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MIGRATION OF RIBOSOMES ALONG THE AXONS OF THE CHICK VISUAL PATHWAY

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Summary

The axoplasmic migration of ribosomes has been detected in the visual system of the chick. Monocular injection of radioactive uridine or an amino acid mixture was followed by sedimentation analysis in sucrose or cesium sulfate density gradients, of ribosomes prepared from the retinae of injected eyes and the left and right optic lobes. By this means both RNA and protein components of ribosomes were found to migrate from the retina to the innervated contralateral optic lobe. Following denervation of the distal nerve segment by eye removal, the stability of the transported RNA was reduced, suggesting its presynaptic location. The transport of RNA was not significantly impaired by intraocular injection of inhibitors of informational RNA or mitochondrial RNA synthesis prior to injection of radioactive uridine but was depressed by a low dose of actinomycin D.

Introduction

There is considerable evidence for the proximodistal migration of macromolecular RNA along the nerve fiber [1—7]. However, the migration of RNA precursors and their possible transfer between axons and surrounding glia has made it difficult to definitively demonstrate the intraaxonal migration of RNA [8—10]. In contrast, the axoplasmic transport of proteins is well established, and very little migration of free amino acids has been detected [11—13]. Also, transneuronal passage of macromolecular protein is known to be minimal [14].

In an attempt to resolve the problem of RNA migration, we have studied the axoplasmic transport of both the RNA and constituent proteins of purified ribosomes. The avian visual pathway has been used because the total crossover of the optic tract leads to each optic lobe being innervated solely from the contralateral eye [15]. We are also reporting on the effect of denervation on the stability of ribosomal RNA that appears to have been transported distally along the axon and the effect of inhibitors on this process.

Experimental procedures

Sucrose and cesium sulfate gradients

Isotopes were monocularly injected into the left eye of 12 two-day-old chicks using a 30 gauge needle and a volume of 10 μl. For studies of protein migration, a total of 2.0—3.1 mCi of gaseously labelled amino acids (New England Nuclear Corp. Boston, Mass.) was used. The composition of the reconstituted mixture was: (values are specific activity in Ci/mmol and the μCi amino acid/mCi mixture, Ala (18.3, 80), Arg (27.3, 70), Asp (23.7, 80), Glu (16.2, 125), Gly (8.6, 40), His (2.9, 15), Ile (73.5, 50), Leu (67.6, 140), Lys (55, 60), Phe (5.6, 80), Pro (35, 50), Ser (3.4, 40), Thr (2.1, 50), Tyr (69.4, 40), Val (4.8, 80).

Ribosomes were prepared by homogenizing tissues in 15 ml 0.32 M sucrose. In studies with [^{3}H]uridine, 4 whole, non-radioactive chick brains were added to each sample as carrier. In protein migration studies, two whole chick brains were added to each retinal sample. Homogenates were centrifuged for 7 min at 21 000 × *g* and 4 ml 0.08 M KCl, 0.0125 M $MgCl_2$, 0.05 M Tris · HCl (pH 7.8) was added to each supernatant together with puromycin to a final concentration of 1.25 mg/ml. This was then incubated at 37° C for 30 min, after which the mixture was brought to 1% sodium deoxycholate and centrifuged for 90 min at 100 000 × *g*. Puromycin incubation was found necessary in order to achieve complete dissociation of the ribosomes. The final precipitate was resuspended in the KCl · $MgCl_2$ · Tris medium and centrifuged for 10 min at 8500 × *g* to remove large aggregated particles. The supernatant was brought to 0.88 M KCl, and 0.6—1.0 ml of this was layered on a sucrose or Cs_2SO_4 gradient.

12-ml linear gradients (10—30% sucrose) were prepared in 0.88 M KCl, 0.0125 M $MgCl_2$, 0.05 M Tris · HCl (pH 7.8) and centrifuged for 105 min at 20° C and 200 000 × *g* in a Beckman SW41 rotor [16]. 5-ml Cs_2SO_4 gradients were generated by centrifugation of a solution of Cs_2SO_4 (1.35 g/ml) in 0.02 M $MgCl_2$ and 0.05 mM Tris · HCl (pH 7.0) for 20 h at 0° C. Preliminary experiments showed brain ribosomes to be much more stable in Cs_2SO_4 than CsCl. Fractions were collected from gradients after puncturing tube bottoms (3 drops from sucrose and 4 drops from Cs_2SO_4). Cs_2SO_4 density was determined at several points along the tube by weighing 10 μl aliquots. 1 ml water was added to each fraction, and the absorbance at 260 nm determined. Samples were then counted in 20 ml 3a70 scintillation fluid (Research Products Int. Corp. Elk Grove Village, Ill.) using a Picker Liquimat Counter at an efficiency of 32—36%. In order to calculate radioactivity per unit absorbance, areas under curves were determined by weighing their cut-out profiles. These values were used to compare specific activities of RNA from left and right optic lobes.

