for isotopic decay) equaled a constant for the experimental and control gels to be compared. This constant was approximately equal to 700,000 cpm-days for SB-5 film and 455,000 cpm-days for the slightly more sensitive XAR film.

Analysis of two-dimensional gel patterns: Initial screening by visual comparison of experimental and control gels

Photographic prints of the Coomassie blue patterns of each gel were made so that the stained spots would superimpose directly on to tracings made of the actual processed gels. The positions of suitable Coomassie blue-stained landmark spots, easily identifiable in all nerve gels, were noted on the fluorographs. Using the positions of each of these spots as references, the fluorographs were then photographed with a 35-mm camera fitted with a macrolens to establish a gridwork of rectangles, each the size of a 35-mm slide. Enlarged prints of these grid elements were then used to catalog each spot on the fluorograph. Each pair of spots on the experimental and control gels were then compared visually for their intensities, using both the enlarged photographs and the original fluorographs. The intensity of each spot on the experimental gel was then designated as being virtually identical, slightly different, or very different from that of the analogous spot on the control gel. Eighty-nine of these spots were selected for subsequent quantitative analysis. These included all spots which showed a consistent visible difference between experimental and control nerve gels, and a sampling of spots which did not appear to differ. For identification, each selected spot was assigned an integer. If the spot appeared in several phases of transport (i.e., co-migrated with another spot whose isoelectric point and molecular weight were indistinguishable from its own), it received the same integer designation for each phase in which it appeared.

Quantitation of two-dimensional gel patterns

The balanced exposure fluorographs from optic nerve gels were scanned on an Optronics rotating drum image scanner, which digitized the average optical density over a 100 × 100 μm image area spaced every 300 μm. This information was then entered on to a PDP 11/60 computer equipped with an IP 5000 image processor and stored on disk. Optical density measurements of the designated spots were made with the aid of two-dimensional gel analysis software (system and software provided courtesy of Dr. Carl Merril, National Institute of Neurological Communicative Disorders and Stroke, Bethesda, MD; see Goldman et al., 1982; and Heydorn et al., 1983 for details). Contours were drawn around each spot to be measured, and around an appropriate background region surrounding the spot. The computer then calculated the average optical density within the designated spot area, subtracted the modal background density, and then multiplied this number by the area of the spot to obtain the integrated spot density (optical density × mm^2).

Because x-ray film becomes nonlinear above an optical density of 2.0, any spots containing more than 10% of their area at a density of 2.0 or more were identified by the computer. Whenever possible, these spots were cut out of the original gel and counted directly in scintillation cocktail (Loh and Gainer, 1975).

The ratios of intensities of spots in experimental versus control gels were calculated directly from the optical density data and then averaged over the gel runs from a given time point. The ratios for saturated spots, counted directly by scintillation counting, were normalized to the total amount of TCA-precipitable radioactivity loaded onto each gel. These data were then averaged for each spot and reported here as the sample mean ± the standard error of the mean.

Determination of the variability inherent in the gel and measurement technique

The sequence of operations involved in two-dimensional gel electrophoresis offers numerous opportunities for experimental variability (Wilson et al., 1977; Bossinger et al., 1979). We dealt with most of these by processing all of our experimental and associated control samples in parallel. In addition, we quantitatively compared labeled proteins in three pairs of gels run on normal right and left optic nerves harvested 18 hr after isotope injections from the same set of frogs (10 frogs for each separate pair of gels). For this comparison, we chose the same proteins as were compared between regenerating and normal nerves for the same 18-hr time point. The mean and variance of these data were used as a standard against which the significance of the change induced by regeneration could be tested.

Distinguishing transported proteins from those locally synthesized

In order to distinguish axonally transported proteins from those locally synthesized by support cells, we compared two-dimensional gels containing labeled proteins from normal distal segments of optic nerves harvested 4 and 18 hr after intraocular isotope injections to similar segments from the contralateral nerve, which had been acutely crushed *at the orbit* 5 to 30 min prior to isotope injection into its own eye. See Szaro et al. (1984) for details and rationale concerning this paradigm.

Results

Regrowth of the optic nerve fibers into the tectum following surgical interruptions of the fibers

Three independent methods were used to confirm that the scratch lesions along the tectal borders successfully transect the optic fibers as they enter the optic lobe, and to chart the initial phases of optic fiber regeneration into the deafferented tectum.

Liquid scintillation counting of tissue sections. Liquid scintillation counting of serial sections through the tectum demonstrated the effectiveness of the lesion and provided an overview of early kinetics of fiber regrowth into the tectum (Fig. 2). Like the scratch lesion itself, the method makes use of the fact that optic fibers approach the tectum in *Xenopus* along its anterior and lateral margins, and course over the tectal surface in a predominantly rostral-to-caudal direction. Thus, we could follow grossly the regrowth of fibers by counting transverse brain sections of intraocularly injected [^{3}H]proline-labeled animals in scintillation cocktail. One eye of each frog was injected at 30 min (0 days), 3 days, 6 days, 10 days, and 13 days after surgery, and fixed 1 day later. The amount of radioactivity in each slice served to indicate the presence of labeled regrowing fibers at each respective rostrocaudal level in the tectum. In the unoperated control animals, labeled fibers were detected through the diencephalon, across the tectal surface, and as far as the caudal edge of the tectum. In frogs injected 30 min after surgery (0 days), the counts in the tectum dropped sharply to background levels and remained there for as long as 3 days. By 6 days, a buildup of label was noted at the lesion site and had begun to cross over into the tectum. This caudal spread over the tectum became more pronounced at 10 days and appeared to cover the full rostrocaudal extent of the tectum between 2 and 3 weeks (Fig. 2). The data suggest (*i*) that the scratch lesion of the tectal borders acutely severs the optic fibers entering the tectum, (*ii*) that a lag period of from 3 to 6 days ensues the lesion, during which little regrowth of optic fibers into the tectum was observed, and (*iii*) that thereafter, fibers gradually regrow, from rostral to caudal, covering the tectal surface over the next week or two.

Light microscopic autoradiography. The effectiveness of optic fiber transections and initial phases of the regrowth of severed optic axons into the tectum was also evaluated by light microscope autoradiography. Figure 3 shows autoradiographs of paraffin sections through the tectum in frogs whose optic nerves had been labeled with intraocularly injected [^{3}H]proline 18 hr prior to fixation. Figure 3, *A* and *B*, shows light- and darkfield photomicrographs of a parasagittal section through the tectum of a normal juvenile frog with an intact visual pathway. Exposed silver grains, demonstrating the presence of optic fibers, filled the tectal optic layers and extended the entire rostrocaudal length of the tectum. Fig. 3, *C* and *D*, shows bright- and darkfield images of a similar animal injected with [^{3}H]proline within 30 min of optic fiber transection via a scratch lesion at the rostral and lateral tectal margins. In contrast to the normal

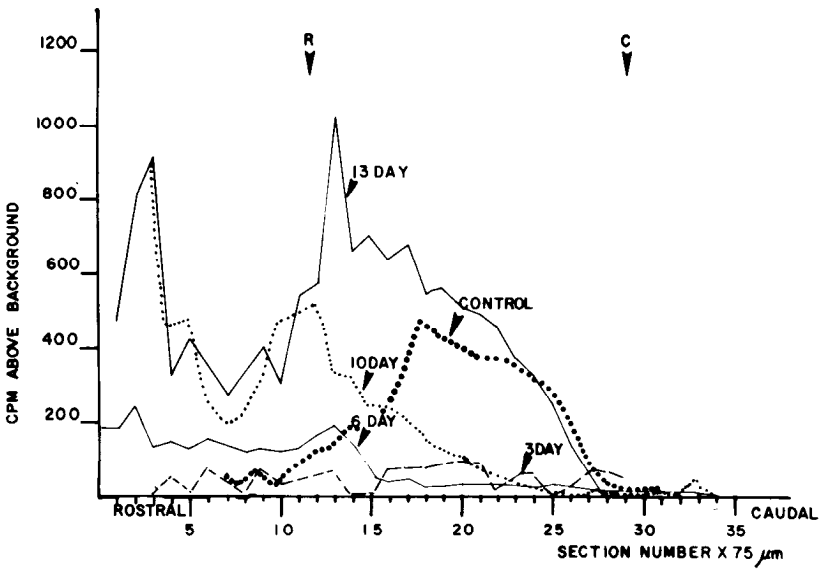

Figure 2. Data from liquid scintillation counting of representative solubilized serial (transverse) sections through the optic tectum at various intervals following the tectal scratch lesion described in the text and in Figure 1. Sections were taken through the complete rostrocaudal extent of the tectum, and included its neighboring rostral and caudal tissue; the approximate rostral and caudal margins (±3 sections) of the tectum appear in the *numbered sections* shown by the *arrows*. The data show that counts are abolished acutely following the lesion, reappear first in the rostal tectum by 6 days, and are detected over the full rostrocaudal extent of the tectum by 13 days. Each section was 75 μm thick.

