STUDIES ON RNA IN GOLDFISH BRAIN. I. ISOLATION AND *IN VIVO* LABELING*

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INTRODUCTION

The metabolism of brain RNA has been the subject of several recent investigations[7,16–19,27]. Interest stems in part from reports of changes in amount of total brain RNA under various functional stresses[4,26,30], changes in labeling patterns or in base ratios in individual brain cells with training[13,14] and from studies showing that various antimetabolites block memory formation[2,5,10].

Studies in this laboratory have shown that long-term memory formation of shock avoidance in the goldfish can be blocked by intracranial injections of puromycin or of acetoxycycloheximide[1]. Recently, we showed that actinomycin D has a similar effect[3]. In order to further study the chemical basis for these effects, we have devised methods for the isolation of various subcellular components of goldfish brain and for extracting RNA from them.

METHODS

Homogenization of brain. Goldfish (*Carassius auratus*) weighing 9–11 g were purchased from Ozark Fisheries, Stoutland, Mo., and were stored in 200 gallon tanks prior to use. The spinal cord was cut with scissors, and a cranial flap was removed. The brain was easily removed with a small spatula. All subsequent treatments were at 0–2°C unless otherwise stated. Brains (usually 15) were pooled in a chilled, loosely fitting manual Teflon–glass homogenizer containing 10 volumes of the following buffer (medium A): 0.32 *M* sucrose; 0.01 *M* Tris buffer, pH 7.35; 0.001 *M* $MgCl_2$; 0.5 mg/ml naphthalene disulfonate; 20 μg/ml polyvinyl sulfate. Following 15 up-down strokes, the homogenate was filtered through 4 layers of cheese cloth and

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centrifuged at 850 × *g* for 10 min. The crude nuclear pellet was suspended by gentle homogenization in 5 ml of medium A, then mixed with 26 ml of medium B (2.39 *M* sucrose; 0.05 *M* Tris buffer, pH 7.35; 0.001 *M* $MgCl_2$). After centrifugation at 25,000 rev./min in an SW 25.1 Spinco rotor for 1 h, the nuclear fraction was recovered as a small pink pellet. The dense sucrose together with a small amount of material at the interphase was decanted by rapid inversion of the tube and the excess sucrose was then wiped out. The 850 × *g* supernatant fraction was centrifuged at 15,000 × *g* for 15 min to sediment the mitochondrial fraction. The latter supernatant fraction was used as the source of cytoplasmic RNA.

Preparation of polyribosomes. Brains were homogenized as described above in 4 volumes of medium C: 0.25 *M* sucrose; 0.005 *M* $MgCl_2$; 0.025 *M* KCl; 0.05 *M* Tris buffer, pH 7.35. After centrifugation of the whole homogenate at 15,000 × *g* for 15 min, the supernatant fraction was pipetted off, made 0.5% with respect to sodium deoxycholate and then centrifuged at 35,000 rev./min in a No. 40 rotor for 90 min. The resulting polyribosomal pellet was washed twice with medium C and then suspended in 1 ml of the same buffer per g of original wet weight of brain.

Extraction of nucleic acids from nuclei. In procedure A, RNA was extracted according to a method described for HeLa cell nuclei[22]. Nuclei from 2 g of brain (25–30 goldfish brains) were lysed with 2 ml of medium HSB: 0.5 *M* NaCl; 0.01 *M* Tris buffer, pH 7.35; 0.05 *M* $MgCl_2$. The resulting nucleohistone gel was digested at room temperature with 200 μg of deoxyribonuclease. To the mixture, sodium dodecyl sulfate, ethylenediaminetetraacetate (EDTA) and polyvinyl sulfate were added to a final concentration of 0.5%, 0.05 *M* and 0.01%, respectively. After addition of an equal volume of phenol, the preparation was heated to 55°C for 3 min. Chloroform, 2 ml, containing 1% isoamyl alcohol was added and the mixture was agitated with a vortex mixer, then heated again to 55°C for 3 min. After centrifugation for 2 min at 2500 rev./min at room temperature, the lower (phenol) phase was removed by pipetting and 2 ml of chloroform–isoamyl alcohol were added to the combined upper phase and interphase. After recentrifugation, the upper phase was carefully removed and RNA was precipitated by the addition of 2 volumes of 95% ethanol containing 2% potassium acetate. After 2 h at —15°C, RNA was sedimented at 15,000 × *g* for 15 min.

