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Enhanced labeling of a retinal protein during regeneration of optic nerve in goldfish

(tubulin/axon outgrowth/microtubule assembly/vinblastine)

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ABSTRACT **Goldfish retinas were examined for changes in the labeling pattern of protein during regeneration of retinal ganglion cell axons following unilateral optic nerve crush. At various times after optic nerve crush the normal retinas were incubated *in vitro* with [^{3}H]methionine and retinas from the opposite side of the treated fish were incubated with [^{35}S]methionine. The incubated retinas were combined and a soluble protein fraction was isolated and separated by sodium dodecyl sulfate–urea polyacrylamide gel electrophoresis. A band on the gel showing an elevated ^{35}S to ^{3}H ratio appeared at 5 days following optic nerve crush, increased to a maximum at 15 days, and was barely detectable at 45 days, during which time vision is known to return. On the basis of several criteria, the protein fraction showing the increased incorporation of methionine in retina after optic nerve crush appears to be of the tubulin class.**

The goldfish retinal ganglion cell responds to transection of the optic nerve or tract by undergoing morphological changes similar to those exhibited by other neurons capable of regeneration of the cut axon (1). Murray and Grafstein (1) presented radioautographic evidence that the morphological changes were accompanied by increased amino-acid incorporation into retinal ganglion cell protein during regeneration of the optic nerve. Studies on mammalian cranial and spinal motor neurons (2–4) and on mammalian spinal ganglion cells (5, 6) have also been interpreted to indicate increased protein synthesis in these neuronal perikarya following axotomy. The increased incorporation of amino acid into protein of the nerve cell body is accompanied by an increased rate of axonal transport in the regenerating axon (7–9).

Following intra-orbital crush of the optic nerve in goldfish, the retinal ganglion cell must grow out a new axon several millimeters long. This process not only implies a substantial increase in the rate of protein synthesis but may also involve a selective increase in the synthesis of the proteins necessary for the renewal of axoplasm and replacement of structural elements of the axon. Formation of cell-specific markers involved in retinotectal recognition may be increased during this period (10). There might, in addition, be an initial decrease in the synthesis of components necessary to maintain the functional integrity of the nerve ending until the growing nerve reaches the optic tectum (11).

This report presents comparisons of the *in vitro* protein labeling pattern of normal goldfish retina with that of retinas removed at various times after optic nerve crush (post-crush retina). A double-labeling technique coupled with sodium dodecyl sulfate–urea polyacrylamide gel electrophoresis (12) revealed an increased amino-acid incorporation into a protein fraction with properties of the microtubule subunit, tubulin.

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; NGF, nerve growth factor.

MATERIALS AND METHODS

Goldfish (*Carassius auratus*), 6–7 cm in body length, were anesthetized with tricaine methane sulfonate prior to an intraorbital crush of the right optic nerve. The left optic nerve remained intact so that the left retina served as a control. Following surgery, the goldfish were stored in groups of 50 to 80 in 30 gallon tanks at 20–22°, and were fed daily.

Fish were dark-adapted for 30 min prior to removal of retinas in order to facilitate separation of the retina from the pigment epithelium. After hemisection of the eye, the retina was floated free from the pigment layer with 0.86% saline and detached by cutting at the optic disk. The retinas were then rinsed and stored in ice-cold saline or incubation medium for up to 1 hr prior to incubation.

***In Vitro* Labeling of Retinal Protein.** L-[*Methyl*-^{3}H]methionine (New England Nuclear, 0.23 or 10.0 Ci/mmol) and L-[^{35}S]methionine (New England Nuclear, specific activity adjusted to equal that of the [^{3}H]methionine) were evaporated to dryness under nitrogen immediately before use. After evaporation, each was resuspended in a small volume of incubation medium. The purity of each isotopic preparation was checked by paper chromatography.

