# 4S RNA Is Present in Regenerating Optic Axons of Goldfish

Abstract. *Regenerating optic axons of goldfish were loaded with [$^3$H]RNA by injecting [$^3$H]uridine into the eye and allowing time for the radioactivity to be delivered to the optic tectum. The axons were subsequently removed from the tecta by cutting the optic nerve and allowing the optic axons in the tectum to degenerate. Analysis of tectal [$^3$H]RNA by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed a selective loss of tritiated 4S RNA and not ribosomal RNA from the denervated tecta. These results support the hypothesis that regenerating optic axons of goldfish grow back into the tectum carrying 4S but not ribosomal RNA.*

Axons can be characterized morphologically as being void of ribosomes and biochemically as being incapable of carrying out protein synthesis. However, the presence of small amounts of RNA within a variety of axons has been reported (*1*). The site of synthesis as well as the molecular species of RNA contained within axons is uncertain. While there have been several reports that all major RNA species (ribosomal, messenger, and transfer RNA's) could be found within vertebrate axons, most of these experiments did not fully account for the possibility that not only axonal RNA, but RNA of supporting cells (glial or Schwann) was assayed as well. Since supporting cells synthesize all RNA species, any RNA from these cells could mask the presence of a single RNA species within the axons. In invertebrates, the predominant RNA in the axoplasm of squid and *Myxicola* giant axons has a 4S sedimentation coefficient and many of the transfer RNA characteristics (*2, 3*).

Axonal RNA has also been studied extensively in the goldfish visual system. When [$^3$H]uridine is injected into the eye of fish in which optic nerves are regenerating, large amounts of [$^3$H]RNA can be detected in the contralateral optic tectum several days later (*4*). Further, examination by electron-microscope autoradiography indicates that approximately 50 percent of the [$^3$H]RNA in the tectum is contained within regenerating axons and axonal growth cones (*5*). These and other data have led us to propose that during regeneration of the optic axons of goldfish, some RNA is synthesized in retinal ganglion cells in the eye and then transported within the elongating axons to the optic tectum.

The question of what species of RNA is axonally transported in these axons has not been completely answered. In all of the experiments in which [$^3$H]uridine was injected into the eye and tectal radioactivity was assayed at various times after injection, the appearance of [$^3$H]RNA in the tectum was preceded by the arrival of [$^3$H]RNA precursors. These precursors are utilized for RNA synthesis by the glial cells surrounding the axon (*5*), and presumably this RNA is composed of all molecular RNA species. Therefore, any attempt to identify [$^3$H]RNA in optic axons must differentiate it from [$^3$H]RNA synthesized in tectal cells. Since physical separation of axons and glia is not technically possible in this system, several indirect approaches have been employed in an attempt to identify the nature of the axonal RNA (*6, 7*). Results of these experiments

