4 S RNA IN REGENERATING OPTIC AXONS OF GOLDFISH¹

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Abstract

Previous experiments have demonstrated that 4 S RNA is transported axonally during the reconnection period of optic nerve regeneration in goldfish. The present experiments were performed to determine whether 4 S RNA is transported axonally during later, maturational stages of nerve regeneration and to examine some of the characteristics of 4 S RNA in regenerating axons.

[³H]Uridine was injected into both eyes of fish 18 days (near the time of reconnection) after bilateral optic nerve crush and the fish were sacrificed at various times up to 180 days after injection. [³H]RNA was isolated by phenol extraction and ethanol precipitation from 24 pooled tecta and fractionated by SDS-polyacrylamide tube gel electrophoresis. Data from these experiments showed that 70% of the [³H]RNA is present as 4 S RNA 12 days after injection, and 60 days after injection, this value is still approximately 50%. When nerves were cut 36 days after injection (54 days post-crush) and allowed to degenerate for 6 days before determining tectal [³H]RNA, the majority of the 4 S [³H]RNA was lost from the tectum. This indicates that up to 36 days after injection, 4 S [³H]RNA remains within regenerating axons and that it is unlikely that significant amounts of axonally transported 4 S RNA are transferred out of the axon to surrounding cells. In other experiments, the time of sacrifice after injection was held constant and the time of injection after nerve crush was varied. Analysis of tectal [³H]RNA showed that the major period of axonal transport of 4 S [³H]RNA was in the early stages of regeneration at 24 and 36 days after nerve crush and that the amount of 4 S [³H]RNA which is transported axonally decreases at later time points. Calculations of the amount of axonal 4 S [³H]RNA versus what is synthesized in periaxonal cells gave results suggesting that 4 S RNA in regenerating axons turns over relatively slowly when compared with 4 S RNA in tectal cells.

Although there is little evidence for protein synthesis in axons, the presence of RNA has been demonstrated in a wide variety of axons (Edstrom, 1964; Lasek, 1970; Peterson et al., 1968; Gambetti et al., 1978). It is likely that the origin of this RNA is the neuronal cell body and that it appears in the axon as a result of axonal transport (Ingoglia et al., 1975; Gambetti et al., 1978; Por et al., 1978; Gunning et al., 1979b). The molecular species of RNA transported is somewhat in doubt. In squid, where axoplasm can be analyzed with relative certainty that it is free of contaminating periaxonal cellular elements, the principal RNA species has been found to be 4 S RNA (Lasek et al., 1973; Black and Lasek, 1977), but recent

evidence reports the presence of small amounts of ribosomal RNA (Giuditta et al., 1980). In vertebrates, there is also some controversy as to which RNA species are contained in axons. Koenig (1979) recently has reported the presence of all molecular species of RNA in Mauthner axons, but other data indicate that, in the regenerating optic axons of goldfish (Ingoglia and Tuliszewski, 1976), in optic (Por et al., 1978) and sciatic nerve axons of chickens (Gunning et al., 1979b), in developing optic axons of rats (Politis and Ingoglia, 1979), and in both intact and regenerating axons of the sciatic nerve of rats (Lindquist and Ingoglia, 1979; Lindquist et al., 1981), the principal, if not the only, intra-axonal RNA species is 4 S RNA. Recent data indicate the presence of 4 S RNA in axons of adult rabbits (R. W. Ledeen, personal communication) and rats (M. F. Zanakakis and N. A. Ingoglia, unpublished findings).

In the regenerating optic nerves of goldfish, 4 S RNA, which is transported axonally from the retina to the tectum, has been found by quantitative EM autoradiography to be present in axonal growth cones as well as in

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regenerating optic axons (Gambetti et al., 1978). Under the conditions employed in these experiments, the time course of regeneration is such that axons reconnect with the tectum approximately 3 weeks after crushing the optic nerve, and myelination and maturation of the fibers occur over the subsequent 2 to 3 months (Murray, 1976). In previous experiments, we have investigated the axonal transport of 4 S RNA during the earliest (reconnection) stage of regeneration (Ingoglia and Tuliszewski, 1976; Ingoglia, 1979). In the present experiments, the axonal transport of 4 S RNA has been examined during later maturational stages of axonal growth with the ultimate purpose of defining its physiological role in the growth of axons.

