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Transcription and Processing of Ribonucleic Acid in *Rhynchosciara* Salivary Glands. I. Rapidly Labeled Ribonucleic Acid†

Hugo A. Armelin* and Nelson Marques‡

ABSTRACT: A method of cell fractionation for *Rhynchosciara* salivary glands is described. The kinetics of uridine-*t* incorporation into nuclear and cytoplasmic RNA have been studied, and are compatible with the transfer to the cytoplasm of only about 10% of RNA synthesized per 10 min. The rapidly labeled RNA was found in large polydisperse particles, with sedimentation coefficient up to 300 S, insensitive to EDTA and exclusively nuclear. The majority of the rapidly labeled RNA, in very different cell physiological states, was found to be very unstable with a half-life of the order of 10 min. Labeled 18S rRNA first appeared in the cytoplasm 20 min after, and 28S rRNA 35 min after the start of uridine-*t* incorporation. Messenger-like RNA species started to label between 20 and 30 min of incorporation. EDTA-sensitive, monodisperse particles, with a sedimentation coefficient of 25 S carrying messenger-like RNA were found in the cytoplasmic fraction.

We have studied RNA synthesis in salivary glands of the Diptera *Rhynchosciara* (Meneghini *et al.*, 1968; Armelin, 1969; Armelin *et al.*, 1969a,b, 1970; Meneghini *et al.*, 1971). This tissue has giant polytene chromosomes and gene activity can be followed by the puff pattern (for details, see review by Pavan and da Cunha, 1969). The salivary gland is composed of a population of cells with synchronized transcription, as indicated by the synchronized development of puffs. Thus we have in this animal a very rare "*in vivo*" event of a homogeneous population of cells, as far as RNA synthesis is concerned, which undergo several states of cytodifferentiation during larval development.

This work is devoted to the study of transcription and processing of RNA. It was possible to study the intracellular compartmentalization of several classes of RNA and of RNA-carrying particles. The results obtained suggest that polydisperse particles containing rapidly labeled RNA (heterogeneous nuclear RNA), with high sedimentation coefficient and insensitive to EDTA, are restricted to the nucleus, and probably localized on the chromosomes. Also we describe kinetic evidence indicating that the major part of the transcribed RNA turns over in the nucleus. The rate of entrance of ribosomal RNA and messenger-like RNA into the cytoplasm was followed. This work provided us RNA fractions of defined cellular origin, which allowed us to make a detailed study of RNA–DNA hybridization, described in the accompanying paper (Armelin and Marques, 1972).

Materials and Methods

Animals. Rhynchosciara angelae (or *Rhynchosciara americana*) larvae were raised under laboratory conditions as previously described (Lara *et al.*, 1965). A thorough analysis of this biological system can be found in a recent review by Pavan and da Cunha (1969). For the purpose of the consideration of this work we provide some minimal information about *Rhynchosciara* salivary glands development in Table I. In view of the synchrony of development, the age of a group of larvae (and also salivary gland physiological state) can be determined from microscopic observation of salivary gland chromosomes of three or four larvae in squash preparations.

Isotope Administration. All incorporations were accomplished *in vivo* through direct injection (2 μl/larvae) into the hemocoele of animals previously anesthetized with ether, as described elsewhere (Armelin *et al.*, 1969b).

Cell Fractionation. Crude chromatin was prepared in a Dounce homogenizer (tight fit) with vigorous homogenization in 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM $MgCl_2$, and 200 mM sucrose (Tris–NaCl–$MgCl_2$–sucrose buffer). After 5-min centrifugation (3500*g*) the pellet, which is the

† From the Departamento de Bioquimica, Instituto de Quimica, Universidade de São Paulo, São Paulo, Brazil. *Received October 12, 1971.*

* Corresponding address: University of California, San Diego, Department of Biology, La Jolla, Calif. 92037.

‡ Predoctoral fellow from Fundacão de Amparo á Pesquisa do Estado de São Paulo.

TABLE I: Development of Salivary Glands[a] at the End of Larval Development in *Rhynchosciara*.