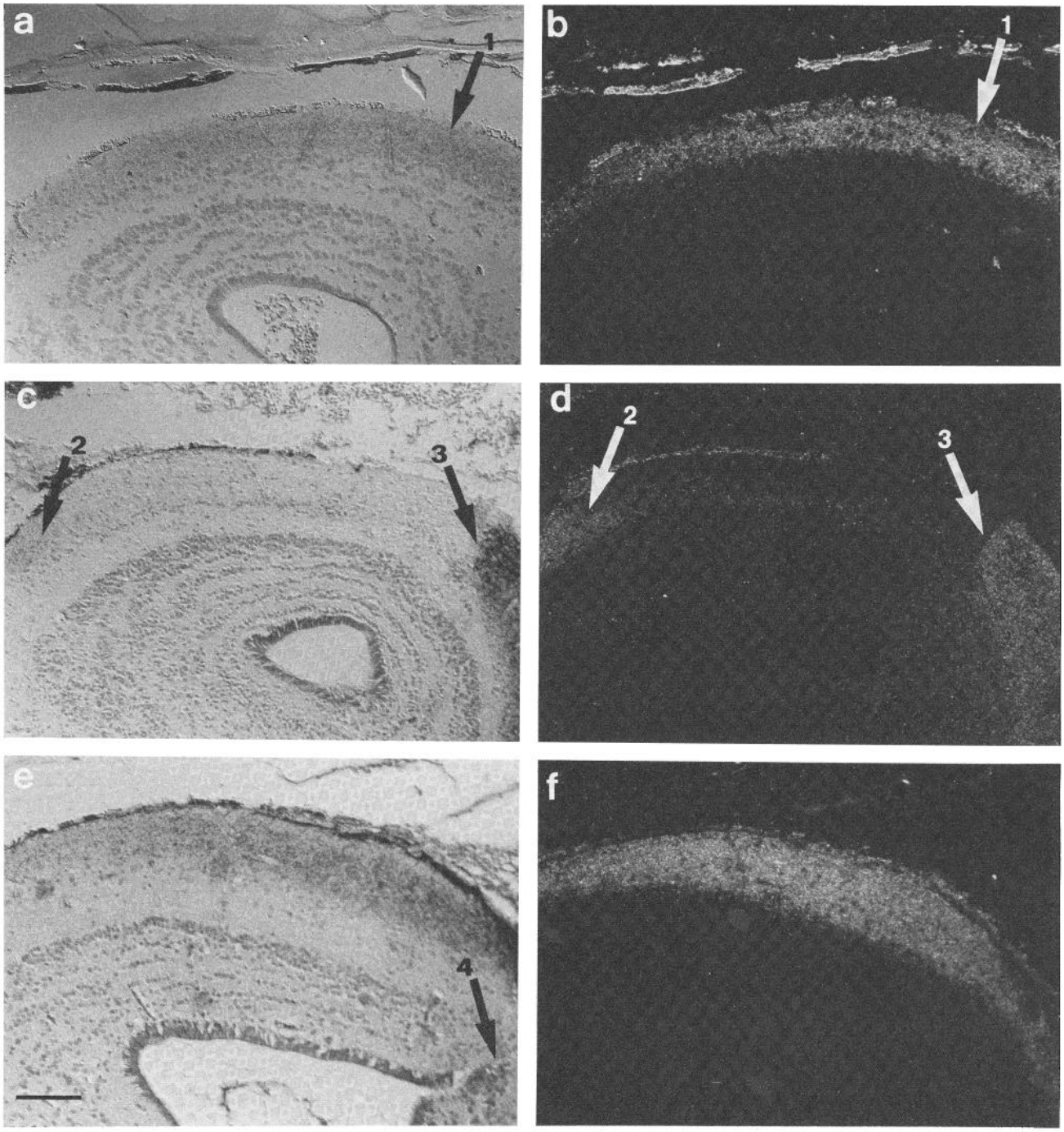

Figure 3. Bright- (*a*) and darkfield (*b*) views of a parasagittal section of a [^{3}H]proline-labeled normal optic tectum. This animal was fixed 22 hr after an intraocular injection of [^{3}H]proline. *Arrow 1* in the anterior region of the tectum highlights the silver grains exposed by the intact, labeled optic fibers in the superficial layers of the optic tectum. Rostral is to the *right*. *c* and *d*, bright- and darkfield autoradiographs of the optic tectum of a frog whose optic nerve had been severed 30 min prior to the injection of [^{3}H]proline into the eye. Note the buildup of grains at the site of the lesion (*3*), and the paucity of grains more posterior to the lesion, except for a small region at *2* in the caudal region of the tectum where a few fibers had been spared. *e* and *f*, bright- and darkfield autoradiographs of the optic tectum of a frog whose optic nerve had been severed 2 weeks prior to the injection of label into the eye. The optic fibers in this section taken from the lateral region of the tectum had regrown over much of the tectal surface. In sections taken more medially, the regrowing axons had completely covered the tectum. *Arrow 4* highlights the lesion, detectable here as a slight fault in the tectum. *Bar* equals 100 μm.

animal, the lesioned frog showed a large buildup of label at the site of transection. The wound itself is visible as a slight fault through the tectum. Caudal to the lesion, the optic tectum was generally devoid of significant label except for occasional patches of residual label contained in the few fibers surviving the surgery (see *arrow* at the back of the tectum in Fig. 3*C*). Fig. 3, *E* and *F*, shows bright- and darkfield autogradiographs of an animal injected 2 weeks after surgery. Note the fault in the tectum produced by the surgery (*arrow 4*). Caudal to the wound, the optic layers of the tectum showed evidence of regrowing optic fibers extending one-half to two-thirds of the distance across the tectum in medial sections (not shown) to nearly the full length of the tectum in sections taken more laterally (3*E* and 3*F*). The autoradiographic data thus confirmed that our lesions achieved the strategic aim of severing the distal tips of the optic fibers some distance away from the region to be harvested for biochemistry, and that optic fiber regrowth was vigorously underway at 2 weeks following the lesion.

Electrophysiological mapping of the visual field projection to the lesioned tectum. Electrophysiological mapping of the visual field projection to the lesioned tectum was the last of the three methods used to evaluate the time course of optic fiber regeneration into the tectum. The extent of topographic organization in the retinotectal projection at each stage of regeneration could be ascertained from the visual field ordering evident within these electrophysiological maps. It may be useful to note the four different classes of visually evoked responses detected in these regenerating animals, since the first three are not routinely observed in frogs with intact retinotectal innervation. First, certain tectal points were unresponsive to gross optical stimulation (i.e., turning the room light off and on), but could be made to respond to gross auditory or tactile stimuli provided by clapping or blowing a thin stream of air onto the animal through a Pasteur pipette. These auditory and somatosensory responses provide an important internal control, indicating that the tectum was alive and capable of evoked responses despite the lesion, and give credence to the negative finding of no visual responses as indicative of the absence of optic fibers. A second class of tectal points responded weakly to gross visual stimulation, but not to localized spots of light. A third class of tectal points responded to gross visual stimulation, and could be driven by moving spots of light. However, unlike the intact tectum, these points did not respond to stationary or flashing spots, and their visual receptive fields were abnormally large, often covering 25% to 50% of the visual field. Since our electrodes favor detection of signals from the high current density of terminal arborizations of optic fibers (George and Marks, 1974), it is likely that these weak and diffuse responses were recorded either from (diffusely innervated) tectal cells or from regrowing optic fibers which had not yet formed a mature terminal arbor. The fourth class of tectal points gave normal responses, both to moving spots and to stationary spots of light, from circumscribed receptive field areas in the visual field, as seen in intact frogs (Maturana et al., 1959; Gaze, 1970).

Figure 4*A* shows a map of the visual field onto the optic tectum of a normal frog. This tectum gave only normal (of the fourth class described above) responses. Moreover, the projection of the visual field over the tectal surface shows a smooth and continuous *topographic* order. As the electrode is moved from rostral to caudal on the tectum, the responses are evoked from successively more temporal visual field, while progressive recordings from lateral to medial on the tectum detect responses evoked from successively more *superior* positions in visual field. Figure 4*B* shows the map of an animal recorded 3 days after surgery. Most of the tectum was unresponsive to visual stimuli except for a few loci at the rostral edge of the tectum, which gave normal responses. These latter responses were probably derived from optic fibers spared by the surgery. Figure 4*C* shows the size and location in the visual field of the receptive fields and the distribution of responsive recording loci in the tectum of a frog recorded 2 weeks after surgery. All four classes of responses were represented on this tectum. In this animal, unresponsive points were distributed over the caudal regions of the tectum. Points which responsed to visual stimulation were more rostral. The overall disorganization is highlighted in Figure 4*D* in which the centers of the visual receptive fields have been plotted, and the nasal-temporal, superior-inferior axes of polarity have been indicated. By 9 weeks, the projections were grossly ordered (Fig. 4*E*) with a few abnormal points, and by 18 weeks the visuotectal projections were essentially normal in response characteristics and topographic order (Fig. 4*E*).