Materials and Methods

Goldfish (*Carassius auratus*, 10 to 12 cm in length) were obtained from Merrit Imports, Paramus, NJ and [^3H]uridine (25 to 30 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). The procedures followed for optic nerve crushes and cuts and intraocular and intracranial injections have been described previously (Ingoglia and Tuliszewski, 1976). [^3H]RNA was extracted from homogenized goldfish tecta with phenol and precipitated with 0.25 M NaCl in ethanol by conventional techniques (see Ingoglia and Tuliszewski, 1976). Electrophoresis of 2.4% and 10.0% acrylamide, bisacrylamide SDS gels was performed according to procedures described elsewhere (Politis and Ingoglia, 1979) but essentially followed the techniques described by Loening (1970) and Weber and Osborn (1969). Upon completion of electrophoresis, gels were scanned for absorbance at 260 nm, cut in 2-mm segments, and dissolved in 10% piperidine as described previously (Ingoglia and Tuliszewski, 1976). Radioactivity was determined in a

Beckman liquid scintillation counter and raw counts were converted to disintegrations per min using equations derived from quench correction curves programmed into a laboratory computer facility.

Results

In the first series of experiments, both optic nerves were crushed in 96 fish, and 18 days later, [^3H]uridine was injected into both eyes. In normal goldfish (optic nerves not regenerating), the amount of [^3H]RNA arriving in the tectum is maximal 6 or 12 days following a single intraocular injection of [^3H]uridine (Ingoglia et al., 1973). Thus, in these and subsequent experiments, this time point has been chosen as a value for non-regenerating controls. Fish were killed at times ranging from 6 to 180 days after injection. RNA was extracted from 24 pooled tecta (12 fish) and RNA species were separated by SDS-polyacrylamide gel electrophoresis as described above.

The radioactivity present in phenol and ethanol fractions was determined and assumed to be composed of nucleosides, nucleotides, and $^3\text{H}_2\text{O}$ (Ingoglia and Pilchman, 1980). This fraction has been termed "non-RNA" radioactivity (Fig. 1). The radioactivity in this fraction reaches maximum levels 12 days after injection and declines gradually thereafter up to 180 days. The radioactivity in the RNA fraction (ethanol precipitable) increases gradually up to 60 days after injection and then declines to near control levels by 180 days after injection (Fig. 1). At each time point, RNA was fractionated by electrophoresis on 2.4% and 10.0% polyacrylamide gels. Typical profiles of 2.4% gels for non-regenerating controls (Fig. 2A) show radioactivity peaks coinciding with optical density peaks, with approximately 15% of the radioactivity associated with low molecular weight RNA. In fish

Figure 1. Radioactivity in the goldfish optic tecta in an RNA fraction ($\blacktriangle$) and a "non-RNA" fraction ($\bullet$), composed of ^3H -nucleosides, nucleotides, and $^3\text{H}_2\text{O}$ at various times after the intraocular (I.O.) injection of [^3H]uridine. Injections were made 18 days after both optic nerves had been crushed, and RNA was extracted from tectal homogenates with phenol and precipitated with ethanol (see "Materials and Methods"). Each time point represents radioactivity extracted from 24 tecta (12 fish).

Figure 2. Typical radioactivity profiles of [^3H]RNA extracted from goldfish optic tecta and layered on 2.4% polyacrylamide gels. Electrophoresis was carried out for 1.5 hr and gels were cut in 2.0-mm slices. Profiles are taken from fish in which [^3H]uridine was injected 6 days prior to killing the fish (*A*, control) and from fish in which both optic nerves were crushed 18 days prior to injection of [^3H]uridine and killed 60 days after injection (*B*). Note the large amount of radioactivity in the low molecular weight region of the gel in *B* compared with *A*. When placed on 10% gels, the majority of this radioactivity co-migrates with 4 S RNA markers.

with regenerating nerves, the RNA profiles 6 or 12 days after injection were similar to those published previously (see Fig. 1, *A* and *C* of Ingolia, 1979), with the most obvious feature being the marked enrichment of radioactivity in the low molecular weight region of the gel. This feature is still obvious in fish in which optic nerves were regenerating for 78 days (60 days after injection of [^3H]uridine (Fig. 2*B*)).