In procedure B, we used a method described for the isolation of RNA from liver cell nuclei[24]. Nuclei from 2 g of goldfish brain were lysed by homogenization in 5 ml of medium D: 0.05 *M* sodium acetate buffer, pH 5.2; 0.3% sodium dodecyl sulfate; 0.14 *M* NaCl; 0.005% polyvinyl sulfate. Following the addition of an equal volume of phenol, the preparation was shaken at 60°C for 10 min and at room temperature for 15 min, and then centrifuged at 15,000 × *g* for 10 min. The aqueous phase was removed by pipet and treated again with phenol at room temperature for 10 min. After separation of the phases by centrifugation, the aqueous phase was treated with 2 volumes of 95% ethanol containing 2% potassium acetate. The RNA was precipitated overnight at 0°C. The ethanol precipitation was repeated twice more.

Extraction of cytoplasmic RNA. The postmitochondrial supernatant fraction was made 0.5% in sodium dodecyl sulfate and treated with an equal volume of phenol.

After shaking for 15 min at 4°C, the sample was centrifuged at 15,000 × *g* for 10 min. The upper phase was removed by pipet. An equal volume of medium A containing 0.5% sodium dodecyl sulfate was added to the remaining combined interphase and phenol phase, and the preparation was shaken at 4°C for 10 min. Following centrifugation, the upper phases were combined and phenol extraction was repeated. The final aqueous phase was treated twice with 0.5 volume of chloroform–isoamyl alcohol (4 : 1, v/v). RNA was precipitated at —15°C following the addition of 2 volumes of ethanol containing 2% potassium acetate as described above.

Density gradient centrifugation. Linear sucrose density gradients, prepared by means of a proportioning pump[9], were used to separate and characterize classes of RNA and polyribosomes. To study size distribution of nuclear or cytoplasmic RNA, the precipitated sample was first dissolved in 0.01 *M* sodium acetate buffer, pH 5.2. The solution was layered over 30 ml of a linear 10–40% sucrose gradient which contained 0.01 *M* sodium acetate buffer, pH 5.2, 0.1 *M* NaCl and 0.001 *M* EDTA. Tubes were centrifuged at 25,000 rev./min in a Spinco SW 25.1 rotor for 16 h at 5°C. Occasionally 4–20% sucrose gradients (in the same buffer) were employed. Samples were centrifuged at 25,000 rev./min for 14 h at 5°C.

Polyribosome suspensions were layered on a 10–25% sucrose gradient (0.05 *M* Tris buffer, pH 7.35, 0.025 *M* KCl; 0.005 *M* $MgCl_2$) and centrifuged at 25,000 rev./min in the SW 25.1 rotor for 2.5 h. In order to characterize monoribosomes and lighter particles, polyribosomes were centrifuged in a 10–30% sucrose gradient at 25,000 rev./min for 5.5 h. Following centrifugation, gradient tubes were punctured and the contents drawn by means of a constant flow pump through a 4 mm light path flow cell (LKB) which recorded transmittance at 254 mμ. Alternatively, gradients were pumped through a 5 mm ISCO flow-through cell fitted in a Gilford spectrophotometer. Absorbance at 260 mμ was recorded. Samples of about 1 ml were collected by drop counting. The dead space between the flow cell and the delivery tip was determined so that radioactivity determinations corresponded to observed transmittance or absorbance. Bovine serum albumin, 100 μg, was added to each tube followed by 3 ml of cold 10% trichloroacetic acid. Precipitates were collected on membrane filters (0.45 μ pore size, Gelman) and washed with cold TCA and ethanol. Filters were dried and counted in 15 ml of toluene containing 0.4% 2,5-diphenyloxazole (PPO) and 0.005% 1,4-bis-(5-phenyloxazolyl-2)-benzene (POPOP). Sedimentation coefficients in the case of RNA analyses refer to 28S assigned to the rapidly sedimenting component. In the case of polyribosome sedimentation, the value of 76S was assigned for the monoribosome peak.