Inhibitor studies

The effect of various compounds on the axonal transport of RNA was determined by injection of inhibitors into the left eye 2 h prior to injection of 10 μCi [^{3}H]uridine into the same eye. Three days after uridine administration, total radioactivity within RNA of the retina from the injected eye and the optic lobes was measured. Tissues were homogenized in cold 5% (w/v) trichlo-

roacetic acid and centrifuged. The precipitate was further washed with cold trichloroacetic acid (twice) and finally with ethanol. The precipitate was then suspended in 2 ml 0.1 M NaOH for 18 h at 37° C in order to hydrolyze RNA. This suspension was then acidified with 0.05 ml 70% perchloric acid to reprecipitate DNA and protein. One ml samples containing hydrolyzed RNA were mixed with 10 ml 3a70. 0.2 ml M HCl was added prior to counting in order to reduce chemiluminescence. The proportion of RNA that migrates distally along the axon was calculated for each bird by use of the formula:

$$\frac{R - L}{E + (R - L)}$$

E, R and L are the counts within RNA of the left retinal, right optic lobe and left optic lobe respectively. Data expressed in this way are independent of (a) the size of the non-radioactive RNA precursor pool in the retina or the brain, and (b) the extent of cerebral uptake of isotopes from the circulatory system.

The mean value and standard error of this figure was calculated, using 6—11 animals for each experiment.

Results

Ribosomal RNA

Thirty days after monocular [^{3}H]uridine injection, the specific radioactivity of both the 60 S and 40 S ribosomal units in the sucrose gradients was around ten times higher in the optic lobes contralateral to the injected eye than in the ipsilateral lobes (Figs 1A, 1B). Relatively little radioactivity remained at the top of the gradient indicating that the bulk of the label in the applied ribosomal suspension was within ribosomes.

In a parallel study, an equal number of chicks had their injected eye removed 3 days after [^{3}H]uridine administration. At this time, the major part of migration of RNA from the retina to the innervated optic lobe has occurred [5]. After a further 4 weeks, the specific activity of RNA of the ribosomes of the optic lobe contralateral to the injected and removed eye was only 31 ± 6% of that of the corresponding lobes of the unoperated chicks (figs 1C, 1D). Degeneration of the distal terminals of the retinal ganglion cells has taken place by 28 days after nerve section [17] while major transneuronal degeneration is not evident. This suggests that the major part of migrating RNA is dependent on the integrity of the presynaptic terminations of the optic nerve.

Ribosomal protein

In order to label ribosomal protein to a relatively high degree, it was necessary to inject 2—3 mCi of a mixture of [^{3}H]amino acids. Six days after injection of such a mixture into the left eye, ribosomes from various regions were subjected to sucrose density gradient sedimentation. Considerable radioactivity was present in the ribosomal subunits from the right optic lobe while those of the left optic lobe contained very little label (Figs 2A, B). Under the dissociating conditions used, the bulk of radioactivity within protein was not within ribosomal subunits but remained toward the top of the gradient. Simi-