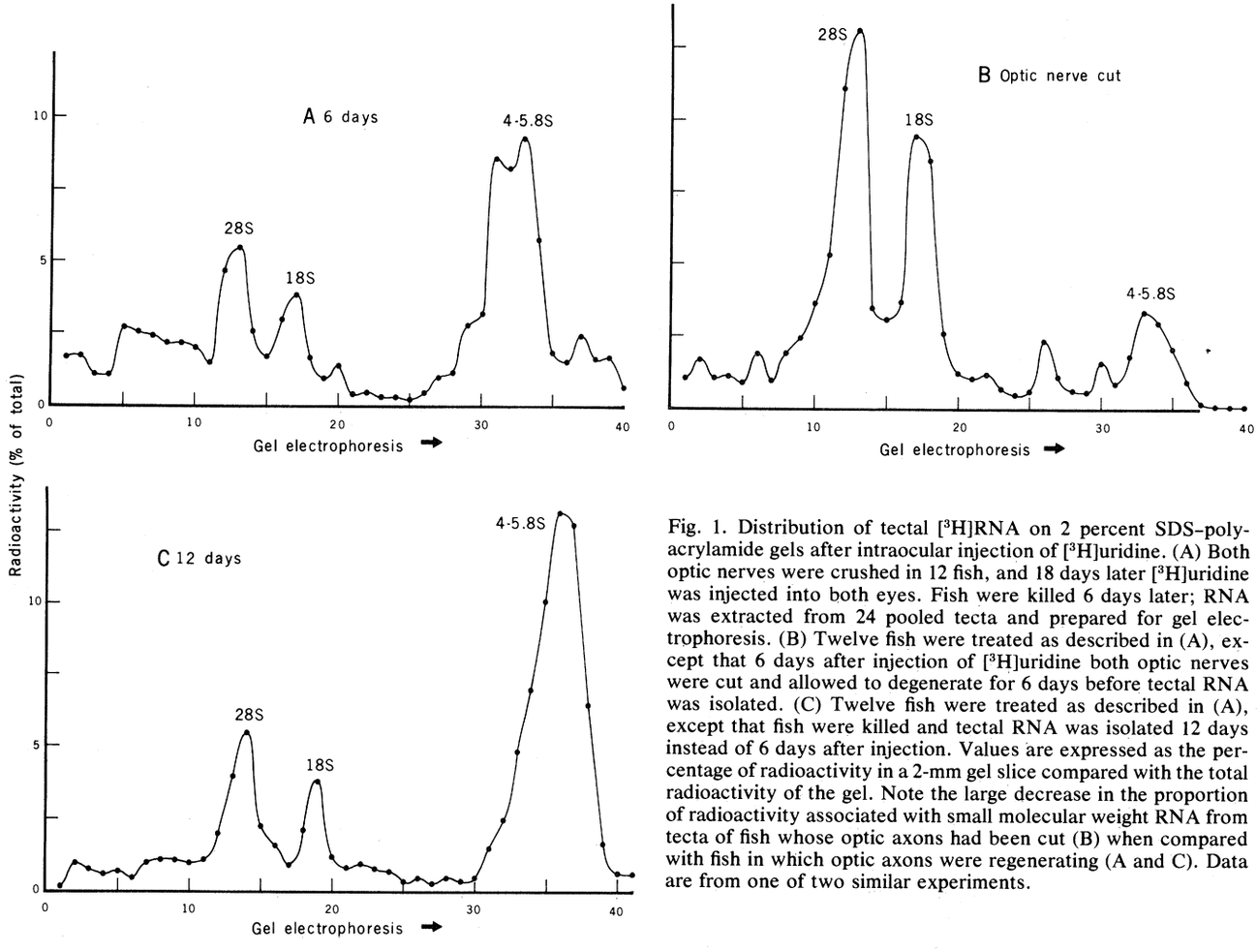


Fig. 1. Distribution of tectal [$^3$H]RNA on 2 percent SDS–polyacrylamide gels after intraocular injection of [$^3$H]uridine. (A) Both optic nerves were crushed in 12 fish, and 18 days later [$^3$H]uridine was injected into both eyes. Fish were killed 6 days later; RNA was extracted from 24 pooled tecta and prepared for gel electrophoresis. (B) Twelve fish were treated as described in (A), except that 6 days after injection of [$^3$H]uridine both optic nerves were cut and allowed to degenerate for 6 days before tectal RNA was isolated. (C) Twelve fish were treated as described in (A), except that fish were killed and tectal RNA was isolated 12 days instead of 6 days after injection. Values are expressed as the percentage of radioactivity in a 2-mm gel slice compared with the total radioactivity of the gel. Note the large decrease in the proportion of radioactivity associated with small molecular weight RNA from tecta of fish whose optic axons had been cut (B) when compared with fish in which optic axons were regenerating (A and C). Data are from one of two similar experiments.


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have led us to propose that as in squid and *Myxicola* axons, the predominant if not the only RNA in regenerating axons of the retinal ganglion cells of goldfish is 4*S* RNA. The present experiments were performed in an attempt to provide more direct evidence bearing on this interpretation of the data.

If only 4*S* RNA is present in regenerating optic axons in the tectum, then cutting the optic axons and allowing them to degenerate, and thus removing them from the tectum, should lead to a larger relative loss of tectal 4*S* RNA than ribosomal RNA. To determine if this is the case, both optic nerves were crushed in 36 goldfish, and 18 days later, 4 $\mu$Ci of [$^3$H]uridine was injected into both eyes of all the fish (*8*). Fish were separated into three groups of 12. The first group was killed 6 days later and RNA was isolated from 24 pooled tecta by the use of hot phenol extraction and ethanol precipitation (*9*). In the second group, both optic nerves were cut 6 days after injection, allowing [$^3$H]RNA to arrive in the tectum before the axons were sectioned. Fish were killed 6 days later, after significant axonal degeneration had occurred (*10*). The third group was killed along with the second group, but received no experimental treatment other than injection of isotope 12 days earlier. RNA was extracted from tecta of groups 2 and 3 as for group 1.