Analytical ultracentrifugal analysis. Sedimentation was followed by schlieren optics in a Spinco Model E ultracentrifuge. S values were corrected to 20°C in water. The viscosity and density of the solvent were taken as that of water.

Materials. [5-^{3}H]Uridine, 8.0 C/mmole was obtained from Schwarz Bio-Research. Crystalline deoxyribonuclease was purchased from Worthington Biochemical Corp. Phenol (AR, Mallinkrodt) was distilled, saturated with water and stored frozen in the presence of 1% 8-hydroxyquinoline. Sodium dodecyl sulfate (Fisher) was recrystallized from hot 95% ethanol[8]. 1,5-Naphthalene disulfonate,

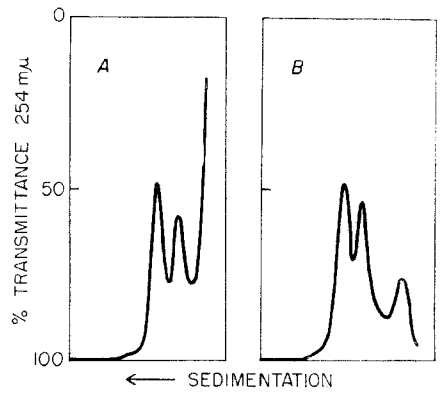

Fig. 1. Sedimentation analysis of goldfish brain nuclear RNA extracted according to two different procedures (see text for details). The direction of sedimentation in the 10–40% sucrose gradient is from right to left.

sodium salt and 8-hydroxyquinoline were obtained from Eastman Organic Chemicals. Polyvinyl sulfate was purchased from K and K Laboratories, Inc. and sodium deoxycholate (special enzyme grade) was obtained from Mann Research Laboratories, Inc.

RESULTS

Isolation of nuclear and cytoplasmic RNA from goldfish brain. Fig. 1 shows results of extraction of nuclear RNA by procedures A and B (see Methods). They

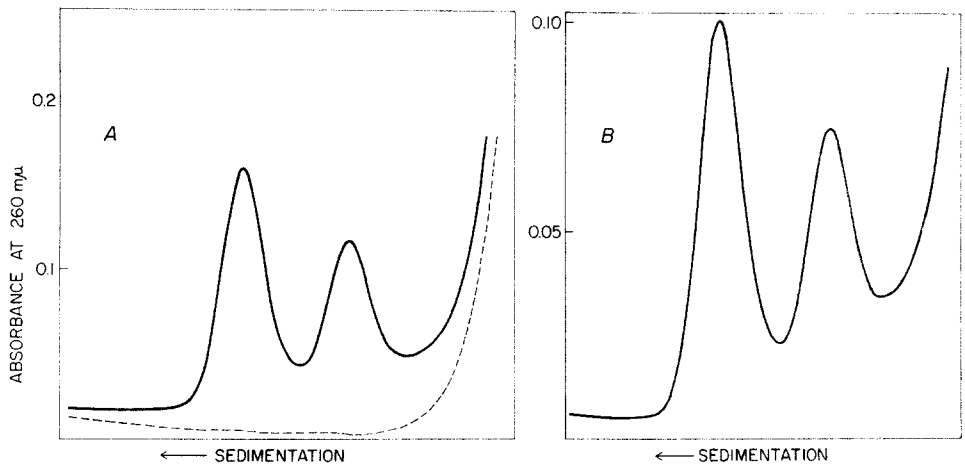

Fig. 2. Effect of detergent on the zonal centrifugation of nuclear RNA in 10–40% sucrose gradients. A, Brains were homogenized in the presence of 0.25% Triton X-100. Nuclear RNA was extracted according to procedure A (see text for details). In the absence of detergent (solid line) and in the presence of detergent (dashed line). B, Brains were homogenized in the presence of 0.25% of a mixture containing 1 part of 10% sodium deoxycholate and 2 parts of 10% Tween 40. Nuclear RNA was extracted according to procedure A.