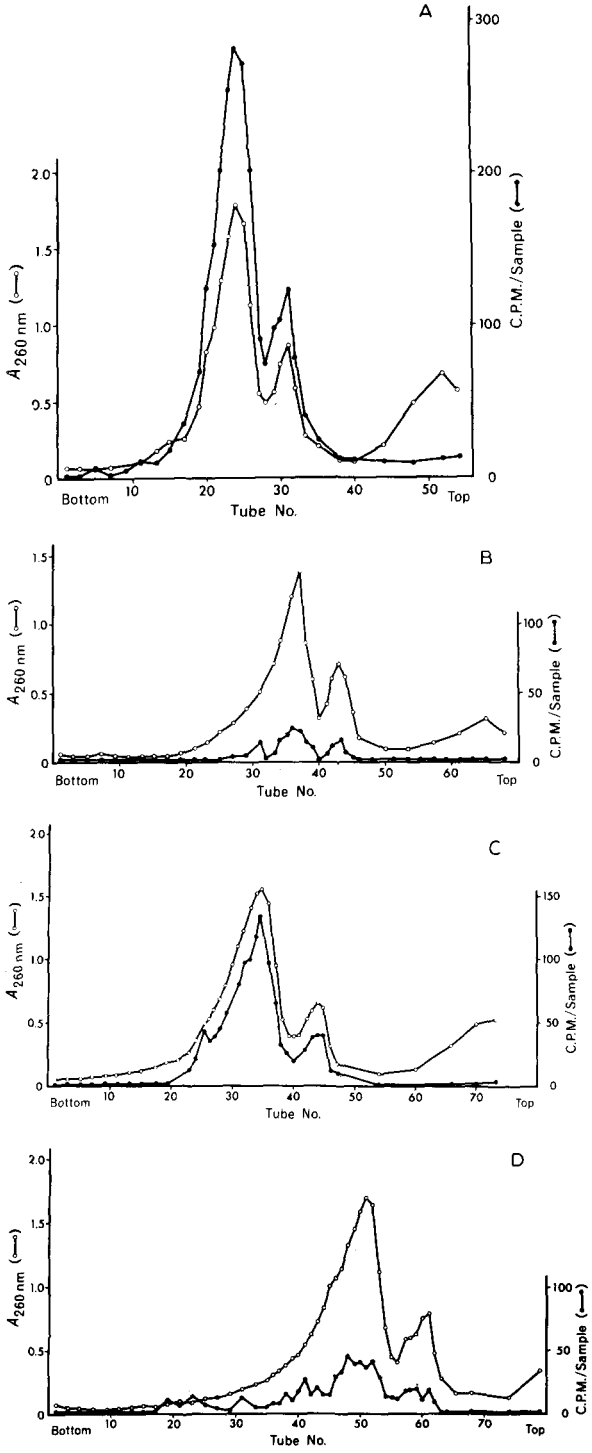

Fig. 1. Sucrose density gradient sedimentation profile of ribosomes prepared from optic lobes, 30 days after injection of [^{3}H]uridine into the left eyes of 2 day old chicks. A, C = right optic lobes, B, D = left optic lobes. In C and D the left eye was removed 3 days after isotope injection (details in Experimental Section). ○———○ absorbance at 260 nm/fraction; ●———● = cpm/fraction.