The incubation medium was that of Dunlop *et al.* (13): *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 25 mM; $MgSO_4$, 1.3 mM; $CaCl_2$, 2.6 mM; K_2HPO_4, 1.2 mM; KCl, 5.9 mM; NaCl, 106.5 mM; glucose, 12 mM; and NaOH to pH 7.4.

Retinas (in groups of 5 to 20) were pre-incubated for 2 min at 25° in 1.9 ml of incubation medium. Labeled precursor (0.1 ml) was then added (1.0–4.0 μCi per retina) and incubation was continued in air in a Dubnoff metabolic shaker at 25°. All incubations using double-labeled methionine were carried out for 1 hr. The reaction was stopped by dilution with 2 volumes of ice-cold incubation medium containing 2 mM L-methionine. After centrifugation at 2000 rpm (International centrifuge, 24 cm at tip) for 5 min, the pelleted retinas were washed again with cold incubation medium. In double label experiments, [^{35}S]- and [^{3}H]methionine-labeled retinas were combined and homogenized in 0.01 M sodium phosphate buffer (pH 7.1) containing 1 mM EDTA and 1 mM phenylmethylsulfonylfluoride (2.0 ml per 20 retinas), using a motor-driven glass-on-glass tissue grinder at 4°. The retinal protein soluble after centrifugation at 100,000 × *g* for 60 min was made 0.2% in sodium dodecyl sulfate ($NaDodSO_4$), heated at 80° for 5 min, and dialyzed 12 hr against 1 liter of water containing 0.2% $NaDodSO_4$ and 0.1% mercaptoethanol. The protein was concentrated by lyophilization followed by resuspension in one-fifth the original volume of 0.1 M sodium phosphate buffer (pH 7.1) containing 1 mM EDTA and 1% mercaptoethanol. After heating at 80° for 5 min, urea was added to a final concentration of 8 M. Bromophenol blue was used as a tracking dye.