involve respectively extraction at pH 7.35 following digestion with deoxyribonuclease and at pH 5.2 without deoxyribonuclease. Absorbance peaks corresponding to 28S and 18S RNA were seen in both instances, while 4S RNA was obscured by oligodeoxyribonucleotides in procedure A. Procedure B always resulted in a lower 28S : 18S ratio than procedure A. Occasionally a shoulder which suggested material sedimenting somewhat faster than 28S RNA was observed. When studies were performed on nuclei from previously frozen brain, gross degradation of nuclear RNA was observed with both procedures A and B.

Effect of detergents. Various detergents have been used during homogenization of cells to prevent contamination of the final nuclear pellet with endoplasmic reticulum[6,15,20]. In goldfish brain, Triton X-100 at a concentration of 0.25% caused gross degradation of nuclear RNA (Fig. 2A). In contrast, when brains were homogenized in the presence of a mixture of sodium deoxycholate and Tween 40 (ref. 22),

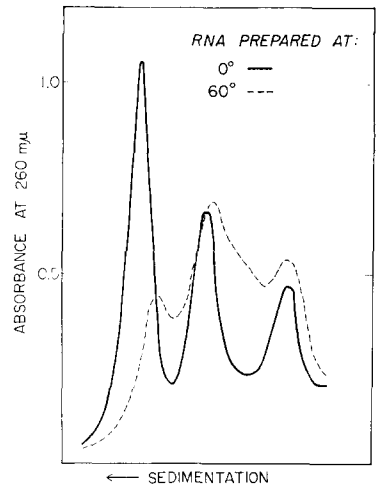

Fig. 3. Effect of temperature on extraction of brain cytoplasmic RNA. RNA preparations were centrifuged on a 4–20% linear sucrose gradient.

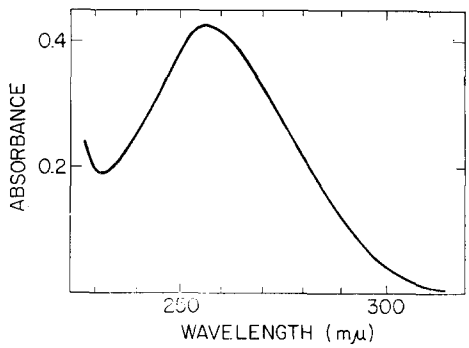

Fig. 4. Ultraviolet absorption spectrum of cytoplasmic RNA extracted with phenol at 0°C.

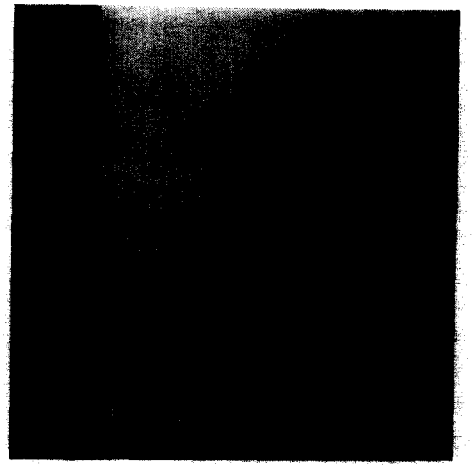

Fig. 5. Sedimentation velocity pattern of brain cytoplasmic RNA (500 μg/ml) in 0.01 *M* sodium acetate buffer, pH 5.2, containing 0.1 *M* NaCl. The photograph was taken 4 min after attaining a speed of 56,100 rev./min.