In summary, the tectal slice counting and the autoradiographic and recording data demonstrated acute deafferentation of the tectum following a scratch lesion at the margins of the tectal lobe. These data further indicated that, by 2 weeks after the lesion, most of the tectum had been invaded by growing optic fibers, but that retinotopic order had not yet been reestablished. We, therefore, selected 2 weeks postlesion as the focus for biochemical studies on optic nerves engaged in active stages of regrowth. Hereafter, in all biochemical observations on regenerating nerves, the interval between lesion and isotope injection was 2 weeks. For the sake of clarity, the usage "time points" is hereafter confined to the intervals between isotope injection and dissection of the tissue for biochemistry.

Changes in optic nerve proteins during regeneration

The general features of axonal transport (e.g., transport rates and the changing composition of proteins within each phase of transport), in normal nerves as revealed by [^{35}S]methionine labeling, have been presented elsewhere (Szaro et al., 1984). In brief, the three fastest phases of transport appear in this short nerve between 1 and 4 hr after injection. Few, if any, proteins present in the nerve at 1 hr are cleared from the nerve by 4 hr. Consequently, 2 to 4 hr following isotope injection, the nerve contains a comprehensive sample of the proteins contained in all three of the fastest phases of transport. More slowly transported proteins, comparable to phase IV of Willard et al. (1974), and SCb phase of Lasek (1980), were observed in *Xenopus* optic nerve at 18 hr, following isotope injection, while proteins moving in the slowest phase of transport were detected in the nerve 5 to 9 days after injection (Szaro et al., 1984). In order to sample each phase of transport, we compared regenerating optic nerves to normal optic nerves within the same animal, pooling 10 nerves in each sample, and harvested the nerves at 2 to 4 hr, 18 hr, and 7 days following isotope injection.

Quantitative changes in bulk amounts of labeled optic nerve material during regeneration. The amount of labeled TCA-precipitable material was measured in regenerating and normal optic nerves at the various time points after [^{35}S]methionine injections into the eyes. Each sample represented a 5% aliquot from a sample harvested from 10 animals. In order to normalize the amount of radioactivity between animals, the amount of radioactivity in the nerve was represented as a percentage of the total radioactivity present in the eye, nerve, and corresponding tectum (e.g., right eye, right nerve, and left tectum) at the time of harvest. The results in Table I represent the ratio of normalized radioactive material present in regenerating nerves relative to normal nerves harvested from the same animals at the same time. In order to compare the differences attributable to regeneration to the differences one might expect as a result of experimental procedure, we compared right to left optic nerves in three sets of normal animals (10 animals each) harvested 18 hr after isotope injection. The sample mean ± SE for this "normal versus normal" comparison was 1.04 ± 0.05, indicating that our experimental procedure was highly repro-

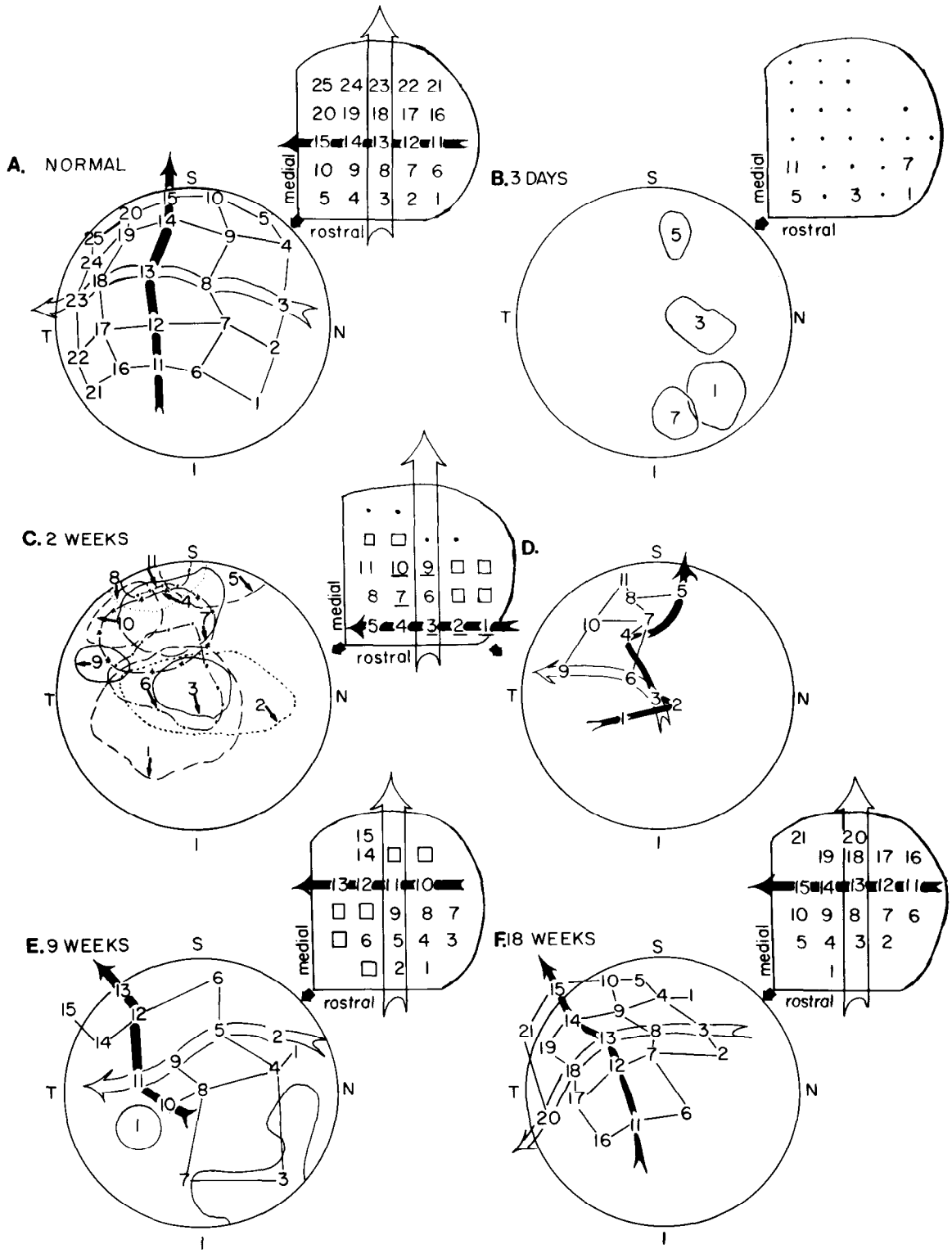

Figure 4. Representative visuotectal projections assayed by electrophysiological mapping showing projections from a normal intact frog (*A*) and frogs at 3 days (*B*), 2 weeks (*C* and *D*), 9 weeks (*E*), and 18 weeks (*F*) following a tectal scratch lesion. The *numbers* on the tectal schematics show electrode-recording positions at which a response to a small spot of light in the correspondingly numbered effective stimulus area of visual space was detected. *Underlined numbers* in *C* represent those regions of the tectum which responded only to moving stimuli rather than to discrete stationary spots of light. These moving responses presumably represent either responses recorded postsynaptically from tectal cells or from overlapping presynaptic axons which had not yet formed a mature dense arbor. The *boxes* represent those regions of the tectum which could be weakly stimulated by gross changes in room illumination but which responded too weakly to spots of light for us to localize the stimuli for these responses in visual space. The *dots* represent tectal recording loci which were unresponsive to visual stimulation (see text). *A* shows a normal visuotectal projection. *B* shows a projection of a frog 3 days after a tectal scratch lesion. The tectum was unresponsive to visual stimulation except for a few loci, presumably spared by the surgery. *C* shows a partially recovered projection 2 weeks after injuring the nerve. *D* shows the same projection as in *C*, but with the centers of the effective stimulus areas indicated by *numbers* in order to illustrate the degree of retinotopy of the retinotectal map. *E* shows a projection at 9 weeks, mostly recovered except for a number of loci with weak responses, an abnormally shaped receptive field at locus 3, and one recording locus (*1*) which received input from two areas of visual field. *F* shows a fully regenerated retinotectal projection 18 weeks after injury.

TABLE I

Ratios of normalized, labeled material relative to that from control nerves

Phases of Transport	Number of Pooled Samples[a]	TCA-precipitable Proteins Regenerating/ Normal[b]
Fast (4 hr)	5	2.49 ± 0.33
Intermediate (18 hr)	3	2.53 ± 0.25
Slow (7 days)	2	1.75 ± 0.40

[a] Each sample consists of a pooling of tissue from 10 separate animals.