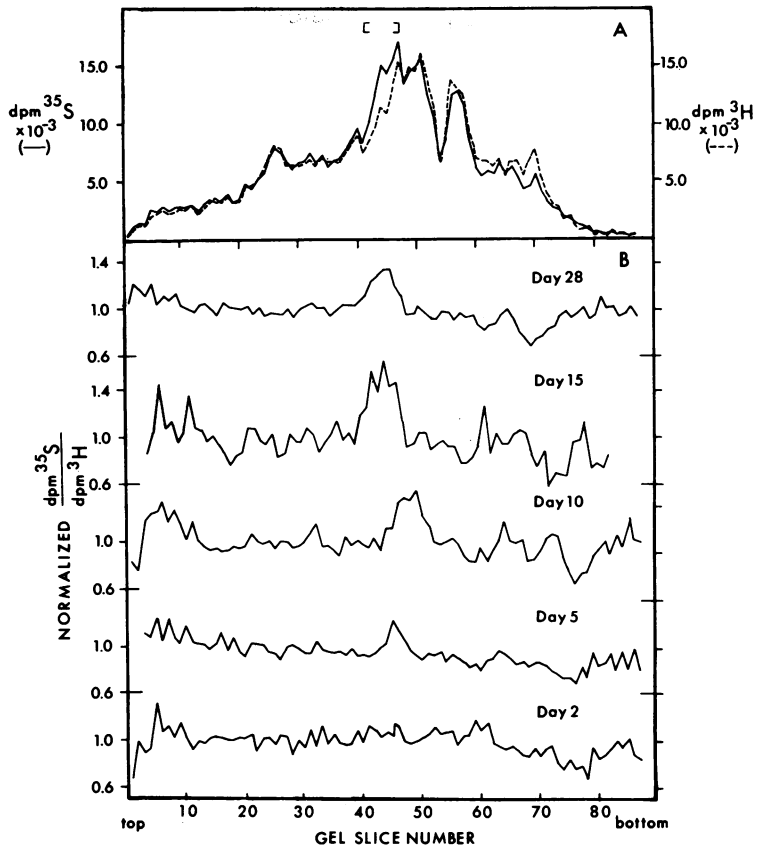

FIG. 1. Comparison of soluble protein labeling pattern on $NaDodSO_4$-urea gel electrophoresis in normal retinas with retinas at various times after optic nerve crush. Retinas from 10 to 25 fish were pooled for each experiment. Each gel contained 300–350 μg of protein. The specific activity of the protein applied to the gels ranged from 84 dpm $^{35}S/\mu g$ of protein (day 15) to 1072 dpm $^{35}S/\mu g$ of protein (day 28). (A) ^{35}S- and ^{3}H-labeling pattern of retinal soluble protein 28 days after optic nerve crush. The brackets enclose the area of the gel showing an increased ^{35}S to ^{3}H ratio. (B) Normalized ratio of ^{35}S dpm to ^{3}H dpm at 2, 5, 10, 15, and 28 days after optic nerve crush. The ^{35}S to ^{3}H ratio (which ranged from 0.85 to 1.5) has been normalized by multiplying by a factor (constant for each gel) to make the average ratio across each gel equal to 1.0.

$NaDodSO_4$-Urea Polyacrylamide Gel Electrophoresis. Gel electrophoresis was performed in 5 mm inside diameter × 12 cm glass tubes filled to a height of 10.5 cm with a solution containing 5% acrylamide, 0.2% bis-acrylamide, 0.1% $NaDodSO_4$, 8 M urea, and 0.1 M sodium phosphate buffer (pH 7.1). Samples containing 150–350 μg of protein (in 25–200 μl) were electrophoresed at 6 mA per tube for 6 hr with 0.1 M sodium phosphate buffer (pH 7.1) containing 0.1% $NaDodSO_4$ in the buffer chamber. Gels were then fixed in water:methanol:acetic acid (6:3:1) and either stained with 1% amido black or sliced into 1 mm sections for measurement of radioactivity. The gel slices were dissolved by incubation for 12 hr at 60° in 0.4 ml of 30% hydrogen peroxide. Radioactivity was determined after adding 10.0 ml of a toluene-based scintillant containing 26% Triton X-100, using a Packard model 3375 liquid scintillation counter. Recovery of the applied radioactive protein in the gel slices was 85–100%.

Radioactivity in [^{3}H]leucine-labeled retina homogenate was determined by a modification of the filter paper method of Mans and Novelli (14). Protein was determined spectrophotometrically (15).

RESULTS

Retinas examined histologically 8 or 21 days after optic nerve crush exhibited the nuclear eccentricity and nucleolar volume increase described by Murray and Grafstein after optic tract transection (1). The retinas appeared normal in other respects and there was no indication of damage to the blood supply as a result of the crush. Goldfish with both optic nerves crushed were blind as determined using an optomotor response as a measure of vision (16). The response returned about 30 days after nerve crush, indicating that regeneration of the optic nerve had occurred.

Effect of Optic Nerve Crush on Amino-Acid Incorporation in Goldfish Retinas. Incubations carried out with L-[4,5-^{3}H]leucine (30 Ci/mmol, 1 μCi per group of five retinas) demonstrated linearity of incorporation of labeled amino acid into retinal protein during a 90-min incubation. Following a 30-min incubation with [^{3}H]leucine, the specific activity of protein of retina from the unoperated side (left) was compared with that of retina from the operated side (14 days after right optic nerve crush). Determinations from five normal groups and five post-crush retina groups revealed an 11% increase in protein specific activity in the latter. The difference, although small, was statistically significant ($t = 1.86$, $P < 0.05$).

Gel Electrophoresis of Double-Labeled Retinal Soluble Protein. In double-label incubations, normal retinas (left eye) were routinely incubated with [^{3}H]methionine and post-crush retinas (right eye) were incubated with [^{35}S]methionine, so that an increase in the ratio of ^{35}S dpm to ^{3}H dpm in an acrylamide gel band would reflect an increased incorporation of methionine into a protein fraction from retinas undergoing optic nerve regeneration.