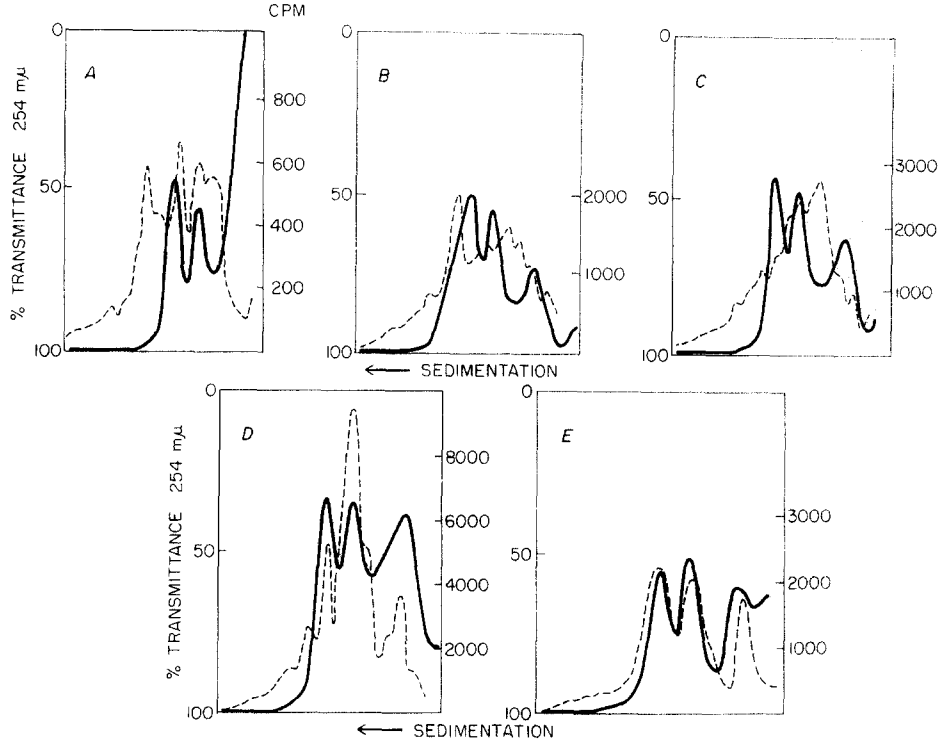

Fig. 6. Pattern of RNA labeling by [5-^{3}H]uridine in goldfish brain nuclei. Zonal centrifugation on a 10–40% sucrose gradient was performed on nuclear RNA from brains obtained 15 min (A), 30 min (B), 1 h (C), 2 h (D), and 17 h (E) after administration of the precursor. Solid line, % transmittance at 254 mμ; dashed line, trichloroacetic acid-insoluble radioactivity. Due to considerable variability in the amount of radioactivity incorporated, particularly for brief pulses, 2 or more experiments were performed for each time point. Representative runs were selected to illustrate the time course. A 3-fold batch of nuclei (from 80 goldfish brains) was used for each experiment. Fish received 10 μC of labeled precursor for the 15 min pulse, 7.5 μC for the 30 min pulse and 5 μC for longer times.

a morphologically clean nuclear fraction free of blood cells was obtained. No breakdown of RNA was observed during subsequent isolation by procedure A (Fig. 2B).

Effect of temperature on isolation of cytoplasmic RNA. When RNA was extracted with phenol at 0°C, three discrete RNA peaks corresponding to 28S, 18S and 4S were obtained (Fig. 3). After phenol extraction at 60°C, there was evidence of degradation. Sodium dodecyl sulfate and polyvinyl sulfate, inhibitors of ribonuclease, did not protect RNA at these temperatures. Cytoplasmic RNA extracted at 0°C gave a ratio of $OD_{258} : OD_{230} = 2.2$, characteristic of undegraded pure RNA (Fig. 4). In the analytical ultracentrifuge, we found sedimentation coefficients of 16.2 and 25.2 (Fig. 5). These values are probably minimal in view of the known concentration dependence of S_{20} values[21].