[b] Ratios are expressed as: R = (% total TCA-precipitable cpm in the regenerating (right) nerve)/(% total TCA-precipitable cpm in the normal (left) nerve) and % total TCA-precipitable cpm in the nerve = (cpm in nerve)/(cpm in nerve + eye + tectum). Ratios are expressed as the sample mean ± SE ($S/\sqrt{n}$).

ducible. Similar statistical tests confirmed that the increase in bulk TCA-precipitable counts, in regenerating versus normal optic nerves, was significant in the fast (2 to 4 hr) and intermediate (18 hr) phases of transport.[4]

Changes in individual labeled proteins during regeneration

The two-dimensional gels of regenerating nerves and normal nerves at each phase of transport were initially compared visually as described in "Materials and Methods." This comparison led to two observations: (*1*) virtually no proteins in regenerating nerves were undetectable in normal nerves; and (*2*) while the intensities of most proteins appeared relatively unchanged, a subset of proteins was observed to increase consistently during regeneration while another subset was observed to consistently decrease.

An attempt was made to correlate spots at one time point with homologous spots of similar isoelectric point and molecular weight at other time points. A total of approximately 630 spots were consistently resolvable in the gels containing proteins harvested at the three time points (120 at 4 hr, 250 at 18 hr, and 260 at 7 days). Based on a rough estimate of the overlap between gels, we estimate that these 630 spots represented some 250 to 300 separate proteins. Of these, 89 were compared quantitatively as described in "Materials and Methods." These 89 included all of the proteins which, on the basis of visual examination, appeared to change consistently between regenerating and normal nerves, and also a large sample of proteins which did not. The remaining 161 to 211 proteins, which we did not measure, were proteins which did not appear, on the basis of visual examination, to differ consistently between regenerating and normal nerve gels.

Figures 5, 6, and 7 show fluorographs made from two-dimensional gels containing proteins from regenerating and normal optic nerves harvested 4 hr, 18 hr, and 7 days after intraocular injection of isotope. The measured spots are designated by integers. Five pairs of gels were included in the analysis of fast (2 to 4 hr) transported proteins, three pairs at 18 hr, and two pairs at 7 days.

In order to estimate the variability attributable to the experimental method itself, spots in three pairs of intact nerve samples (10 animals each) harvested 18 hr after isotope injection were compared as described in "Materials and Methods." A total of 208 measurements were made of these control spots distributed over the three pairs of gels. The sample mean (± SE) for this "normal versus normal" comparison was 1.13 ± 0.03 ($S = 0.49$, $n = 208$).

This statistical variability in the normal versus normal sample was used to test the significance of the ratios obtained for single specific proteins. In general, the variances in measurements for most of the specific proteins (as obtained by the F statistic) were not significantly different from the normal versus normal sample at $p = 0.05$. Consequently, for these, the standard two-tailed t test for comparing two samples with equal variances was used to measure the differences in means ($\mu_1 - \mu_2$, $p = 0.01$ and 0.05) of each specific experimental protein from the mean obtained from the normal versus normal nerve comparisons (Blalock, 1972). In the few cases where the variances were not equivalent, we used the corrected degrees of freedom calculation shown in Footnote 4.

Table II lists those spots and their respective time points, molecular weights, and ratios (regenerating/normal), for those pairs of proteins which differed significantly ($p < 0.05$) during regeneration from the normal versus normal sample. We have listed separately those spots significantly different at the 99% confidence level ($p \leq 0.01$) from those significant at the 95% confidence level ($0.01 < p \leq 0.05$). Six spots were found to increase at the 99% level. They were: *1*, 240; *3*, 135; *11*, 65, *12*, 58; *17*, 54; and *23*, 56 kilodaltons (kDa). Four more increased at the 95% level. They were: *8*, 64; *31*; 31; *34*, 26; and *62*, 58 kDa. Of these, four were in fast transport (*1, 3, 11,* and *23*); the rest were found in the nerve at 18 hr, while two were also found at 7 days (*12* and *17*). Three spots decreased at the 99% level: *41*, 15; *42*, 17; and *66*, 29 kDa. Spot *66* was found to be significant at all time points, while spot *42* was significant both at 18 hr and 7 days, and spot *42* at 7 days only. Six additional spots decreased at the 95% confidence level; *43*, 15 kDa, 18 hr; *53*, 49 kDa, 18 hr; *59*, 72 kDa, 7 days; *60*, 56 kDa, 7 days; *64*, 42 kDa, 18 hr; and *75*, 22 kDa, fast and 18 hr. Seventy other spots quantitated were found not to change significantly, by these criteria, during regeneration.

Identification of transported proteins and locally labeled proteins. Previously, we have distinguished transported proteins from those synthesized locally by glia and other support cells of the nerve sheath, based on a comparison of gels containing labeled proteins from distal segments of normal optic nerves and distal segments of optic nerves crushed 5 to 30 min before isotope injection (Szaro et al., 1984). Figures 8 and 9 show two-

[4] Comparisons of the variances between experimental ratios and the normal versus normal ratios were made using the F statistic ($F = S_H^2/S_L^2$, where S_H^2 is the experimental sample variance, and S_L^2 is the normal versus normal sample variance). This comparison showed that the biological variability in the experimental samples was significantly greater ($0.01 < p < 0.05$) than that expected from the experimental procedure alone. Perhaps some animals responded more than others to the trauma, either as a result of intrinsic animal variability or as a result of imperceptible variations in surgical techniques.

In order to estimate the significance of the increases seen in bulk TCA-precipitable material, we calculated the statistic $\mu_1 - \mu_2$, for $p = 0.05$. Here, $\mu_1 - \mu_2$ represents the difference in means between experimental samples and control samples at the 95% confidence level. The estimated error in the difference $\mu_1 - \mu_2$ at the same 95% confidence level was found using the equation:

$$\mu_1 - \mu_2 = |\chi_1 - \chi_2| \pm t_c \times S(1/n_1 + 1/n_2)^{1/2}$$

Here, χ_1 and χ_2 are the experimental and control sample means, respectively, and n_1 and n_2 are the number of measurements made in each sample. S is the sample covariance and t_c is the estimated two-tailed Student's t statistic, at the 95% confidence level, found using the method of the corrected degrees of freedom for samples with unequal variances (Blalock, 1972).

On the basis of these calculations, the increases seen for fast transport (which increased by a factor of 2.49 ± 0.33 (SE)), and for phase IV at 18 hr (2.53 ± 0.25) were significant ($0.01 < p < 0.05$). The increase for the slowest phase of transport at 7 days was less (1.75 ± 0.40), and under the criteria outlined above was not as significantly different ($0.2 < p < 0.3$) as the ratios found for the samples at 2 to 4 and 18 hr.