Arbitrary Division of 4th Instar[b]	Approximate Age (Days)	Markers Used to Define Larval Age in This Work	Important Known Events in Salivary Glands
Second period	40–47	40 days after hatching and before cocoon making	Low level of RNA synthesis (Armelin, 1969). Low level of DNA synthesis (Meneghini, 1969)
Third period	47–52	Cocoon formation starting	Large increase in RNA and DNA content per cell; highest rate of rRNA synthesis (Lara and Hollander, 1967; Armelin *et al.*, 1969a,b)
Fourth period and fifth period	52–57	Development of giant "DNA puffs" in B and C chromosomes of salivary gland cells	Development of DNA puffs (Breuer and Pavan, 1955; Ficq and Pavan, 1957; Rudkin and Corlette, 1957); highest levels of DNA and RNA per cell; rRNA synthesis stops (Lara and Hollander, 1967; Armelin *et al.*, 1969a); highest level of protein synthesis (Bianchi and Lara, 1969); highest level of DNA synthesis (Meneghini, 1969; Simões, 1970); evidence for gene amplification (Meneghini *et al.*, 1971)
Sixth period	57–60	Definitive cocoon ready; no DNA puffs	Deep and fast fall in DNA and RNA content per cell (Lara and Hollander, 1967)
Pupal molt	60–61	Pupal molt	Hystolysis during metamorphosis

[a] The salivary gland is composed of a limited number of large cells, which grow without cell division (chromosome polytenization). These large cells, which represent practically 100% of the tissue mass, have synchronized chromosomal activity as far as puff pattern development is concerned. [b] This division was originally introduced by Guaraciaba and Toledo (1967) and later modified by Armelin *et al.* (1969a).

crude chromatin, was collected; the supernatant is called the "chromatin supernatant."

In order to fractionate cells in such a way as to preserve nuclear structure, some modifications were made on a previously described method (Armelin *et al.*, 1969b). After dissection the glands were placed on a nylon net held in a polyethylene ring in physiological solution from which they were transferred to Tris–NaCl–$MgCl_2$–sucrose buffer containing 10 mg/ml of Pronase and maintained for 3–5 min at 0°. This treatment allows partial destruction of gland envelope without affecting the cells (conditions standardized by microscopic observation). Immediately after proteolytic treatment the net was immersed in Tris–NaCl–$MgCl_2$–sucrose buffer containing bovine serum albumin fraction V (25 mg/ml) for washing. Finally the polyethylene ring holding the net was placed over the rim of an appropriate vial with the above buffer plus 1% Nonidet P40 (a nonionic detergent from Shell do Brazil) at 0°. In this solution, 100% of the cells is lysed in 10–20 min liberating the nuclei without damage (see also O'Brien, 1964, and Borun *et al.*, 1967). The material in the net was then gently stirred in order to liberate the nuclei from the residual mucoprotein gland envelope which is not dissolved by the detergent. The net was then withdrawn and the suspension of nuclei centrifuged at 3000*g* for 5 min. The resulting pellet was washed once with the same buffer and recentrifuged. The combined supernatants comprise 100% extraction of cytoplasm and the pellet is a nuclear fraction but with a low yield (due to gland envelope residues that trap many nuclei in the nylon net). The nuclei in the detergent solution show a very well-preserved structure under the phase microscope as one can see in the microphotograph of Figure 1. It is possible to keep the nuclei in this solution for 2 days at 5° without apparent alteration. Centrifugation at 3000*g* causes severe aggregation of nuclei though without apparent disruption.

RNA Extraction. We previously described conditions of pH, salt concentration, and temperature that are adequate for optimizing the fractionation of *R. angelae* RNA (Armelin, 1969; Armelin *et al.*, 1969b, 1970). In summary, this procedure involves: first tissue homogenization in 50 mM Tris–50 mM NaCl–1 mM $MgCl_2$ (pH 7.4) and extraction with equal volume of buffer-saturated phenol for 15 min at 0° with strong agitation; second, reextraction of the phenol phase and interphase with the same buffer, now with 1% sodium dodecyl sulfate; third, reextraction of the phenol phase and interphase, now with 50 mM Tris–50 mM NaCl–1 mM $MgCl_2$–1% sodium dodecyl sulfate (pH 8.3) and at 65°. The RNA in the aqueous phase are obtained by precipitation with ethanol, redissolved, treated with deoxyribonuclease (50 μg/ml, 30 min, 37°) and Pronase (20 μg/ml, 60 min, 37°), and finally deproteinized again by two phenol extractions in the same conditions as before. The extractions at 0° allow the recovery of 95% of cellular RNA; at 65°, only about 1–5% of cellular RNA is extracted; the radioactivity extracted at 0° plus that extracted at 65° represents 98–100% of total radioactivity incorporated into cellular RNA. In RNA–DNA hybridization experiments, the RNA was deproteinized with the mixture suggested by Kirby (1965): 500 ml of phenol, 70 ml of *m*-cresol, 0.5 g of 8-hydroxyquinoline plus 50 mM Tris-HCl, 50 mM NaCl, and 1 mM $MgCl_2$ (pH 7.4 or 8.3) to saturate.