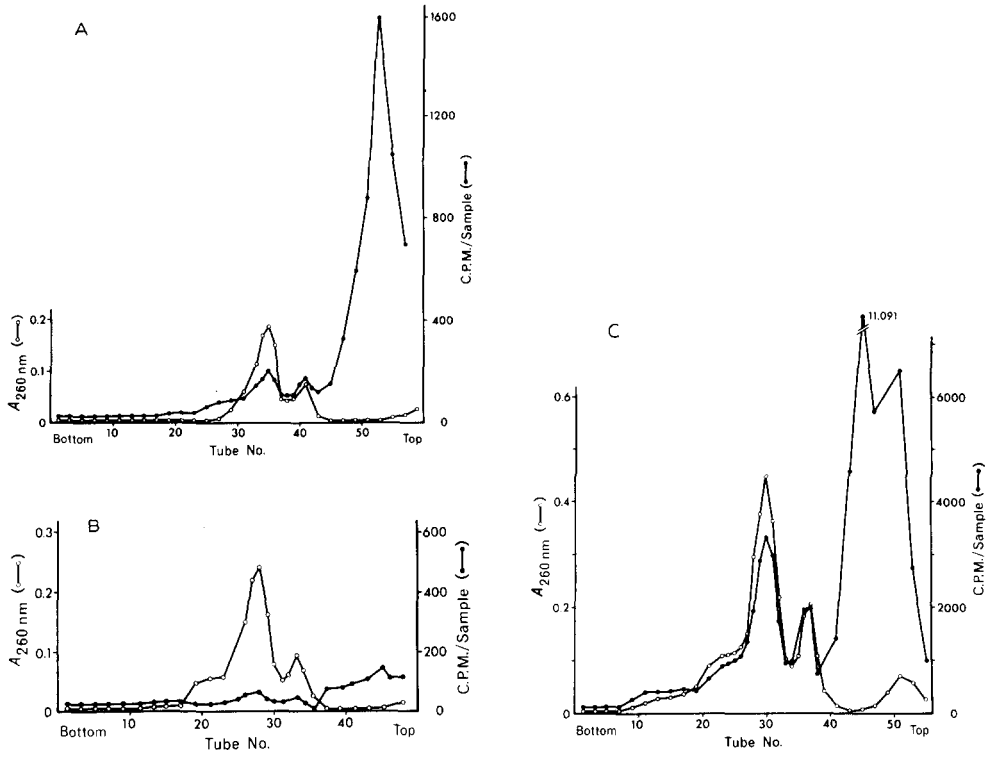

Fig. 2. Sucrose density gradient sedimentation profile of ribosomes prepared A, right optic lobes, B, left optic lobes, C, left retinae, 6 days after injection of [^{3}H]-labelled amino acids into the left eyes of 2 day old chicks (details in Experimental Section) ○———○ absorbance at 260 nm/fraction; ●———● = cpm/fraction.

larly, sedimentation analysis of the retinal ribosomal preparation (Fig. 2C) revealed that over 70% of the radioactive protein was not ribosomal. This non-ribosomal peak from the retina or right optic lobe migrated some distance into the gradient, and the radioactivity was precipitable with 10% trichloracetic acid. Thus it did not consist of free amino acids. Furthermore, no significant asymmetry of trichloracetic-acid soluble materials from left and right optic lobes was present 6 h and 6 days after isotope injection. The proportion of true ribosomal protein migrating to the optic tectum relative to that remaining within the retina was around 16%.

In order to more vigorously separate ribosomes from contaminating proteins, deoxycholate-treated ribosomal pellets were centrifuged in a Cs_2SO_4 gradient. Prior fixation of ribosomes with formaldehyde was not carried out. While some ribosomal protein is stripped off by non-fixation, maximally dissociating conditions [18] the possibility of non-ribosomal protein binding to ribosomes is minimized [19]. A single ribosomal peak was apparent in all centrifugal runs, and this peak had an equilibrium density of 1.33—1.34 g/ml (Figs 3A, B, C). This is similar to previously reported values for eukaryotic ribosomes in Cs_2SO_4 [19]. The peaks had an absorption minimum at 240 nm and a maximum at 260 nm.