The RNA (50 to 100 $\mu$g) was layered onto the surface of 2 or 10 percent sodium dodecyl sulfate (SDS)–polyacrylamide gels and subjected to electrophoresis for 1.5 and 4.5 hours, respectively (*11*). Optical density profiles were determined by using a Beckman ACTA III spectrophotometer. Gels were frozen, cut into 2-mm slices, and assayed for radioactivity (*9*). In all three groups, radioactivity comigrated on 2.0 percent gels with the major stable RNA components of 28*S*, 18*S* (ribosomal RNA), and 4 to 5.8*S* (Fig. 1). Radioactivity on the 10.0 percent gels remained at the origin (high molecular weight RNA) or comigrated with a 4*S* yeast RNA marker, with small amounts of radioactivity in the 5 and 5.8*S* positions (small molecular weight ribosomal components). In all cases $>$ 70 percent of the radioactivity that did not remain at the origin of the 10.0 percent gel was associated with 4*S* RNA. Thus, changes in the proportion of radioactivity associated with the furthest migrating peak on the 2.0 percent gel primarily represent changes in 4*S* RNA radioactivity.

The distribution of radioactivity in the major RNA peaks for fish killed 24 days after optic nerve crush and 6 days after intraocular injection (Fig. 1A) is similar to published results (*6*, *7*). The most obvious feature of this profile is the large amount of radioactivity in the 4*S* RNA peak. Twelve days after injection, a large proportion of the radioactivity is still associated with 4*S* RNA (Fig. 1C). However, in group 2, in which optic axons were cut and allowed to degenerate before tectal RNA was analyzed (a procedure that removes axonal [$^3$H]RNA from the tectum), there is a selective loss of 4*S* RNA (Fig. 1B) (*12*). These results indicate that a relatively large amount of 4*S* [$^3$H]RNA was probably within the regenerating optic axons of the tectum.

It can be argued, however, that when optic axons are regenerating, tectal cells selectively increase the synthesis (or decrease the degradation) of 4*S* RNA (Fig. 1, A and C), and that when axons are degenerating this pattern with regard to 4*S* RNA is reversed (Fig. 1B). Thus the changes in [$^3$H]RNA in the present experiments could be due entirely to incorporation of axonally transported [$^3$H]RNA precursors into RNA by tectal cells rather than changes in the [$^3$H]RNA content of optic axons. To rule out this possibility the procedure for experi-