Distribution of rapidly labeled nuclear and cytoplasmic RNA. Groups of 10 goldfishes were each injected intracranially[2] with 5 μC of [5-^{3}H]uridine in 10 μl of 0.15 *M* NaCl. They were returned to their tanks and groups were sacrificed at various times indicated in Fig. 6. Nuclear RNA was labeled at the earliest times examined. There was no apparent correspondence with ultraviolet absorbance. A significant amount of radioactivity appeared in molecules heavier than 28S. Radioactivity

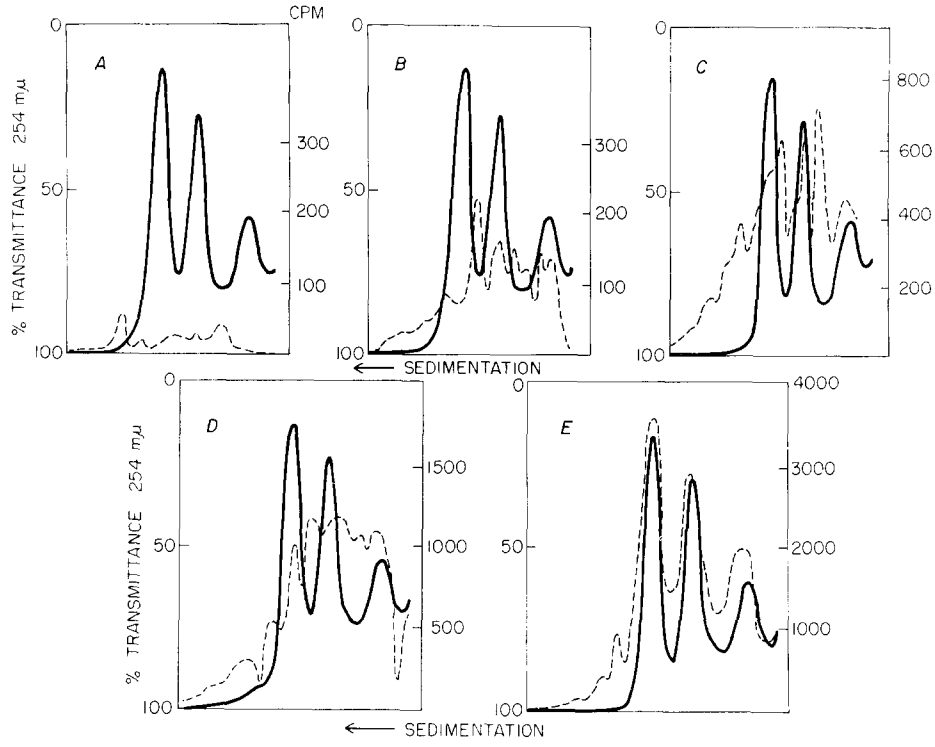

Fig. 7. Zonal centrifugation patterns of brain cytoplasmic RNA after labeling *in vivo*. The supernatant fraction following sedimentation of nuclei (see Fig. 6) was centrifuged at 15,000 × *g* for 15 min and about one-third of the resulting postmitochondrial fraction was used for extraction of cytoplasmic RNA. Half of this amount (10–12 OD_{260} units) was applied to the sucrose gradient.

sedimenting slightly faster than 28S was observed 30 min after injection. In addition, there was material of apparent high specific activity in the 4S–18S region. After 2 h, correspondence between ultraviolet absorbance and labeling appeared, the 18S RNA showing initially a higher specific radioactivity than 28S. Even after longer times, some radioactivity sedimenting more rapidly than 28S was present. Fig. 7 shows the labeling of cytoplasmic RNA at corresponding times. Radioactivity begins to appear in the cytoplasm 30 min after injection and is rather broadly distributed through the gradient. Correspondence with ultraviolet absorbance was not seen in samples taken earlier than 17 h. Rapidly sedimenting radioactivity (faster than 28S)