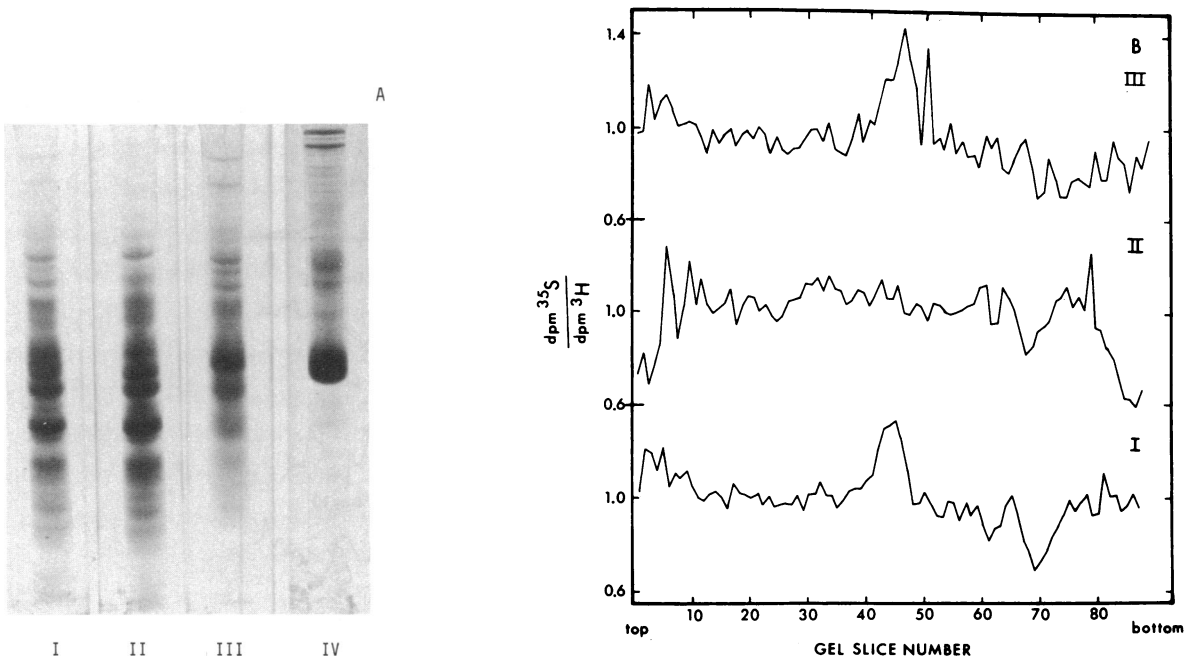

FIG. 2. Vinblastine treatment of retinal soluble protein. One third of the soluble protein was treated with $NaDodSO_4$ and prepared for gel electrophoresis. The remainder (6560 μg of protein) was incubated with 2 mM vinblastine sulfate at 37° for 60 min, followed by centrifugation at 100,000 × g for 30 min at 4°. The vinblastine-soluble and precipitable fractions were each treated with $NaDodSO_4$, dialyzed, and prepared for $NaDodSO_4$-urea gel electrophoresis. (A) $NaDodSO_4$-urea gels stained with amido black. I, Total soluble protein; II, vinblastine-soluble protein; III, vinblastine-precipitable protein; IV, mouse brain tubulin purified by assembly-disassembly. Gels I, II, and III contained 150 μg of protein; gel IV contained 60 μg of protein. (B) Ratio of ^{35}S dpm to ^{3}H dpm in $NaDodSO_4$-urea gels. I, Total soluble protein; II, vinblastine-soluble protein; III, vinblastine-precipitable protein. Each gel contained 300 μg of protein.