For sedimentation analysis of RNA from cytoplasmic fraction or "chromatin supernatant," the suspension was simply made 1% in the dodecyl sulfate and 3% in diethyl pyrocarbonate (Solymosy *et al.*, 1968) and the temperature was raised to 35° for 3–5 min then an aliquot was placed on top of a 15–30% sucrose gradient at 2–5° (named sodium dodecyl sulfate–diethyl pyrocarbonate–EDTA sucrose gradient) and centrifugation was carried out at the same temperature. The low temperature of centrifugation causes sodium dodecyl

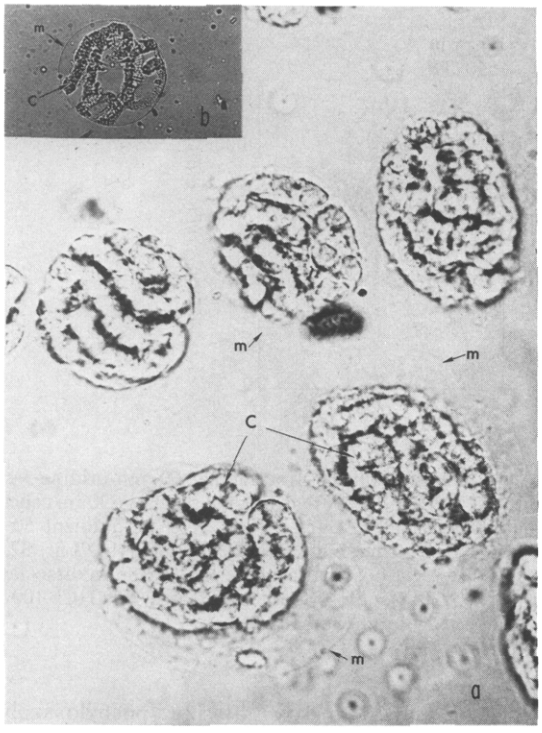

FIGURE 1: Nuclei collected by centrifugation at 200*g* for 5 min. (a) Fresh preparation in fractionation buffer (under phase-contrast microscope) consists of nuclei with membranes (m) and intact polytene chromosomes (c), the nucleoli are not visible. The same procedure, but with 500 mM NaCl, gave nuclei with intact membranes but "empty" of chromosomes, because the chromosomal structure is completely disrupted in a buffer with this ionic strength. (b) In fractionation buffer the isolated nuclei remain morphologically intact even after 4 hr at room temperature (under photomicroscope). Lenses magnification ×500.