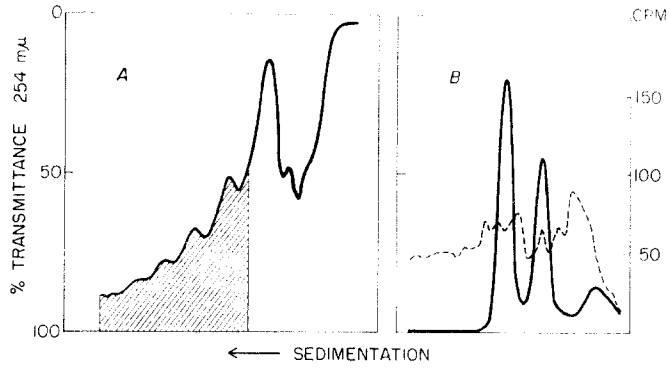

Fig. 8. Zonal centrifugation of goldfish polyribosomes and polyribosomal RNA. Twenty goldfish were each injected with 10 μC of [5-^{3}H]uridine and sacrificed 30 min later. Polyribosomes were fractionated on a 10–25% sucrose gradient (A). Heavy particles (shaded region) were pooled. Phenol-extracted RNA from this region is shown in (B). Solid line, % transmittance (254 mμ); dashed line, trichloroacetic acid-insoluble radioactivity.

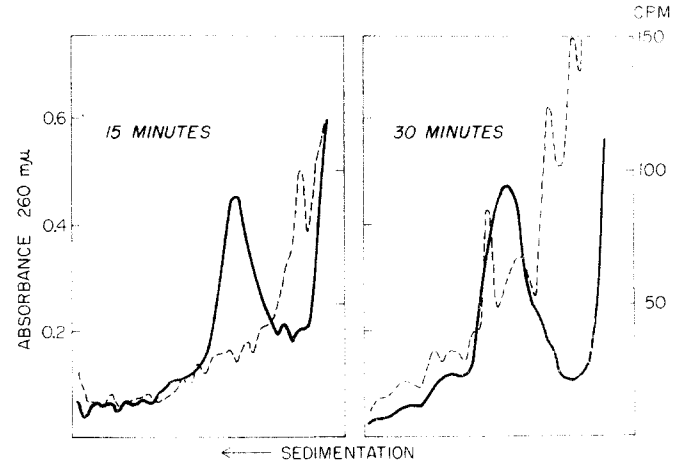

Fig. 9. Distribution of radioactivity in sodium deoxycholate-treated cytoplasmic fractions 15 and 30 min following administration of [5-^{3}H]uridine. Postmitochondrial supernatant fractions were made 0.5% with respect to deoxycholate sodium and centrifuged on a 10–30% sucrose gradient at 63,600 × g for 5.5 h. Solid line, absorbance at 260 mμ; dashed line, trichloroacetic acid-insoluble radioactivity. Twenty fishes were each injected with 10 μC of labeled precursor for each time point.

was seen in all fractions. Since it was possible that this material represents messenger RNA, further studies were performed with labeling of polyribosomes.

Isolation and labeling of polyribosomes. Goldfish brain polyribosomes (Fig. 8A, B) were prepared as described under Methods. The ratio of OD_{258}/OD_{236} was 1.43 and the ratio of OD_{260}/OD_{280} was 1.77. Size distribution of goldfish brain polyribosomes (Fig. 8A) were similar to that seen in other vertebrates. Following phenol extraction and gradient centrifugation of polyribosomal RNA, a heterogenous labeling pattern was seen (Fig. 8B). A considerable amount of radioactivity was found to sediment faster than 28S RNA as was observed in labeling of total cytoplasmic RNA (Fig. 7).

Labeling of ribonucleoprotein particles. Studies were performed to study the nature of early labeled particles in the polyribosomal fraction. Experiments were performed as in Fig. 8 but fish were killed 15 or 30 min following intracranial injection of labeled uridine and polyribosomes were centrifuged on a 10–30% sucrose gradient for 5.5 h. It can be seen (Fig. 9) that after 15 min, particles of approximately 45S became labeled. After a 30 min pulse an increased amount of radioactivity was observed sedimenting at approximately 45S together with considerable labeling in the 45S–80S region.