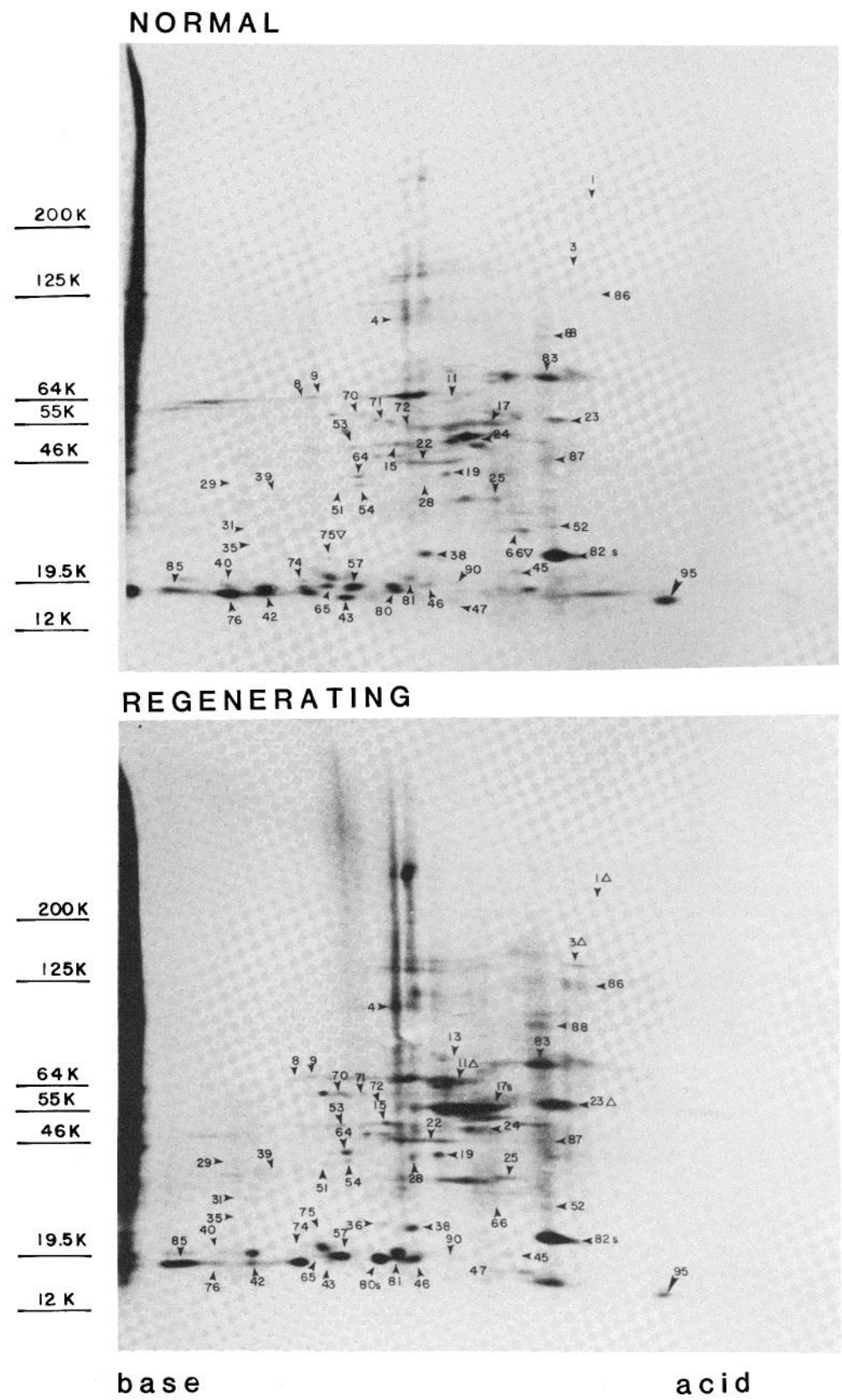

Figure 5. A visual comparison of fluorographs made from two-dimensional gels run on labeled optic nerves harvested 4 hr after intraocular injection of [^{35}S]methionine (fast transport). The top gel was run on material from 10 labeled intact nerves, while the bottom gel was run on material from 10 optic nerves from animals injected with radioactive methionine 2 weeks after an optic nerve lesion. The x-ray film for each gel was exposed for 700,000 cpm-days. Molecular masses were obtained by comparing the migrations of prominent Coomassie blue-stained spots on the left side of the gel and within the gel itself to prominent Coomassie blue bands contained in one-dimensional gels run on unlabeled optic nerves (see Szaro et al., 1984 for details). The acid end of the gel was at approximately pH 3.5; the basic end was at pH 8.5. The *numbered spots* represent those spots selected for quantitative measurements, either by computer or by direct scintillation counting of the physically excised spots (marked by *s* next to the *number*; see text for details). This figure shows that, while most spots remained relatively constant, a select few either decreased or increased during regeneration. Those spots which increased during regeneration are designated by Δ next to the appropriate number on the gel run on regenerating nerves (*bottom panel*). Those spots which were greater in gels run on normal nerves are designated by ∇ in the *top panel*. *K*, kilodalton.

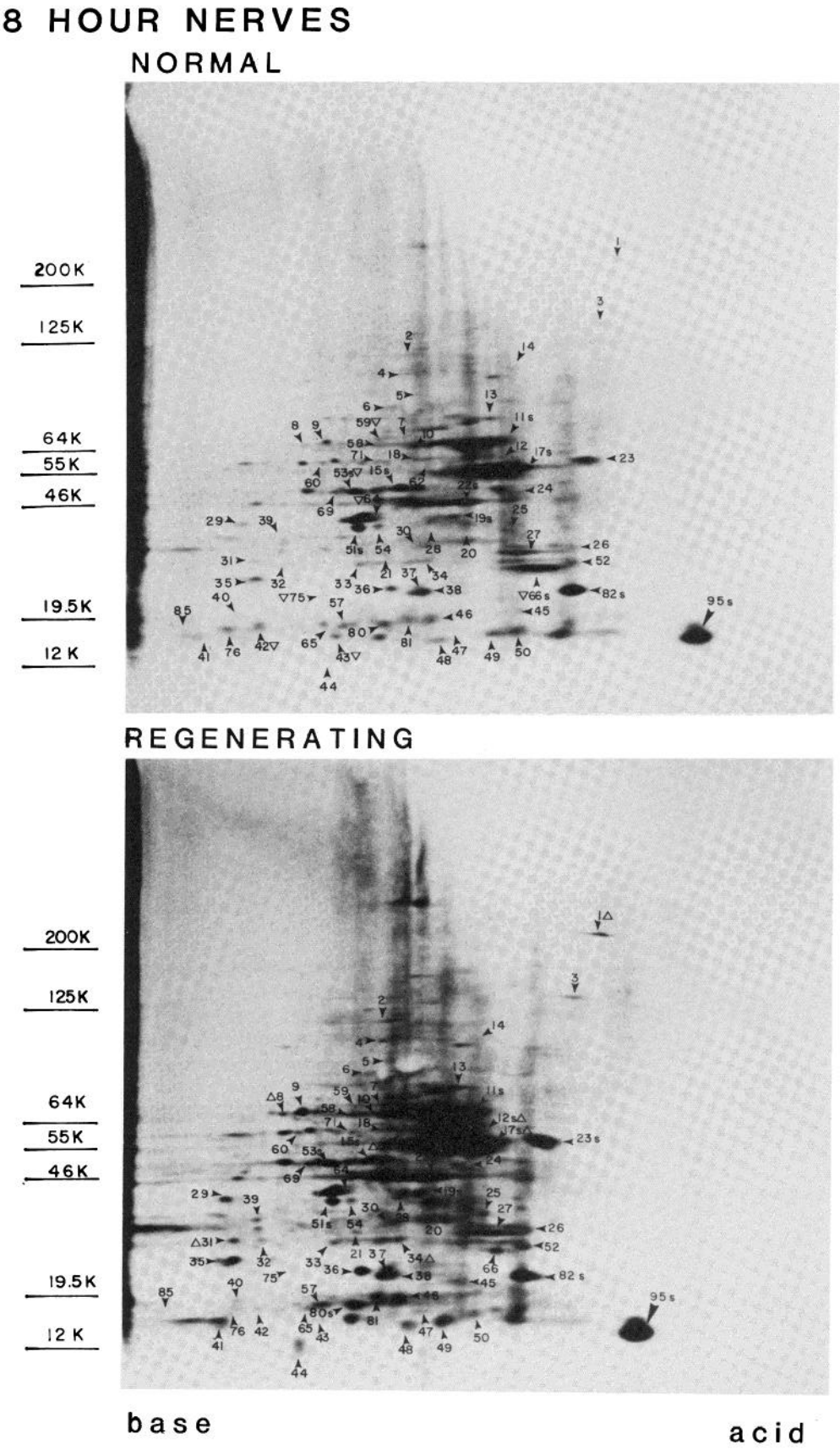

Figure 6. A visual comparison of fluorographs made from two-dimensional gels of electrophoretically separated labeled proteins from 10 normal (*top*) and 10 regenerating (*bottom*) optic nerves harvested from the same animals 18 hr after intraocular injection of [^{35}S] methionine. See the legend for Figure 5 for further details concerning the gel parameters. *K*, kilodalton.