Incubations and preparation of the combined ^{3}H- and ^{35}S-labeled retinal soluble protein for $NaDodSO_4$-urea gel electrophoresis were carried out as described in *Materials and Methods*. An example of the pattern of ^{3}H and ^{35}S labeling of soluble protein from the combined retinas (28 days after unilateral optic nerve crush) is shown in Fig. 1A. The ratio of ^{35}S dpm to ^{3}H dpm across this gel and for samples analyzed at various times after optic nerve crush is shown in Fig. 1B. Increased incorporation of methionine in the 50,000–60,000 molecular weight region of the gel (calibrated with bovine serum albumin, purified mouse brain tubulin, and ovalbumin) is apparent at day 5 after optic nerve crush, appears to reach a maximum at day 15, and may be somewhat decreased by day 28. The $^{35}S/^{3}H$ peak is not detected at day 2 and is not observed in a control experiment in which ^{3}H- and ^{35}S-incubated normal retinas were combined (not shown).

Vinblastine Precipitation. Since the protein fraction which shows an increased incorporation of labeled methionine during optic nerve regeneration has a mobility on $NaDodSO_4$-urea gels similar to that of tubulin, the effect of treatment with vinblastine was investigated. Incubation with 2 mM vinblastine sulfate causes aggregation and almost complete precipitation of tubulin (17, 18). Double-labeled soluble protein was prepared from goldfish retinas 28 days after optic nerve crush. Each group of 25 retinas was incubated with 55 μCi of [^{35}S]methionine (operated eye) or of [^{3}H]methionine (unoperated eye), each at 10 Ci/mmol. After incubation, the retinas were combined, the soluble protein fraction was isolated, and an aliquot was incubated with 2 mM vinblastine sulfate. The protein staining pattern after $NaDodSO_4$-urea acrylamide gel electrophoresis of the vinblastine-soluble and precipitable retinal protein is shown in Fig. 2A, as well as the pattern from the crude 100,000 × g soluble protein and from tubulin purified from mouse brain by two cycles of assembly-disassembly (19). The vinblastine precipitate is heterogenous but it is enriched in a band with the same mobility as tubulin. Examination of the ^{35}S to ^{3}H ratio (Fig. 2B) indicates that the protein fraction that shows increased methionine incorporation in post-crush retina is completely precipitated on treatment with vinblastine. The protein fraction enriched in [^{35}S]methionine has the same mobility as ^{3}H-labeled retinal protein co-purified with mouse brain tubulin through 2 cycles of assembly-disassembly (not shown).

Purification of Double-Labeled Retinal Tubulin by Assembly-Disassembly of Microtubules. The protein fraction showing increased labeling in post-crush retina was similar to tubulin in its molecular weight and behavior on vinblastine treatment. Since neither of these properties is unique to tubulin, further identification was attempted, making use of the ability of tubulin to participate in the assembly and disassembly of microtubules (19).

Tubulin was prepared from double-labeled retinal soluble protein 28 days after right optic nerve crush (^{35}S, right; ^{3}H, left) with added carrier mouse brain soluble protein, by two cycles of assembly and disassembly of microtubules. The purified tubulin sample was treated with $NaDodSO_4$, dialyzed, lyophilized and subjected to $NaDodSO_4$-urea gel electrophoresis (Fig. 3). Most of the radioactivity moves with the tubulin band (see Fig. 2A). The ^{35}S to ^{3}H ratio of the retinal protein which is co-purified with mouse brain tubulin is more than three times that of the crude soluble retinal protein (Table 1).