Radioactivity profiles obtained from 10.0% gels were used to determine the proportion of radioactivity associated with 4 S RNA at each time point. On these gels, low molecular weight RNAs were separated into distinct 4 S, 5.0 S, and 5.8 S RNA peaks with the majority of the radioactivity co-migrating with 4 S RNA. The profiles (not shown) were, in all cases, similar to those reported previously (Ingolia and Tuliszwski, 1976). The fraction

of radioactivity associated with 4 S RNA has been expressed as a percentage of the total radioactivity on a gel (Fig. 3). At its peak (12 days after injection), greater than 70% of the tectal RNA-associated radioactivity is present as 4 S RNA. This value then decreases gradually, approaching control levels 180 days after injection, but is still approximately 35% of the total RNA radioactivity 4 months after a single intraocular injection (Fig. 3). Two sets of control fish, in which injections were made into the eye in the absence of nerve regeneration (*control I*, Fig. 3) or injections were made intracranially during regeneration (*control II*), had no more than 10 to 20% of the radioactivity present as 4 S RNA.

In all experimental fish, [^3H]RNA in the tectum is found in both axons and periaxonal cells (Gambetti et al., 1978). A fundamental question related to intra-axonal 4

Figure 3. Results of fractionation of [^3H]RNA (from experiments described in Fig. 1) by electrophoresis on 2.0% and 10.0% polyacrylamide gels. Data show the proportion of radioactivity associated with 4 S RNA for experimental fish, fish in which optic nerves were not crushed (non-regenerating controls), and fish in which optic nerves were crushed but injections of [^3H]uridine were made intracranially rather than into the eye, thus labeling tectal cells but not axons. The open bars show the percentage of radioactivity in 4 S RNA in experiments in which optic nerves were cut 6 days prior to killing the fish. In the latter experiments, note that the proportion of radioactivity in 4 S RNA is similar to control levels. Since optic axons are not present in these tecta, it is likely that 4 S [^3H]RNA which is transported axonally is lost from the tectum along with degenerating axons and that 4 S [^3H]RNA remains within axons following its axonal transport.

S RNA is whether it remains intra-axonal following transport or if it is transferred to periaxonal cells. To answer this question, an axonal degeneration experimental paradigm was followed similar to that described previously (Ingolia, 1979; Lindquist et al., 1981). Essentially, this involves repeating the experiments described in Figure 3 except that optic nerves are cut 6 days prior to killing the fish. This procedure removes optic axons from the tectum, and it is assumed that, if 4 S RNA remains intra-axonal following axonal transport, it too will be lost as a result of axonal degeneration. This was found to be the case (see Fig. 3, open bars) regardless of whether optic nerves were cut 6 or 36 days after injection. The data suggest that, at least up to 36 days after injection, axonally transported 4 S [^3H]RNA remains intra-axonal following its migration in regenerating axons.

Using this information, we have attempted to discriminate axonal from perikaryal tectal [^3H]RNA. To do this, we assume that only 4 S RNA is intra-axonal, that it remains intra-axonal during and after its transport, and that approximately 15% of the periaxonal cellular RNA is 4 S RNA (as is the accepted level in other cells and is demonstrated to be the case in the tectal cells of the fish; see control II, Fig. 3). Thus:

$$\text{perikaryal } [^3\text{H}]\text{RNA} = \frac{\text{total } [^3\text{H}]\text{RNA} - 4 \text{ S } [^3\text{H}]\text{RNA}}{0.85} \quad (1)$$

and

$$\text{axonal } [^3\text{H}]\text{RNA} = \text{total } [^3\text{H}]\text{RNA} - \text{perikaryal } [^3\text{H}]\text{RNA} \quad (2)$$

This calculation shows that the amount of 4 S RNA in the axon increases dramatically between 6 and 12 days

after injection but then remains constant up to 60 days after injection (Fig. 4). The amount of periaxonal [^3H] RNA gradually increases with time, up to 60 days. Since this fraction represents the [^3H]RNA synthesized in the tectum from axonally transported ^3H precursors, one would predict that cellular [^3H]RNA would reflect the level of radioactive precursors in the tectum. This appears to be the case and the data indicate a gradual utilization of axonally transported precursors for RNA synthesis with a relatively slow turnover for RNA in tectal cells.