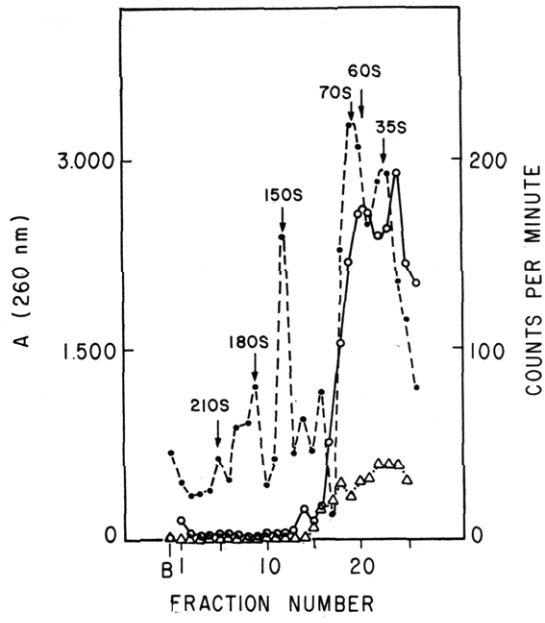

FIGURE 2: Sedimentation profile of particles containing orotic acid ^{3}H-labeled RNA. Fifty third-period larvae; 30-min orotic acid-*t* (5.7 Ci/mmole, 1 mCi/ml) incorporation. Chromatin–supernatant fractionation in 10 mM Tris-HCl–500 mM NaCl–1 mM $MgCl_2$–200 mM sucrose (pH 7.4) buffer as in Methods. Two equal aliquots of the chromatin supernatant (with 1% Nonidet P40) were placed over two 15–30% sucrose gradient in 10 mM Tris-HCl–500 mM NaCl–1 mM EDTA (pH 7.4; 32,000 rpm, 40 min); the fractions of one gradient were treated with 10 μg/ml of RNase for 10 min at 0° before precipitation; both gradients showed essentially the same absorbance profile measured before radioactivity determinations (○) Absorbance at 260 nm; (●) cpm; (△) cpm after RNase treatment.

sulfate precipitation but this does not disturb the gradient.

DNA Extraction. The salivary gland nuclear DNA was extracted by a process based on a method described by Meneghini (1969). In summary, this process involves the following steps: (a) preparation of crude chromatin in 100 mM NaCl–10 mM EDTA pH 8.3 buffer; (b) treatment of the chromatin in NaCl–EDTA buffer, with 1 mg/ml of Pronase and 0.5% sodium dodecyl sulfate per 15 min at 60°; then the solution is made 1 M NaCl (with 5 M NaCl), and two volumes of alcohol are added. The fibrous precipitate is spooled out with a glass rod. The precipitation step is repeated in the same manner; (c) treatment with 100 μg/ml of α-amylase and 100 μg/ml of ribonuclease (prepared as recommended by Marmur, 1961) for 30 min at 37°. Then the Pronase treatment is repeated as before. After addition of NaCl to 1 M the material is deproteinized two or three times with chloroform–isoamyl alcohol (24:1, v/v), each time with careful agitation for 10 min and precipitation as before. This process gives a yield of 70–80% of a DNA with the following ultraviolet (uv) spectrum relationships: 260 nm/232 nm 2.3–2.2 and 260 nm/280 nm 1.8–1.7. The purified DNA was stored in 10 mM Tris-HCl (pH 7.4) buffer with some drops of chloroform at 5°.

Sedimentation Analysis. Particles were examined by sedimentation analysis in linear 15–30% sucrose gradients in 50 mM Tris-HCl–100 mM NaCl–1 mM $MgCl_2$ (pH 7.4) buffer (named magnesium–sucrose gradient) or linear 15–30% sucrose gradient in 50 mM Tris-HCl–100 mM NaCl–1 mM EDTA (pH 7.4) buffer (named EDTA–sucrose gradient); EDTA–sucrose gradient was always used for purified RNA. The centrifugations were made in SW39, SW50, or SW65 rotors of the Spinco ultracentrifuge at 2–5°.

RNA sedimentation coefficients were estimated using *Rhynchosciara* 28S and 18S rRNA as internal standards; for particles the reference was 75S *Rhynchosciara* ribosomes in magnesium–sucrose gradient and 60S and 40S *Rhynchosciara* ribosomal subunits in EDTA–sucrose gradient. The sedimentation coefficients of *Rhynchosciara* ribosomal particles are rough estimates from sucrose gradient with *Escherichia coli* ribosomes as reference. In a few experiments we did not have internal standards; in these cases, sedimentation coefficients were estimated from identical reference gradients centrifuged together and collected in the same way.

DNA was centrifuged to equilibrium in CsCl gradients according to the method of Flamm *et al.* (1966). Gradients of 4.5 ml were prepared by adding 4.271 g of optical grade CsCl to 3.500 g of 10 mM Tris-HCl (pH 7.4) buffer containing the DNA. Before centrifugation, the DNA was sonicated for 2 min at 3 A in 10 mM Tris-HCl (pH 7.4) buffer at 50 μg/ml. Centrifugations were carried out in a Spinco 50 Ti rotor at 42,000 rpm for 36 hr at 15°.

Nucleic Acids and Proteins Determinations. For nucleic acid determinations, the method of Fleck and Munro (1962) was used. RNA was measured in alkaline hydrolysate by the absorbance at 260 nm, but in the case of nuclear fractions the colorimetric method of Mejbaum (1939) was used, taking purified *R. angelae* rRNA as standard. DNA in acid hydrolysates was determined by the colorimetric method of Giles and Myers (1965) with purified *R. angelae* DNA as standard.