As with the sucrose density gradients, ribosomes from the left optic lobe

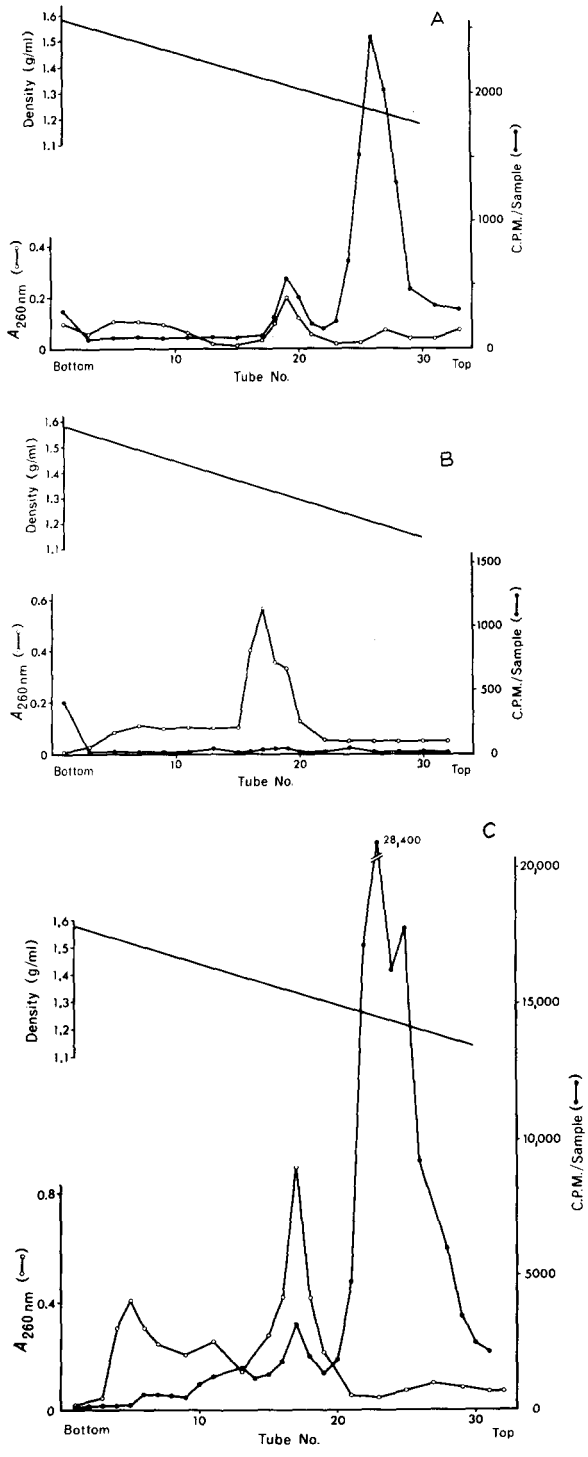

Fig. 3. Cesium sulphate sedimentation profile of ribosomes prepared from A, right optic lobes, B, left optic lobes, C, left retinae, 6 days after injection of [^{3}H]-labelled amino acids into the left eyes of 2 day old chicks (details in Experimental Section). ○——○ absorbance at 260 nm/fraction; ●——● = cpm/fraction; ——— gradient density g/ml.

TABLE I

PERCENTAGE OF RETINALLY SYNTHESIZED RNA APPEARING IN THE CONTRALATERAL OPTIC LOBE 3 DAYS AFTER INTRAOCULAR INJECTION OF INHIBITORS AND [^{3}H]URIDINE

Inhibitors were monocularly injected into the left eye 2 h prior to injection of [^{3}H]uridine (see Experimental Section). After 3 days radioactivity in RNA of paried optic lobes and left retinal was determined and the extent of migration calculated using the formula $[R—L]/[E + (R—L)] \times 100$. E, R and L are the total amounts of label in RNA from the left retina, right optic lobe and left optic lobe respectively. Standard errors of the mean are given.

Inhibitor (μg)	Proportion of RNA migrating distally
None	1.9 ± 0.2
Ethidium bromide (10)	2.1 ± 0.6
Rifampcin (10)	2.5 ± 0.5
α-Amanitin (10)	3.0 ± 0.4
Actinomycin D (0.1)	0.7 ± 0.2

were virtually devoid of radioactivity (Fig. 3C) while those from the right optic lobe showed a clear radioactive peak coinciding with the ribosomal absorbance peak (Fig. 3B). The separation of radioactive ribosomes from the highly radioactive non-ribosomal peak of retinal and right optic lobe preparations, was clearer than that obtained with sucrose gradients. The proportion of retinally synthesized ribosomal protein that had migrated to the contralateral optic tectum was 22% of that remaining within the retina.

Inhibitors

Various materials were injected into the left eye 2 h prior to the intraocular injection of [^{3}H]uridine. Preliminary experiments ensured that the amount of each inhibitor injected did not reduce the rate of retinal RNA synthesis by over 60%. Compounds used included relatively specific inhibitors of eukaryotic mitochondrial RNA synthesis (rifampicin and ethidium bromide) [20—22], an inhibitor of RNA polymerase II, the non-nucleolar polymerase involved in mRNA synthesis (α-amanitin) [23], and actinomycin D which, at the concentrations used, predominantly inhibits rRNA synthesis [24]. Three days after drug treatment and isotope injection, the proportion of retinally synthesized RNA migrating to the optic tectum was determined (Table I).

Rifampicin and ethidium bromide have no significant effect on the proportion. However, actinomycin D at a concentration of 0.1 μg/eye reduced the value considerably. α-Amanitin at the concentration used was lethal to 25% of the chicks but did not reduce the proportion of RNA migrating distally in surviving birds. The apparently increased export of the total retinal RNA in this case may be due to the difficulty in completely removing the retina which was very fragile after α-amanitin treatment. The specificity of some of these pharmacological agents, notably rifampicin and α-amanitin has been questioned [25—26].

Discussion

The time period used for RNA studies was considerably longer than that used for the protein studies. This was because the RNA data included an

enucleation study. In this case we wanted to be sure that the primary synapses of the optic pathway had completely degenerated, for which we allowed 30 days. The shorter time used in the protein studies was because the small amount of radioactive ribosomal protein might have decayed and not been detectable after a month.