In the next series of experiments, the time of injection after crushing was varied, while the time of sacrifice after injection was held constant. Thus, both optic nerves of 48 fish were crushed, and 18, 30, 45, or 60 days later, [^3H] uridine (4 μCi in 4 μl) was injected into both eyes. [^3H] RNA was isolated and fractionated by gel electrophoresis as described above. Total transported "non-RNA" radioactivity (including [^3H]uridine and its derivatives) is greatest during the early stages of regeneration, at 24 and 36 days after crushing (Fig. 5A), and the level of 4 S [^3H] RNA is also greatest at these time points, although more than 20% of the radioactivity is present as 4 S RNA at the two later time points (51 and 66 days; Fig. 5B). Determinations of cellular [^3H]RNA and axonal [^3H] RNA were made as was done for the data shown in Figure 4. The results show that the greatest amount of 4 S [^3H]RNA transport occurs at the early stage of regeneration (close to the time of reinnervation) and that the amount transported to the tectum decreases to near normal levels by 66 days after the crush (Fig. 6). [^3H] RNA synthesis in tectal cells is somewhat variable. However, it appears that there is a gradual decrease in the amount of precursor transported to the tectum (Fig. 6),

Figure 4. Data are taken from the experiments shown in Figures 1 and 2. The values for axonal and perikaryal RNA were determined as described in the text. Note that the level of RNA in the axon reaches a maximum 12 days after injection and continues at high levels up to 60 days later. The amount of perikaryal RNA increases steadily up to 60 days and then decreases sharply and behaves in a predictable precursor-product fashion when compared with unincorporated tectal radioactivity (●).

returning to control levels 66 days after the crush, with a corresponding decrease in the amount of incorporation of precursor into [³H]RNA. The exception to this is at 51 days after the crush when there is a peak of perikaryal RNA synthesis (greater than 3 times controls) at a time when precursor levels are near normal (Fig. 6).

Discussion

The purpose of these experiments was to examine some of the characteristics of RNA present in the axons of the regenerating optic nerves of goldfish. Previous experiments have shown that this RNA is primarily, if not exclusively, 4 S RNA (Ingolia and Tuliszewski, 1976; Ingolia, 1979) and that it is transported axonally during optic nerve regeneration (Gambetti et al., 1978).

The results of the present experiments indicate that 4 S RNA remains within the axon following transport and is not transferred intact to surrounding cells (see Fig. 3). This result might have been predicted from experiments studying transcellular transfer of molecules in metabolically coupled cells (Pitts and Simms, 1977), but studies in squid axons have shown that the transfer of macromolecules does occur between glia and axons (Lasek et al., 1977). Other experiments have shown that protein transfer from glia to axons does not occur in goldfish (Gambetti et al., 1980), but phospholipids are reported to be transferred from the axon to surrounding cells in both peripheral (Droz, 1979) and central (Haley and Ledeen, 1979) axons. The finding that 4 S RNA is not transferred from axons to surrounding cells is important since it rules out one of the possible roles of 4 S RNA, i.e., that it is acting as transfer RNA in glia surrounding the axon and that the axon is a supplier of tRNA to glia (Lindquist and Ingolia, 1979). It now seems reasonable to conclude that, whatever biochemical role 4 S RNA is playing, it is playing that role within the axon.

One of the most intriguing aspects of this study is the data showing that, 60 days after a single intraocular injection of [³H]uridine, there is approximately the same amount of 4 S [³H]RNA within the axons of the optic tectum as there was 6 days after injection (Fig. 4). This could be due either to a continual supply of axonal 4 S RNA from the retina coupled with a turnover rate approximately equal to the retinal supply or to the delivery of a large amount of 4 S RNA 6 or 12 days after injection coupled with a slow turnover of 4 S RNA. Two lines of evidence argue for the latter explanation. First, if there was a continual delivery of 4 S [³H]RNA from the retina, one would predict relatively high or at least stable levels of [³H]RNA synthesized in the retina. In previous experiments, this was found not to be the case, as retinal levels of [³H]RNA drop by approximately 50% within 20 days after a single intraocular injection of [³H]uridine (Ingolia et al., 1973). The second and more convincing reason for concluding that axonal 4 S RNA has a long half-life in the tectum comes from the data shown in Figure 6. These experiments show that the amount of RNA delivered to the tectum is not constant during nerve regeneration. The largest amount is delivered at or near the time of reinnervation (24 days after the crush), and at 66 days post-crush, the amount of axonal 4 S RNA is near normal levels. Thus, the high level of axonal RNA 42 or 60 days after injection (60 or 78 days post-crush; see Fig. 4) could not be due to transport of 4 S RNA 6 days earlier but must be due to a slow turnover of 4 S [³H]RNA which arrived in the tectum by 12 days after injection.