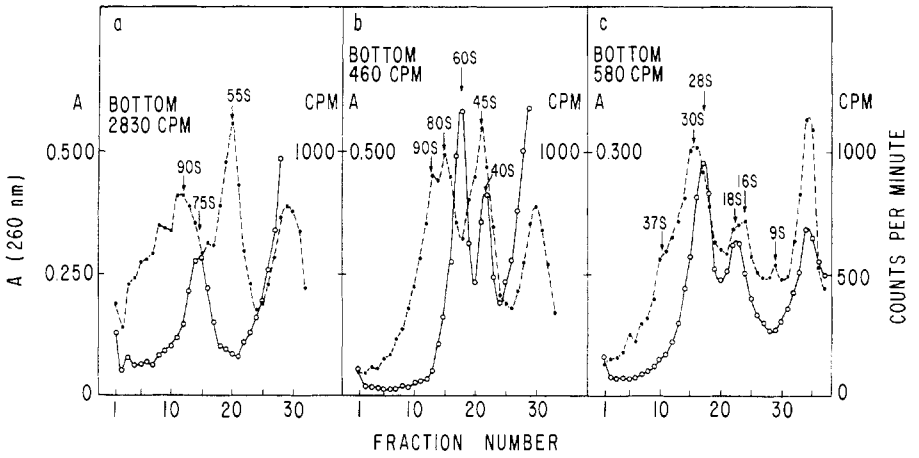

FIGURE 3: Sedimentation profile of particles containing uridine-5-*t*-labeled RNA. Two-hundred third-period larvae; 60-min uridine-5-*t* (19 Ci/mmole, 1 mCi/ml) incorporation. Chromatin–supernatant fractionation in 50 mM Tris-HCl–10 mM NaCl–1 mM $MgCl_2$–230 mM sucrose (pH 7.4) buffer; postmitochondrial supernatant made 1% Nonidet P40, two equal aliquots placed on a 15–30% sucrose gradient 50 mM Tris-HCl–10 mM NaCl–1 mM $MgCl_2$ (pH 7.4) buffer (a) and on a 50 mM Tris-HCl–10 mM NaCl–1 mM EDTA (pH 7.4) buffer (b) (3 hr, 32,000 rpm). The sum of the areas under the 60S and 40S peaks in part b is 2.2 times the area under 75S ribosomal peak in part a, due to dissociation of polysomes in EDTA. Phenol-purified RNA of postmitochondrial supernatant in 15–30% sucrose gradient (10 mM Tris-HCl–100 mM NaCl–1 mM EDTA (pH 7.4) buffer) (3.5 hr, 45,000 rpm, 2–5°) (c). Absorbance at 260 nm (○); radioactivity (●).

With pure RNA the determinations were made simply by the absorbance at 260 nm where an RNA solution of 45 μg/ml at pH 7.4 shows an absorbance of 1.00. Proteins were assayed by the colorimetric method of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

Radioactivity Determinations. Samples were precipitated with an equal volume of cold 10% trichloroacetic acid, after addition of 100 μg/ml of commercial yeast RNA as carrier; the precipitates were collected on nitrocellulose filters by filtration, dried at 60°, and counted in a liquid scintillation spectrometer with 5 ml of a 2,5-diphenyloxazole–1,4-bis[2 (5-phenyloxazolyl)]-benzene–toluene solution (4 g of 2,5 diphenyloxazole and 100 mg of 1,4-bis[2-(5-phenyloxazolyl)]-benzene per 1 l. of toluene). Samples of acid or alkaline nucleic acid hydrolysates were neutralized and an aliquot was placed in 10 ml of Bray's solution (Bray, 1960).

Hybridization RNA–DNA. See description in Methods of accompanying paper (Armelin and Marques, 1972).