This assembly-disassembly experiment was repeated with retina from goldfish 14 days after right optic nerve crush. In this case, the assignment of labeled methionine was reversed, so that normal retinas were incubated with

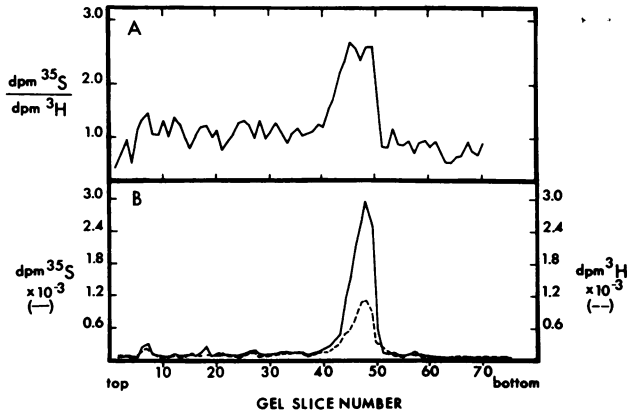

FIG. 3. NaDodSO$_4$-urea gel electrophoresis of purified tubulin. Retinas were removed from 25 fish, 28 days after crushing of right optic nerve. After the standard double-label incubation the right and left retinas were combined and homogenized in 1.5 ml reassembly buffer (0.1 M 2-[*N*-morpholino]ethane sulfonic acid, 1 mM EGTA, 0.25 mM $MgCl_2$, 1 mM GTP, pH 6.4). After centrifugation at 100,000 × *g* for 60 min one-fourth of the soluble protein was treated with NaDodSO$_4$ and prepared for gel electrophoresis; the remainder (4944 μg of protein) was added to 1.8 ml of mouse brain soluble protein (31.8 mg of protein) prepared similarly from the brains of five mice. An equal volume of 8 M glycerol in reassembly buffer was added and the tubulin was purified through two cycles of assembly and disassembly (19). The purified tubulin (111 μg of protein) was subjected to NaDodSO$_4$-urea gel electrophoresis. (A) Ratio of ^{35}S dpm to ^3H dpm across gel. (B) Distribution of ^{35}S dpm and ^3H dpm across gel.

[^{35}S]methionine and post-crush retinas were incubated with [^3H]methionine. The ^3H to ^{35}S ratio in the purified tubulin showed the same 3-fold increase over that of the crude soluble protein, ruling out any possible artifact in the scintillation counting procedure as a factor in the previously observed increased ^{35}S to ^3H ratio.

DISCUSSION

Following transection of the goldfish optic nerve, the retinal ganglion cell axons regrow and reinnervate the optic tectum with return of function. The regenerative process is accompanied by increased incorporation of amino acid in the region of the ganglion cell bodies (1), estimated by radioautographic grain counts to be 3-fold. Comparison of the specific activities of total retinal protein following *in vitro* incubations reported here indicates an 11% increase in labeling. Taken together, the results suggest that the ganglion cells contribute 4% of the total labeling in the incubated retinas. This value seems to correspond reasonably with a crude estimate of the ganglion cell volume relative to other retinal cells and with the estimate of Grafstein (20) that ganglion cells represent 10% of the neurons in goldfish retina.

Fractionation of double-labeled soluble retinal protein by NaDodSO$_4$-urea polyacrylamide gel electrophoresis revealed the presence of a protein fraction in post-crush retina exhibiting a 30–70% increase in incorporation of labeled methionine. Further studies are necessary to determine more exactly the onset following nerve crush and the time of maximum increase of methionine incorporation into this protein band. Preliminary experiments (data not shown) indicate that the difference is barely detectable at day 45 after optic nerve crush and has disappeared by day 75. The radioautographic grain density over retinal ganglion cells after intraperitoneal administration of [^3H]leucine was reported to be slightly increased at day 4 after optic tract transection, doubled at day 7, reached the peak (3-fold) at day 20, and remained elevated (2.5-fold) at least until day 40, in goldfish maintained at 21 ± 1° (1).