The slow turnover rate for 4 S RNA is not a general feature of cellular 4 S RNA but may be true only for 4 S RNA which is within the axon. Following intracranial injections of [³H]uridine (to label cells but not optic axons; Gambetti et al., 1978), the percentage of radioactivity present as 4 S RNA decreases with time, indicating

Figure 5. Both optic nerves were crushed in a series of fish, and 18, 30, 45, and 60 days later, [^3H]uridine was injected intraocularly (*I.O.*). Fish were killed 6 days later and RNA was extracted from 24 pooled tecta as described earlier. RNA species were separated by polyacrylamide gel electrophoresis. *A* indicates total radioactivity in the RNA fraction and in unincorporated radioactivity, while *B* indicates the percentage of the [^3H]RNA in the tectum which is 4 S RNA.

that, if anything, the turnover of 4 S RNA is more rapid than ribosomal RNA (see *control II*, Fig. 3). In other experiments, the apparent half-life for 4 S RNA in chick brain was found to be on the order of 7 days, similar to that of 29 S (7 days) and 18 S (8 days) ribosomal RNAs (Gunning et al., 1979a). We conclude that the 4 S RNA transported axonally in regenerating optic nerves of goldfish reaches maximal levels by 12 days after injection and that this intra-axonal component degrades very slowly. The finding that the amount of intra-axonal 4 S [^3H] RNA remains relatively constant from 12 to 60 days after injection (Fig. 4) may indicate that, during this active period of reinnervation, synapse formation, maturation of axons, and myelin formation (Murray, 1976), there is little turnover of axonal 4 S RNA. Later, between 60 and 120 days after the injection, the axonal 4 S RNA turns over with a half-life of approximately 22 days (calculated by assuming 100% radioactivity at 60 days postinjection and approximately 15% of the radioactivity remaining at 120 days after injection).

[^3H]RNA synthesized in periaxonal cells gradually accumulates with time after a single injection of [^3H]uridine (Fig. 4). This perikaryal RNA fraction represents RNA

synthesized from axonally transported and transcellularly transferred precursors as described previously (Peterson et al., 1968; Gambetti et al., 1973, 1978; Lindquist et al., 1981; Politis and Ingoglia, 1979). We have proposed that this process represents the physiological route for the acquisition by periaxonal cells of some of the precursors necessary to build RNA molecules, and in this way, axons may control or influence periaxonal cell metabolism (Ingoglia and Pilchman, 1980). Thus, the "burst" of RNA synthesis seen 51 days after nerve crush (Fig. 6) may be the result of the axon meeting the metabolic demands of periaxonal cells by increasing the axonal delivery of RNA precursors at a particularly critical point in nerve regeneration.

In conclusion, these studies have shown that the 4 S RNA which is transported axonally during regeneration of the optic nerve of goldfish is: (1) transported maximally at early stages of nerve growth but is still transported 60 or more days later (Fig. 6), (2) not transferred to periaxonal cells but apparently functions intra-axonally, and (3) turning over at a slow rate, with a biological half-life likely to be greater than 22 days.

Recently, we have demonstrated that, when axonally

Figure 6. Perikaryal and axonal RNA as determined in Figure 3. The data are taken from the values shown in Figure 4. Note the "burst" of RNA synthesis in perikaryon apparent 51 days after crushing the nerve. This is presumed to be due to increased periaxonal cell utilization of axonally transported RNA precursors. Also, the largest amount of axonal RNA is seen 24 days after the crush, with the amount decreasing steadily up to 60 days after crushing the nerve.

transported 4 S [³H]RNA is passed through a benzoylated DEAE-cellulose column, its chromatographic profile is similar to *Escherichia coli* tRNA and tRNA isolated from the goldfish tectum (Zanakos et al., 1981). Other experiments indicate that, in the same system, axonal 4 S RNA forms a complex with axonally transported spermine (Ingolia et al., 1982). The association of spermine and 4 S RNA has been shown to be necessary for the biological activity of transfer RNA (Lovgren et al., 1978; Cohen, 1978; Takeda, 1978). Thus, it seems likely that the 4 S RNA transported axonally in regenerating optic axons of goldfish, like 4 S RNA in squid axons (Black and Lasek, 1977), is transfer RNA. This conclusion also was reached by Gunning et al. (1979b) in studies of chicken sciatic nerves. One of the possibilities which has been raised for the function of tRNA in axons is that it is acting in the absence of ribosomes as a donor of amino acids to axonal proteins and thereby is modifying proteins after translation has occurred (Black and Lasek, 1977; Ingolia, 1979; Gunning et al., 1979b). These reactions have been described in a variety of tissues (Soffer, 1974, 1980), including brain (Barra et al., 1973). The current findings are consistent with this role for tRNA in regenerating optic axons of goldfish.

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