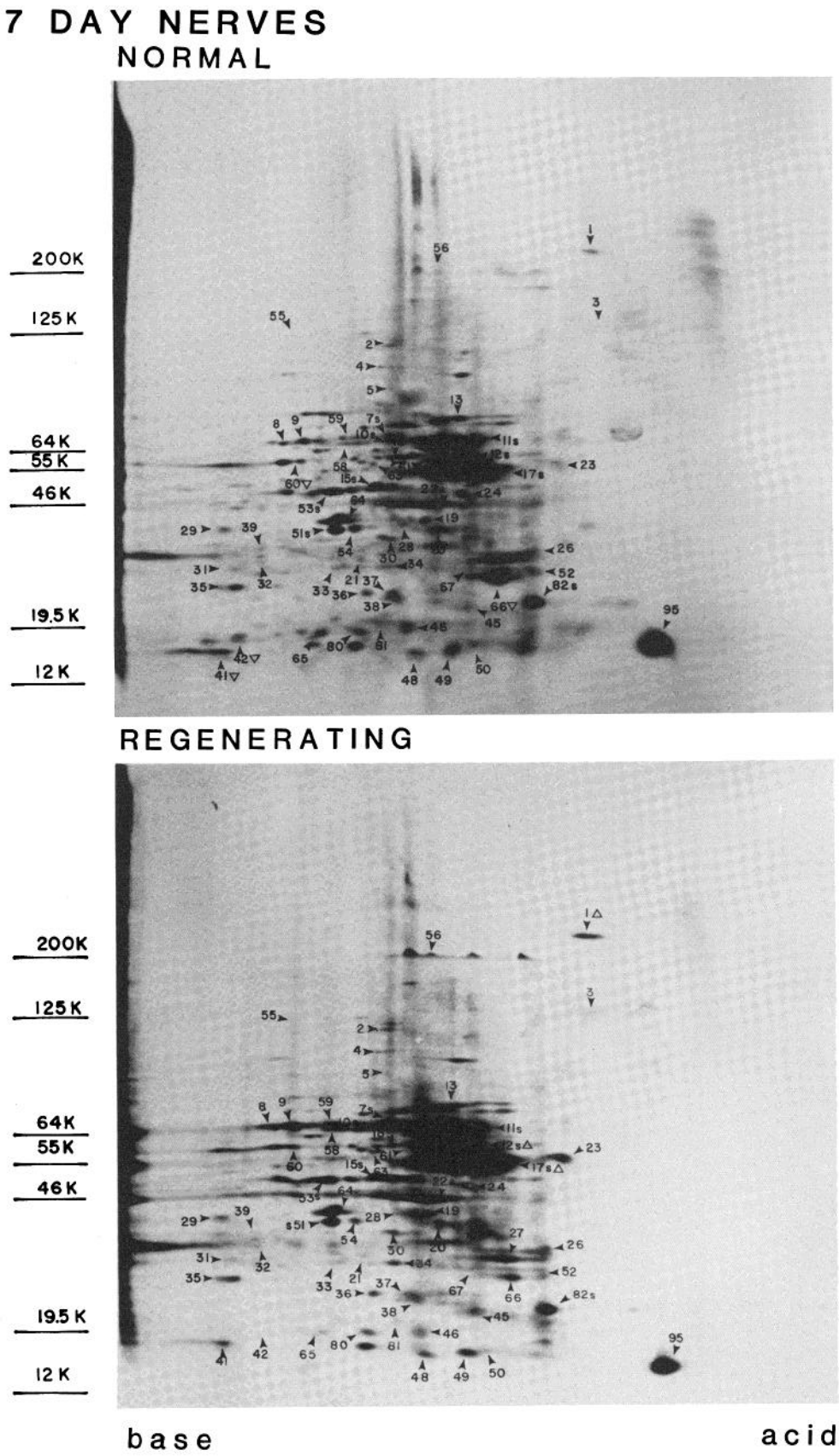

Figure 7. A visual comparison of fluorographs made from two-dimensional gels of electrophoretically separated labeled proteins from 10 normal (*top*) and 10 regenerating (*bottom*) optic nerves harvested from the same animals, 7 days after intraocular injection of [^{35}S]methionine. See the legend for Figure 5 and the text for further details concerning the gel parameters. *K*, kilodalton.

TABLE II

Axonally transported proteins which changed significantly during regeneration

The ratios between regenerating and normal nerves are represented as the sample mean ± the standard error of the mean. The differences between the experimental ratio sample mean and the normal versus normal ratio mean is expressed as a 95% confidence interval. Ratios expressed in parentheses indicate that the same protein is significant at the $0.01 \leq p \leq 0.05$ level at the indicated time point.

Spot	Mass	Ratio	Transport Phase[a]	$\mu_1 - \mu_2$	n
	kDa				
A. Increases					
$p < 0.01$					
1	240	2.08 ± 0.11	Fast	0.95 ± 0.69	2
		(2.71 ± 0.62)	Intermediate-slow	(1.58 ± 1.24)	3
3	135	1.87 ± 0.25	Fast	0.74 ± 0.44	5
11	65	1.78 ± 0.19	Fast	0.65 ± 0.49	5
12	58	1.78 ± 0.46	Intermediate-slow	0.65 ± 0.57	3
17	54	1.92 ± 0.23	Intermediate-slow	0.79 ± 0.56	3
23	56	2.07 ± 0.21	Fast	0.94 ± 0.44	5
$0.01 \leq p \leq 0.05$					
8	64	1.77 ± 0.17	Intermediate	0.64 ± 0.56	3
31	31	1.80 ± 0.32	Intermediate	0.67 ± 0.57	3
34	26	1.80 ± 0.36	Intermediate	0.67 ± 0.57	3
62[b]	58	2.54 ± 1.10	Intermediate	1.41 ± 1.31	3
B. Decreases					
$p \leq 0.01$					
41	15	0.21 ± 0.8	Slow	0.92 ± 0.69	2
42	17	0.18 ± 0.01	Slow-intermedi-	0.95 ± 0.69	2
		(0.53 ± 0.21)	ate	(0.60 ±0.56)	3
66	29	0.19 ± 0.03	Intermediate-slow	0.94 ± 0.56	3
		(0.61± 0.11)	Fast	(0.52 ± 0.44)	5
$0.01 \leq p \leq 0.05$					
43[b]	15	0.33 ± 0.09	Intermediate	0.80 ± 0.69	2
53	49	0.42 ± 0.04	Intermediate	0.71 ± 0.69	2
59[b]	72	0.42 ± 0.06	Intermediate	0.71 ± 0.70	2
60	56	0.42 ± 0.06	Slow	0.71 ± 0.70	2
64	42	0.40 ± 0.04	Intermediate	0.73 ± 0.69	2
75[b]	22	0.38 ± 0.15	Fast-intermediate	0.75 ± 0.69	2
		0.45 ± 0.17		0.68 ± 0.56	3

[a] Fast, intermediate, and slow phases of transport = proteins seen in the nerve 4 hr, 18 hr, and 7 days after intraocular injection of isotope in the eye, respectively.

[b] These proteins do not appear to be axonally transported as determined by crush control experiments (see text).

dimensional gel comparisons on such nerve segments harvested 4 and 18 hr after isotope injection. Those proteins present in normal nerves, but not in acutely crushed nerves, were deemed to be axonally transported. On this basis, we found that of those spots which changed significantly during regeneration, 15 of the 19 were transported. A complete catalog of transported proteins versus locally synthesized nerve proteins is beyond the scope of the present paper, and we confine our present comments to the 89 spots which had been measured quantitatively in regenerating versus normal nerves.

Among the transported proteins, those which increased during regeneration were: *1,* 240; *3,* 135; *12,* 58; *23,* 56; *8,* 64; *31,* 31; and *34,* 26 kDa. Two additional proteins which showed increases during regeneration (*11,* 65, and *17,* 54 kDa) were not entirely absent from the gels of crushed nerves, but showed marked decreases. Perhaps these proteins represent transported neuronal proteins which are nonetheless present in glia as well. Among those proteins which showed decreases during regeneration and were found to be transported were: *41,* 15; *42,* 17; *66,* 29; *53,* 49; *60,* 56; and *64,* 42 kDa. Of the four proteins which changed significantly during regeneration, and which could not be classified as transported, three of them (*62, 59,* and *75*) were too faint to be seen in the normal controls, perhaps because these gels were run only on the distal segments of the nerves. The remaining protein (*43*) which decreased relative to the rest of the proteins during regeneration, appeared to be a glial protein.

We have also examined, on two-dimensional gels, labeled proteins in the optic tectum at intervals following [^{35}S]methionine into the eye (data not shown). Labeled proteins corresponding in electrophoretic mobility to many of the transported proteins which increased significantly duiring regeneration were found in the tectum. They were *1,* 240; *3,* 135, *12,* 58; *17,* 54; *23,* 56; *31,* 31; and *34,* 26 kDa. These proteins were seen in the tectum at a time later than seen in the nerve and probably represent transported proteins which reached the tectum.

Discussion

Before discussing our results, it may be useful to summarize the aims and principal findings of the present study. We have examined labeled proteins in the optic nerve in juvenile *Xenopus* clawed frogs, at three time points (2 to 4 hr, 18 hr, and 5 to 9 days) following [^{35}S]methionine injection into the eye, with special reference to labeled proteins that differ between intact nerves and nerves whose distal axon tips had been transected at the margin of the tectal lobe 2 weeks prior to isotope administration. Three methods were used to demonstrate that this 2-week interval was sufficient for regrowth of the severed optic fibers into the tectum; one of these methods (electrophysiological mapping of the visual field projection) further established that the regenerating optic fibers, at 2 weeks of regrowth, had not yet sorted into their terminal retinotopic pattern. Two-dimensional gel separation of labeled nerve proteins revealed 250 to 300 identifiable proteins, 89 of which (including *all* spots which differed consistently upon direct comparison of regenerating versus normal nerves) were selected for quantitative treatment. Nineteen of these were shown to either increase or decrease significantly as a correlate of regeneration. By using a crush control developed elsewhere (Szaro et al., 1984), and by tracking the labeled proteins into the tectum over time, we were able to (*i*) show that 15 of these 19 proteins had been synthesized in the eye and axonally transported into the nerve and (*ii*) assign each to the rapid, intermediate or slow phase of transport.