Because of co-migration of contaminating protein, the increased ^{35}S to ^3H ratio observed after gel electrophoresis of crude soluble retinal protein (Fig. 1) is a minimal estimate of the increased labeling of this protein fraction. The material resembles protein of the tubulin class in its mobility on NaDodSO$_4$-urea gels, precipitability with vinblastine, and copurification with tubulin by assembly-disassembly of microtubules. The observed 3-fold increase in methionine incorporation into soluble retinal tubulin (about 1% of total labeled retinal protein) following optic nerve crush accounts for less than one-fifth of the 11% increase in protein specific activity seen in post-crush retina, indicating increased incorporation of amino acid into other retinal proteins. It is possible that some of the unaccounted-for enhanced labeling represents membrane-bound tubulin of post-crush retina. Reports that a substantial fraction of brain tubulin is particulate (21) and that some tubulin-like protein may be found in nerve endings (22) have recently been confirmed and extended (23, 24), with tubulin tentatively being localized at the synaptic junction (25) and post-synaptic density (26).

The increased amino-acid incorporation into soluble goldfish retinal tubulin during axonal regeneration reflects the acknowledged role of microtubules in maintaining the structural integrity of axons (27) and is consistent with a probable role for microtubules in axoplasmic flow (28, 29). Both neurite outgrowth (33–35) and rapid axonal transport (29–32) are inhibited by agents that disrupt microtubules, such as colchicine, vinblastine, and exposure to low temperature. Such treatments can lead to retraction of neurites (33–35), which has been correlated with a decrease in microtubules (36, 37). Application of colchicine over the cut optic tract of the goldfish results in cessation of outgrowth of the axon (8).

Studies with neuroblastoma cell cultures and chick embryo dorsal root ganglia explants have attempted to define the relationship between neurite extension and microtubule subunit synthesis or assembly. Initial neurite formation in

Table 1. Purification of retinal tubulin by assembly and disassembly of microtubules

	Specific activity (dpm/μg of protein)			$^{35}S/^3H$ tubulin
	^{35}S	^3H	$^{35}S/^3H$	$^{35}S/^3H$ soluble protein
Retinal soluble protein	1072	1195	0.90	—
NaDodSO$_4$-gel crude tubulin	—	—	1.22	1.35
Retinal and mouse brain soluble protein	166	185	0.90	—
Purified tubulin	335	198	1.68	1.86
NaDodSO$_4$-gel purified tubulin	—	—	2.77	3.07

differentiating mouse neuroblastoma cells is not affected by protein synthesis inhibitors (33) and there is no difference in the amount of tubulin subunits between undifferentiated and differentiated mouse neuroblastoma cells (38). The latter, however, show a higher density of microtubules than the undifferentiated cells, indicating that during neurite outgrowth new microtubules are assembled from a pool of preformed tubulin subunits (33).

In contrast, the increase in the microtubule population in growing neurites of both human neuroblastoma (39) and chick embryo dorsal root ganglia cultures (41) after treatment with nerve growth factor (NGF) appeared to be correlated with increased synthesis of tubulin subunits, measured by increased colchicine-binding activity. Stimulation of neurite outgrowth by NGF does not occur in the presence of inhibitors of protein synthesis. In the dorsal root ganglia cultures, inhibition of NGF-stimulated outgrowth by addition of vincristine (which disrupts microtubules), does not prevent the NGF-induced stimulation of tubulin synthesis (40). Neurite extension in dibutyryl-cyclic-AMP-treated dorsal root ganglia is not accompanied by an increase in microtubule subunit concentration, but may involve stabilization of microtubules or promotion of microtubule assembly by the cyclic nucleotide (41).

It appears, then, that enhanced microtubule subunit synthesis can occur in the absence of *in vitro* neurite extension and *vice versa*. The relevance of tubulin synthesis to axon outgrowth requires further clarification. Their physiological interrelationship is suggested by the present investigation. In a related study, we have found that prior cut of the optic nerve of the *Xenopus* tadpole greatly enhances subsequent neurite outgrowth from the cultured retina (42), providing an *in vitro* model of regeneration. We infer that the incubated post-crush goldfish retina similarly retains characteristics of the regenerating state, as indicated by the observed selective enhancement of tubulin labeling.

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