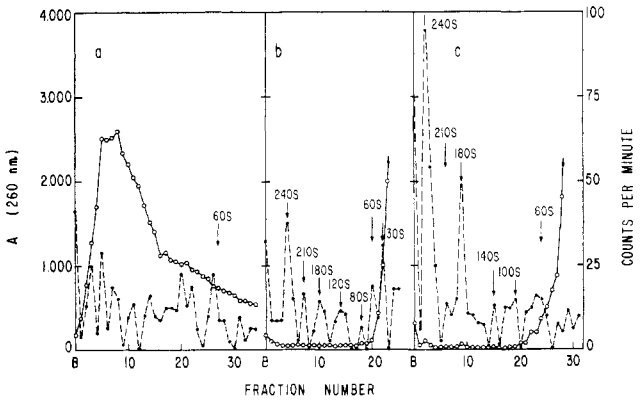

FIGURE 4: Sedimentation profiles of glutaraldehyde-fixed particles. One-hundred and twenty fourth-period larvae; 30-min cytidine-*t* (26.8 Ci/ml; 1 mCi/ml) incorporation. Chromatin–supernatant fractionation in 10 mM Tris-HCl (pH 7.4)–100 mM NaCl–1 mM $MgCl_2$–200 mM sucrose–10 mg/ml of bentonite. Postmitochondrial supernatant plus 1% Nonidet P40 centrifuged for 2 hr at 200,000*g* through 2 ml of 500 mM sucrose. Pelleted particles resuspended in pH 7.4 buffer 1 mM $MgCl_2$ and in pH 7.4 buffer 10 mM EDTA; in both suspensions particles fixed with 33% glutaraldehyde according to Baltimore and Huang (1968). (a) Mg-fixed particles in Mg–sucrose gradient; fraction 8 presented uv absorption spectrum characteristic of ribosomes; (b) Mg-fixed particles made 20 mM EDTA and centrifuged in an EDTA–sucrose gradient; only 25 fractions were collected; (c) EDTA-fixed particles in EDTA–sucrose gradient (32,000 rpm, 40 min); (○) absorbance; (●) cpm.

Results

Particles Containing Rapidly Labeled RNA. In *Rhynchosciara* salivary glands rapidly labeled RNA was found associated to polydisperse particles whose sedimentation coefficients extended from 30 to 300 S (Figures 2, 3, and 4). The heavy particles observed in EDTA–sucrose gradient were completely digested with DNase-free pancreatic RNase at 10 μg/ml 0° for 10 min (Figure 2). These labeled particles were also completely eliminated when the preparation was treated with RNase (10 μg/ml, 0°, 10 min) before centrifugation (see Table III, expt 1); however, RNase-free pancreatic DNase (50 μg/ml, 37°, 5 min) had no effect. Polysomes and monosomes are quantitatively dissociated by 1 mM EDTA (Figure 3a,b) but the large rapidly labeled RNA carrying particles are not affected (Figures 2 and 3b) even at 20 mM EDTA (Figure 4). The NaCl was used at 10 mM (Figure 3a,b), 100 mM (Figure 4), and 500 mM (Figure 2) without detectable variation in sedimentation behavior of the rapidly labeled particles. In the chromatin supernatant (see Methods) high yield of (a) labeled RNA only extractable at 65° (as we reported, Armelin *et al.*, 1970; see also Table III, expt 1); (b) labeled polydisperse RNA with sedimentation coefficient up to 60 S (Figure 3c); or (c) EDTA-insensitive large particles (Figures 2, 3, and 4) depended on severe homogenization of the tissue; mild homogenization always gave poor yield of heavy-labeled particles, with the majority of rapidly labeled RNA being associated to the chromatin fraction. All of these results suggested that the bulk of rapidly labeled RNA is contained in large particles probably localized in the nucleus; these particles are likely to be ribonucleoproteic but a detailed analysis of chemical composition was not performed.

Nature and Turnover of Rapidly Labeled RNA. We reported elsewhere that the salivary gland rapidly labeled RNA is