Transection and regeneration of the optic nerve. The regeneration of crushed or transected nerve fibers is a complex process believed to involve activity in the nerve both proximal and distal to the site of axonal injury. Distally, the glia undergo a number of changes which include astrocyte proliferation as well as changes in the nerve sheath (Wolburg, 1981) thought to be important in clearing axonal debris and preparing a pathway for the regenerating axons. In previous work (Szaro et al., 1984), we found that if the optic nerve was crushed, support cells in the vicinity of the nerve crush showed transient changes in protein synthesis which can obscure the analysis of transported proteins down the regenerating axon. In addition, in some visual systems, the proximal stumps of severed optic nerve fibers themselves die back a considerable distance toward the cell bodies, though this process has not been observed to any significant degree in *Xenopus*. The salamander optic nerve provides an extreme example; following nerve crush, the retina itself may degenerate, and optic nerve regeneration may begin only after repopulation of the retinal ganglion cell layer from pigmented retinal epithelia (Stone and Zaur, 1940). For these reasons, it seemed prudent to trigger regeneration by transecting the optic fibers at the margins of the tectum to (*i*) harvest regeneration-associated proteins from an intact nerve conduit,

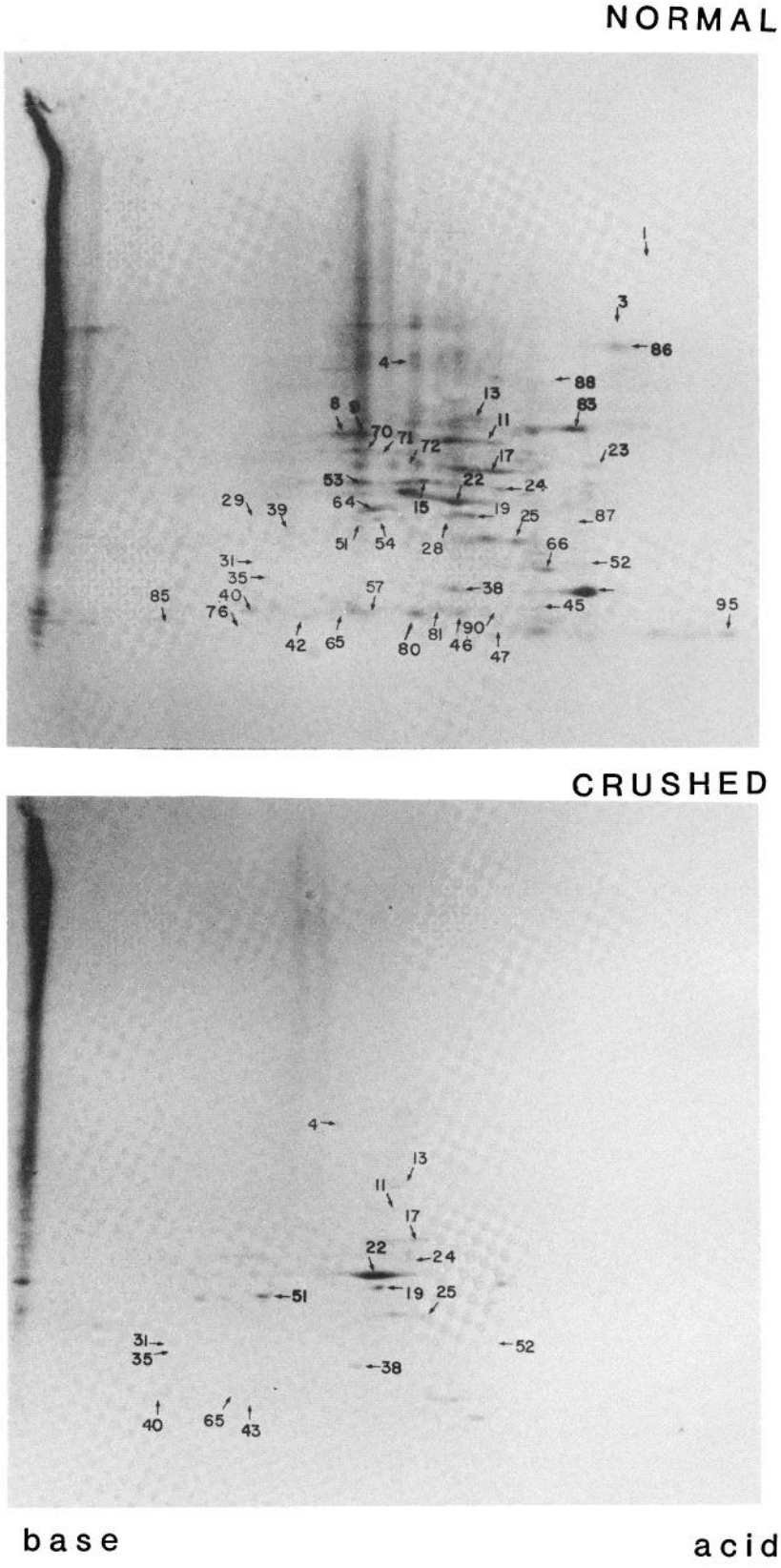

Figure 8. Two-dimensional gel fluorographs of labeled proteins from normal nerve segments extending from roughly one-third the distance from the eye to the chiasm up to the chiasm itself. The top gel contains proteins from 10 such nerve segments which were left intact (left optic nerves) and harvested 4 hr after the eyes had been injected with labeled methionine. The bottom gel contains 10 such labeled nerve segments from the same animal (right optic nerves), but which had been acutely crushed at the orbit 5 to 30 min prior to injection. The *numbered spots* in the top gel indicate those spots corresponding to spots which were measured in the regeneration studies. The *numbered spots* in the bottom gel show those spots which survived the acute crush procedure, and presumably represent proteins labeled by local synthesis rather than those which arrived in the nerve by axonal transport. The normal nerve fluorograph was made on X-AR film exposed for 455,000 cpm-days. The crushed nerve gel was exposed for the same number of days. The gel parameters are otherwise the same as in Figure 5.

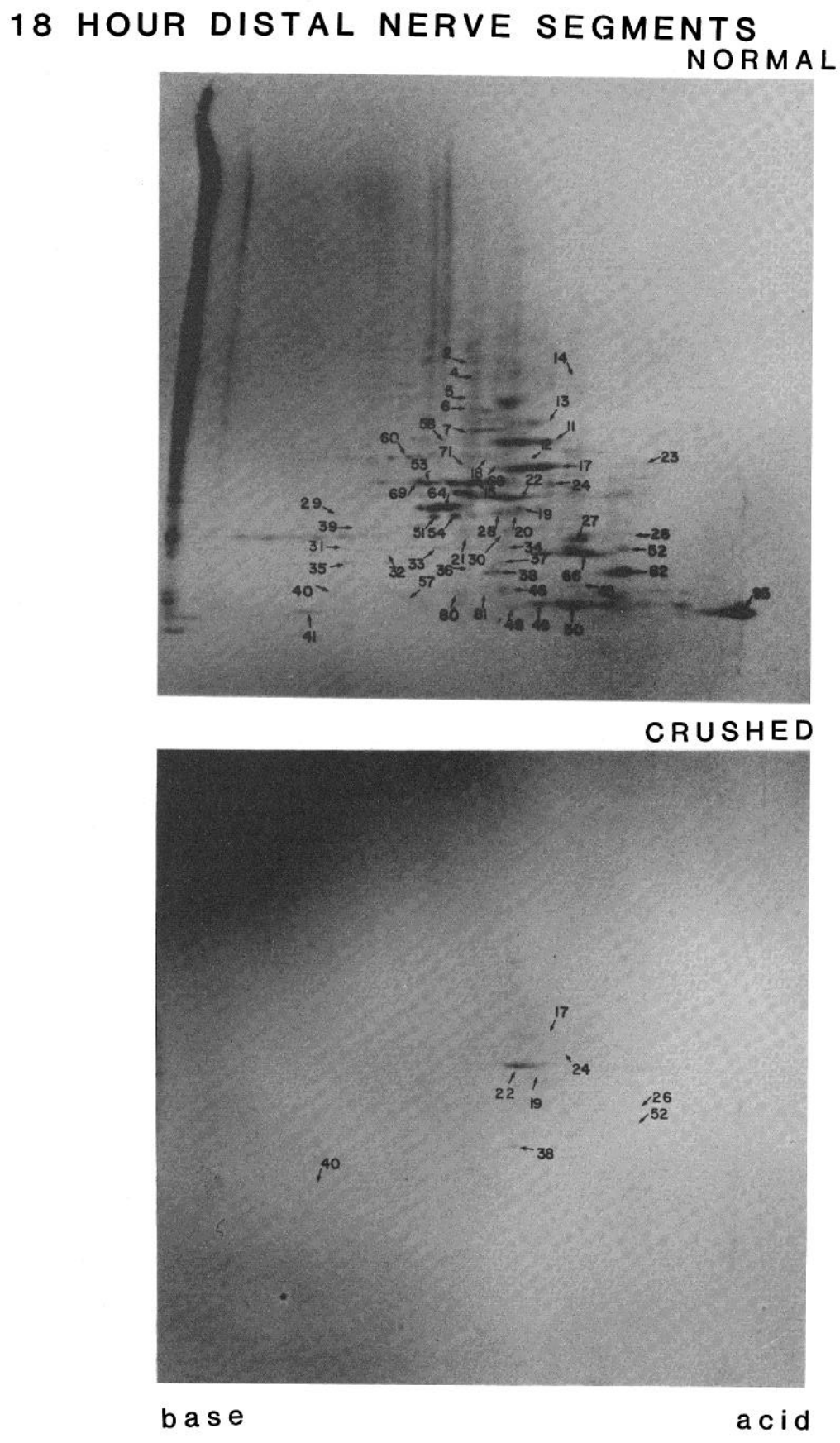

Figure 9. Two-dimensional gel fluorographs of labeled proteins from 10 normal and 10 acutely crushed nerve segments harvested from the left and right sides, respectively, of frogs whose eyes had been injected with [^{35}S]methionine 18 hr prior to dissection. The surgical methods and gel parameters were otherwise identical to those illustrated in Figure 8.