DISCUSSION

Although the goldfish has been used extensively for neurophysiological and behavioral studies, very little is known about the biochemistry of its brain. The present report in fact is the first extensive study on macromolecular RNA in teleost tissue. As with rat[16,21] and rabbit[27] brain, we find the three major species of RNA which are characteristic of eukaryotes, 28S and 18S ribosomal RNAs and 4S transfer RNA. While Triton X-100 has been used successfully for the preparation of nuclei free of endoplasmic reticulum[6,20], this detergent caused complete destruction of nuclear RNA during homogenization. The sodium deoxycholate–Tween mixture used for isolation of HeLa cell nuclei[22] caused no apparent degradation. Unlike observations in HeLa cells nuclei, we found that this mixture did not produce an increase in ratio of 28S to 18S nuclear RNA. The possibilities that the nuclear 18S peak represents cytoplasmic contamination, or alternatively that it is a degradation product of a heavier nuclear RNA are not distinguished by the present experiments.

Studies in mammalian cells[11,12,22,24,25,28,29] with brief pulses of labeled precursors indicate that radioactivity is associated mainly with a nuclear fraction. Partial characterization of this rapidly labeled material within the nucleus has indicated the presence of RNA molecules with approximate sedimentation coefficients of 45S and 32–35S. It has been established[22] that the 45S RNA, which is rapidly synthesized in the nucleolus, represents the ribosomal precursor RNA and is converted within the nucleus to 32S RNA and 16S (18S) RNA which then rapidly leaves the nucleus. The conversion of 32S RNA to 28S RNA takes place at a later time within the nucleus. It has also been shown that the new ribosomal RNA first appears in the cytoplasm in structures that are indistinguishable in sedimentation behavior from ribosomal subunits derived from functioning ribosomes[25,28,29]. In the cytoplasm,

messenger RNA has been found associated with the polyribosomes, sedimenting heterogeneously from 6S to > 45S. It is believed that this polyribosomal RNA fraction represents the functional messenger of the cell.

In goldfish brain, nuclear RNA following a 15 min pulse was polydisperse from 10S to over 70S, with no apparent maximum at 45S. At pulses greater than 30 min, a sharply defined 30–32S peak appeared. A great deal of heterogeneity remained. At later times, the amount of rapidly sedimenting RNA declined and peaks at 18S and 28S appeared. These results are compatable with the appearance of precursors of ribosomal RNA as well as heterogeneously sedimenting messenger, although further experiments such as base analyses and hydribization studies would strengthen the present evidence. Labeling of cytoplasmic fraction in the goldfish resembles that found following RNA extraction of polyribosomes. Radioactivity seems broadly distributed throughout the gradient with a considerable amount in the rapidly sedimenting fractions. Similar polydisperse RNA in the cytoplasmic fraction has been reported in rabbit brain[27]. In the case of both nuclear and cytoplasmic fractions > 28S RNA remained labeled at the longest times examined. These results could be explained on the basis of different turn-over rates for species of RNA which sediment together. They might come from different subcellular pools, from different cell classes (such as from neurons and from glia) or from different regions within the brain. Another possibility is that the heavy material represents artifactual aggregation of small molecular weight RNAs such as are known to form during extraction at high temperature. The latter explanation would not explain the relatively large amount of heavy RNA we found in the cytoplasmic fraction after cold phenol extraction. The labeling pattern could also reflect reutilization of precursor uridine from RNA in the brain or elsewhere in the body.

The labeling of ribonucleoprotein particles lighter than the 76S monomer in goldfish brain is similar to that reported in rat[23] brain as well as in other organs[12,25]. From these reports as well as the present one, it appears likely that the association of ribosomal RNAs with small ribonucleoprotein particles is a general mechanism by which newly formed RNAs are transported from their site of synthesis into the cytoplasm.

SUMMARY

Methods are presented for the isolation of high molecular weight RNA from nuclear and cytoplasmic fractions of goldfish brain following intracranial injection of [5-^{3}H]uridine. Nuclear RNA is labeled first and is followed by labeling of cytoplasmic RNA. The pattern of labeling in the nuclear fraction reflects the formation of broadly distributed RNA species some of which sediment more rapidly than 28S RNA. Radioactivity appears later in the cytoplasm and sediments heterogeneously from 4S to > 50S. Evidence is presented for the presence of a 45S ribosomal RNA precursor and for messenger RNA in the goldfish brain.

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