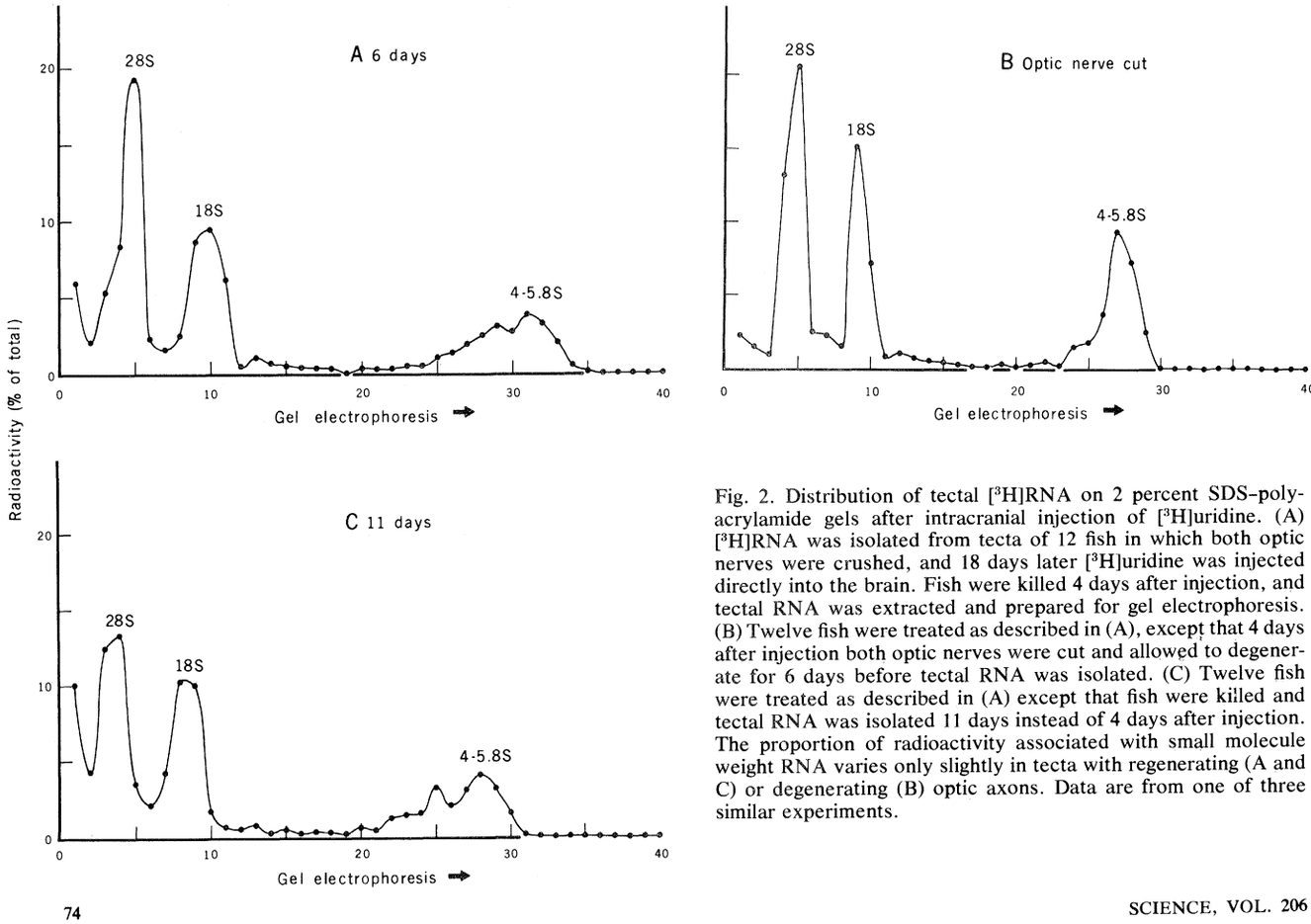


Fig. 2. Distribution of tectal [$^3$H]RNA on 2 percent SDS-polyacrylamide gels after intracranial injection of [$^3$H]uridine. (A) [$^3$H]RNA was isolated from tecta of 12 fish in which both optic nerves were crushed, and 18 days later [$^3$H]uridine was injected directly into the brain. Fish were killed 4 days after injection, and tectal RNA was extracted and prepared for gel electrophoresis. (B) Twelve fish were treated as described in (A), except that 4 days after injection both optic nerves were cut and allowed to degenerate for 6 days before tectal RNA was isolated. (C) Twelve fish were treated as described in (A) except that fish were killed and tectal RNA was isolated 11 days instead of 4 days after injection. The proportion of radioactivity associated with small molecule weight RNA varies only slightly in tecta with regenerating (A and C) or degenerating (B) optic axons. Data are from one of three similar experiments.

ments shown in Fig. 1 was repeated except that [$^3$H]uridine was injected intracranially rather than in the eye, thus labeling only tectal cells and not optic axons (*13*).

Typical profiles of the radioactivity on 2 percent gels are shown in Fig. 2. With minor variations, radioactivity profiles were similar in all experiments and the proportion of radioactivity falling in each of the major stable RNA peaks was not altered by the experimental procedure. These results show that the selective loss of 4*S* RNA demonstrated earlier (Fig. 1) was probably caused not by changes in RNA metabolism in tectal cells, but by a loss of intraaxonal 4*S* RNA.

To determine the fraction of radioactivity present in the 4*S* peak, the total radioactivity on a 10 percent gel was determined and the portion falling in the 4*S* peak was compared with the total radioactivity on the gel for groups of fish injected either intraocularly or intracranially. After intraocular injection, greater than 50 percent of the radioactivity was associated with 4*S* RNA regardless of whether that radioactivity was measured 6 days (53 percent) or 12 days (67 percent) after injection. However, after degeneration of the optic nerve only about 21 percent of the radioactivity was associated with 4*S* RNA. After intracranial injections, the proportion of radioactivity in the 4*S* peak was approximately 25 percent whether optic axons were present or not.

These results, considered with those of earlier studies (*4*–*7*), strongly indicate that a large portion of 4*S* [$^3$H]RNA present in goldfish optic tecta during optic nerve regeneration and after intraocular injection of [$^3$H]uridine is due to the presence of 4*S* [$^3$H]RNA in regenerating optic axons. It is likely that this RNA is synthesized in retinal ganglion cells and then axonally transported in the growing optic axons.

Some evidence suggests that small amounts of RNA are transported in normal optic axons (*4*), but we have not been able to confirm this by autoradiographic or biochemical studies. Therefore, the question of whether RNA is axonally transported in nongrowing optic axons of goldfish remains unanswered. It also remains to be seen if the 4*S* RNA in goldfish optic axons is transfer RNA, as has been shown for squid axons (*3*). Other experiments indicate that 4*S* RNA is transported axonally in the chick visual system (*14*), in both normal and regenerating rat sciatic nerves (*15*), and in optic axons of neonatal rats (*16*). The observation that 4*S* RNA is the only RNA species found in a variety of axons may require a reappraisal of the function of 4*S* RNA in nervous tissue. It may be that the 4*S* RNA is transfer RNA and either donates an amino acid to existing axonal polypeptides, thus modifying axonal proteins (*3*), or is transferred to surrounding tectal cells where it may participate in or regulate protein synthesis (*15*). Alternatively, axonal 4*S* RNA may serve a function in growing axons separate from its role in protein synthesis, a function yet to be described.