(*ii*) minimize the contribution of support cells to changes in the protein profiles seen when regenerating and normal nerves are compared, and (*iii*) provide reasonable evidence that the changes in nerve proteins are associated directly with the process of axonal regrowth rather than with the degenerative events that precede regrowth.

Regrowth of severed axons into the tectum was monitored by scintillation counting of tectal slices, by [^{3}H]proline tissue autoradiography and by electrophysiological mapping of the visual field onto the lesioned tectum. These data (Figs. 2, 3, and 4) confirmed that the tectal scratch lesions had in fact severed the optic fibers, and further showed few signs of optic nerve encroachment on to the tectum for the first 3 to 6 days after lesioning the nerve at the tectal margins. By 2 weeks, the optic fibers had regrown over much of the tectal surface, but had not yet reestablished their original retinotopic order. Coverage continues to increase as did the degree of retinotopy, so that by 18 weeks, essentially normal maps were observed (Szaro, 1982). These observations are consistent with other reports that nerve regeneration consists of an early phase of axon extension and reinnervation followed by a more protracted phase in which the fibers reestablish fine topographic order (Gaze and Jacobson, 1963; Gaze and Keating, 1969). Electron microscopy of tectal synaptic terminals during nerve regeneration (Ostberg and Norden, 1979), and observations on the reappearance of visually evoked responses in the indirect ipsilateral optic lobe projection (an indirect projection which requires transsynaptic communication between the regenerating optic fibers and the cells of their own contralateral tectum) suggest that regenerating optic fibers begin to make synapses soon after they arrive in the tectum (Keating and Gaze, 1970; Adamson and Grobstein, 1982). Consequently, our observations of changes in proteins 2 weeks into regeneration are likely to include not only proteins necessary for regrowth, but also may include proteins necessary for early phases of synaptogenesis. Because we have injured only the distal tips of the optic fibers, it is also possible that the contribution of structural proteins may be less in our experiments than in those paradigms which crush the nerve in the orbit, or at the chiasm, and harvest proteins from a nerve that itself has been rebuilt during the regeneration process.

Qualitative and quantitative changes in labeled optic nerve proteins during regeneration. At each phase of transport, sampled in the nerve at 2 to 4 hr, 18 hr, and 7 days postinjection, we found a 1.8- to 2.5-fold increase in the amount of labeled TCA-precipitable proteins in the nerve, even when the increased amounts in the nerve were normalized against the general increase in protein synthesis observed in the eye during regeneration. These differences were seen to be highly significant for the fast phase and the intermediate phase (18 hr) of transport ($0.01 \leq p \leq 0.05$), but less so for the slow phase ($0.2 < p < 0.3$; 7 days). Because these increases are expressed relative to the amount of newly labeled synthesis in the eye, which also increased dramatically during regeneration, they are likely to underestimate the absolute increases in transported materials which occur during regeneration. These absolute increases are difficult to measure precisely because amino acid pools may change significantly during protracted periods of regeneration.

Upon analysis of the axonally transported proteins during regeneration of the optic nerve, two-dimensional gel electrophoresis revealed increases in nine proteins by factors ranging from 1.77 ± 0.17 to 2.71 ± 0.62. An additional six proteins were decreased by factors ranging from 0.18 ± 0.01 to 0.61 ± 0.11 (see Table II). These proteins spanned a wide range of molecular mass from 15 to 240 kDa. Of the nine axonally transported proteins that increased, three were in the fast component and three were in the intermediate component (Table 2A). Another three were in the slow component (proteins *8*, *12*, and *17*). While none of these proteins have been identified, protein *17*, with a molecular mass of 54 kDa and pI of about 5 may represent tubulin (51 to 55 kDa, pI = 5.4; Strocchi et al., 1981). Indeed, this cytoskeletal protein has been shown to increase in a variety of regenerating systems (Hoffman and Lasek, 1975; Heacock and Agranoff, 1976; Giuliane et al., 1980). It is conceivable that some of the proteins, notably those observed to increase in the fast and intermediate components of transport, may be involved in synaptogenesis—since the stage of regeneration examined represents one at which retinotectal connections are reforming (Adamson and Grobstein, 1982; Ostberg and Norden, 1979) but the retinotopic projection is still disorganized (Fig. 4). It will also be of interest to examine earlier and later time points of regeneration, to assess how transported proteins are modulated upon contact with the target cells (Sonderegger et al., 1983; Benowitz et al., 1983).

Changes in specific proteins during optic nerve regeneration in goldfish (*Carassius auratus*) and toads (*Bufo marinus*) have also been reported by others. Giuliane et al. (1980) using a lesion similar to ours to sever the optic fibers in adult goldfish showed that, 10 to 35 days into regeneration, there were in addition to changes in tubulin and actin an increase in several slowly transported [^{3}H]leucine- and [^{35}S]methionine-labeled proteins with masses of 70, 90, and 300 kDa. Benowitz et al. (1981) and Benowitz and Lewis (1983) crushed the goldfish optic nerve at the orbit, and showed increases in rapidly transported proteins of 210, 24, 110 to 140, and 44 to 49 kDa in size. These may correspond to some of the ones seen in *Xenopus*. Similarly, they found decreases in proteins of 130, 58, 21 to 36, and 27 to 30 kDa, while we had decreases at 56 (*90*) and 29 (66) kDa. Skene and Willard (1981a, 1981b) have examined changes in rapidly transported proteins in the toad, *B. marinus*, using two-dimensional gels. Crushing the nerve of the toad at the chiasm, Skene and Willard found 8- to 20-fold increases in a 43-kDa, pI 4.3 protein, 15-fold increases in a basic 50-kDa protein, and 80-fold increases in a 24-kDa protein. While we see some homologies between the goldfish data and our own findings in *Xenopus*, the species and gel systems used and, more importantly, the surgical paradigms employed to trigger regeneration of the optic fibers must be considered. For example, crushing the nerve at the chiasm (Skene and Willard, 1981a, 1981b) or the orbit (Benowitz et al., 1981) may provide a greater stimulus for regeneration, because of the longer distance from the site of injury to the tectum, and may also reveal support cell proteins made in reaction to the local axonal injury (Szaro et al., 1984). The identification of growth-associated proteins is likely to be a cumulative process, as different gel systems are brought to bear upon the problem.

Transported proteins which decreased in our study were primarily in the intermediate component (Table IIB). These may include functional proteins whose production is temporarily halted due to disruption of function in the severed nerves.

In addition to changes in proteins which were axonally transported, there were changes in four other proteins which were not eliminated in distal segments of crushed nerves (control for transported proteins, Szaro et al., 1984). These are possibly glial proteins and included protein *62* (58 kDa) which increased and protein *43* (15 kDa), *59* (72 kDa), and *75* (22 kDa) which decreased. Our previous studies have shown rapid changes in local protein synthesis within glial and nerve sheath cells following a nerve crush (Szaro et al., 1984).

It is of some interest that we failed to detect any proteins unique to regenerating nerves, and even those which increased most dramatically were nevertheless present at low levels in nonregenerating normal nerves. This may reflect the fact that *Xenopus* eyes continue to grow throughout adult life, and that the normal optic nerves contain modest numbers of actively

growing fibers (Straznicky and Gaze, 1971; Jacobson, 1976). There is also evidence that even established optic nerve connections in the tectum are tuned and refined in the young adult animal, such that even the oldest fibers present—fibers which first synapsed in the tectum at very young tadpole stages—may continue to sprout new endings and withdraw old ones (Gaze et al., 1979; Fraser, 1983; Scott and Lazar, 1976). The retinal ganglion cells may thus find it necessary to transport low levels of growth-associated proteins to their terminals, even when most of their terminal arbor is connected to postsynaptic tectal neurons.

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