An unexpected result was the large amount of radioactivity in protein found at the top of both sucrose and Cs_2SO_4 gradients one week after injection of radioactive amino acids. 69—82% of total radioactivity was not within ribosomal subunits in the sucrose gradients, while 87—94% of radioactivity was not within the ribosomal peak in the Cs_2SO_4 gradients. This difference may be because the proteins of unfixed ribosomes are partly removed in Cs_2SO_4 gradients. The non-sedimenting proteins which were largely of non-ribosomal origin, had a high specific activity as their absorbance at 280 nm as well as that at 260 nm was very low. They appear to bind very strongly to ribosomes under most preparative conditions. The pellet obtained after high speed centrifugation of deoxycholate-treated postmitochondrial supernatants has been used to determine the half life of cerebral ribosomal protein [27,28]. In such reports it is likely that the turnover of these non-ribosomal proteins rather than that of true ribosomal protein was determined. In order to get sufficient incorporation into ribosomal protein, we have had to use large amounts of labelled amino acids. It may be that the rates of synthesis and decay of cerebral ribosomal protein are lower than has been reported.

Two major and interrelated questions arise concerning the possible axoplasmic transport of RNA. These are, whether the precise location of migrating species is truly within the axon and whether such molecules are high molecular weight RNA or soluble nucleotide precursors.

Autilio-Gambetti et al. [9] reported that after intraocular injection of [^{3}H]uridine in the rabbit, most radioactive RNA in the optic nerve was periaxonal rather than intraaxonal. However radioautography of optic nerve segments cannot readily reveal the location of those RNA species that are transported to the terminations of the optic pathway. The RNA migration rate reported by Autilio-Gambetti and co-workers was 37 mm/day, considerably faster than the rate of 10—15 mm/day we have obtained [5]. In contrast to our data, these workers did not find a discrete peak of RNA migrating along the optic pathway. Major biochemical differences between these visual systems are perhaps not surprising in view of the greater emphasis in the avian brain, on the primary areas innervated by the optic nerve.

A very rapid migration of free leucine (410 mm/day) along the sciatic nerve has been observed [29] so that the possibility exists that our data are due to such a transport, followed by incorporation of labelled amino acids into ribosomal protein by postsynaptic elements within the optic lobe. We consider this to be unlikely for the following reasons.

(1) We have not been able to detect a significant migration of free amino acids within our system. There is a considerable delay before increased radioactivity within ribosomes of the optic tectum, after intraocular injection of tritiated protein or RNA precursors. It is unlikely that such tritiated compounds could persist in a soluble form for several days without extensive dilution and conversion to ^3H_2O [30].

(2) The proportion of labelled ribosomal protein that appeared at the contralateral optic tectum a week after monocular isotope injection was between 13 and 17% of retinal ribosomal label in sucrose gradient studies and 22% of retinal ribosomal label in Cs_2SO_4 studies. This is similar to the proportion of total retinal protein migrating distally along the axon [31]. Thus although ribosomal protein is not rapidly labelled, the amount apparently migrating is not especially low. This further suggests a true migration of protein rather than precursor molecules.

The migration of ribosomal RNA along the axon to the presynaptic junction, rather than major migration of precursor nucleotides followed by postsynaptic RNA synthesis is suggested by

(1) The instability of ribosomal RNA following enucleation and consequent degeneration of the distal nerve. This suggests that this RNA is largely within the rapidly disappearing presynaptic structures.

(2) The selective inhibition of axoplasmic transport of RNA by low concentrations of actinomycin D and the lack of inhibition of this process by inhibitors of mitochondrial or messenger RNA synthesis. This is in contrast to the lack of inhibition of RNA labelling in the optic nerve of the rabbit following intraocular injection of actinomycin D and [^{3}H]uridine, found by Autilio-Gambetti et al. [9], again indicating a major biochemical difference between the chick and rabbit visual systems.

(3) The largely intra-axonal and presynaptic location of migrating RNA in the chick optic tectum following intraocular [^{3}H]uridine injection, as determined by electron microscopic radioautography (Bonnet and Bondy, in preparation).

For these reasons, we feel that a true migration of ribosomes takes place in a distal direction along the axons of the chick optic nerve. While the axoplasm of invertebrate giant axons contains predominantly RNA of low molecular weight [32] free extramitochondrial ribosomes have been observed within the axons of mammalian sensory neurons [33].

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