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### References and Notes

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8. Optic nerves were crushed by first submerging goldfish (*Carassius auratus*), 10 to 13 cm in length (Millbrook Farms, Upper Saddle River, N.J.), in ice water for 10 minutes. Optic nerves were then exposed in the anesthesized fish and crushed by compression with curved jewelers' forceps.
9. Details of the extraction procedure and methods for recovering radioactivity from gel slices are given in (*6*).
10. Electron microscope studies of the goldfish optic tectum 6 days after optic nerve section have shown marked degenerative changes in retinal fibers [M. Murray, *J. Comp. Neurol.* **168**, 175 (1976)]. In other studies we have shown that 6 days after optic nerve section 95 percent of rapidly axonally transported proteins are lost from the tectum [N. A. Ingoglia, B. Grafstein, B. McEwen, I. McQuarrie, *J. Neurochem.* **20**, 1605 (1973)]. Thus it appears that a large portion of the contents of optic axons in the tectum are removed from the tectum within a week of cutting the optic nerve.
11. The procedures followed were essentially those of U. E. Loening [*Methods Med. Res.* **12**, 359 (1970)] for 2 percent gels and of K. Weber and M. Osborn [*J. Biol. Chem.* **244**, 4406 (1969)] for 10 percent gels.
12. While Fig. 1 shows relative changes in radioactivity in each of the RNA fractions, there is also a clear decrease in the amount of radioactivity in the 4*S* RNA peak. The actual disintegrations per minute associated with each of the RNA peaks are as follows. Group 1: 28*S*, 1348; 18*S*, 855; and 4*S*, 3647. Group 2: 28*S*, 6424; 18*S*, 4300; and 4*S*, 1741. Group 3: 28*S*, 4984; 18*S*, 2525; and 4*S*, 17,022.
13. Both optic nerves in 36 fish were crushed, and 18 days later 8 μCi of [$^3$H]uridine was injected intracranially. Four days later, one group of 12 fish was killed and the optic nerves of a second group of 12 were cut. The second group and a third group of 12 fish were then killed 11 days after injection. The RNA was extracted from tecta of all three groups as described above and layered on 2 and 10 percent SDS-polyacrylamide gels.
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17. This work was supported by a grant from the Foundation of the College of Medicine and Dentistry of New Jersey and by grant EI-02887 from the National Institutes of Health.

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## Shark Heart Mitochondria: Effects of External Osmolality on Respiration

Abstract. *Shark mitochondrial respiration was studied in media with osmolalities between 160 and 1500 milliosmoles. The respiratory control ratio, a marker for functional integrity of the isolated mitochondria, was maximal at 1000 milliosmoles and decreased during hypotonic or hypertonic exposure. Shark mitochondria function best at their native tonicity, a value that produces abnormal function in mammalian mitochondria.*

Isolated mammalian mitochondria show striking changes in respiratory control when osmolalities of suspending media are less (hypotonic) or greater (hypertonic) than the normal value of approximately 300 mOsm (*1*, *2*). In the studies reported here we measured respiratory control ratios (RCR) and adenosine diphosphate/oxygen (ADP/O) ratios in isolated shark heart mitochondria exposed to external osmolalities varying from 160 to 1500 mOsm. We found that respiratory control is maximal at 1000 mOsm, the usual osmolality in shark cells. This suggests that shark mitochondria are functionally adapted to an osmolality considerably different from that of mammalian mitochondria.

Pelagic sharks approximately 1 m long, indigenous to the San Diego Bay area (*3*) were killed by decapitation. The heart was minced in isolation medium (*4*) and homogenized by hand in a Ten Broeck glass tissue homogenizer. The suspension was centrifuged at 1000*g* for 2 minutes and the resultant supernatant at 17,000*g* for 5 minutes. The final pellet was suspended in isolation medium to a concentration of approximately 10 mg of protein per milliliter.

Mitochondrial oxygen consumption ($\dot{Q}O_2$) was determined polarographically at 25°C with a Gilson oxygraph and Clark platinum electrode assembly, as described in (*1*). Heart mitochondria were added to the reaction chamber, which contained the incubation medium of the experimental osmolality (*4*) and the sub-


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