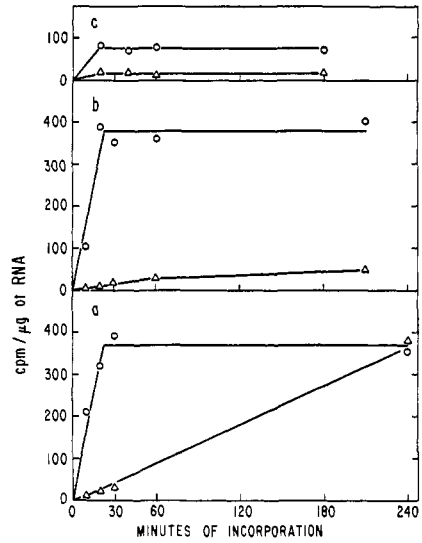

FIGURE 5: Kinetics of uridine-*t* incorporation into RNA extracted at 0 and 65° from salivary glands of different larval developmental periods. Groups of 10 larvae of each period; uridine-*t* (7.2 Ci/mmole 1 mCi/ml). Homogenization in 50 mM Tris-HCl–50 mM NaCl–1 mM $MgCl_2$ (pH 7.4) buffer with 1% sodium dodecyl sulfate at 0°. Two reapeted extractions at pH 7.4 and 0° combined. Phenol phase and interphase reextracted (third time) with pH 8.3 buffer at 65°. The RNA fractions obtained were hydrolysated (300 mM KOH, 50 min, 37°); RNA and radioactivity determined in hydrolysate. RNA content per gland determined according to Fleck and Munro (1962). The combined cold extractions yielded 87–92% and the hot extraction yielded 2–6% of total cellular RNA. (a) Third-period larvae; (b) late fourth-period larvae; (c) larvae during pupal molt. (△) Cold extraction; (○) hot extraction.

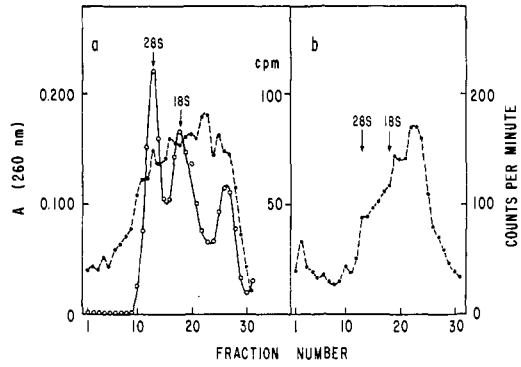

FIGURE 6: Sedimentation profiles of rapidly labeled RNA. (a) Fifteen sixth-period larvae (after development of DNA puffs; 14 μg of RNA/salivary gland; 1–2 days before pupal molt); 60-min uridine-*t* (7.2 Ci/mole, 1 mCi/ml) incorporation. Phenol extractions at 0 and 65° were combined; final preparation treated with ribonuclease-free deoxiribonuclease (50 μg/ml, 30 min, 37°) and preincubated Pronase (20 μg/ml, 60 min, 37°). (b) Ten third-period larvae; 10-min uridine-*t* (7.2 Ci/mole; 1 mCi/ml) incorporation. 65° phenol-extractable RNA obtained as in Figure 5, mixed with 100 μg of cold carrier RNA and enzymatically treated as in part a. Centrifugation in 15–30% sucrose gradient (50 mM Tris-HCl–100 mM NaCl–1 mM EDTA (pH 7.4) (37,000 rpm, 5 hr); (○) absorbance; (●) cpm.

poorly extracted with phenol at low temperature (Armelin, 1969; Armelin *et al.*, 1970; see Methods). The procedure of successive phenol extractions has been useful for partial purification of a rapidly labeled RNA fraction, which is only extracted at 65°, whereas the bulk of cellular RNA is easily extracted at 0°. We studied the kinetics of uridine-*t* incorporation in the RNA extracted at 0° and at 65°; the purpose of these experiments was to obtain information about the overall turn over of the RNA fractions extracted under these conditions. Salivary gland cells at three very distinct physiological states were chosen: (a) beginning of the third larval development period, a period of active cellular growth with 8.0 μg of RNA/salivary gland; (b) late fourth period of larval development when the cells reach their highest content of RNA, with 19 μg of RNA/salivary gland; (c) end of larval development, during the pupal molt; at this point in the development the salivary glands are in an advanced state of histolysis, with 7.5 μg of RNA/gland. The results obtained are presented in Figure 5. The uridine-*t* incorporation into RNA cannot be taken as an actual measure of the rate of synthesis among different periods, since we have no information about the specific activity of the uridine triphosphate pool during these periods.

The kinetics of uridine-*t* incorporation in hot extracted RNA (Figure 5) was very similar for the three cellular states: the specific activity remains constant after 20-min incorporation. The results of Figure 5 suggest that this RNA population has a half-life of the order of 10 min. Obviously, if a small part of this population was more stable, it would not be detected in this experiment. But there is a clear-cut indication that the majority of this RNA is very unstable. Of course, turnover here does not mean exclusively degradation, since part of the population can be changed to the fractions extracted at 0°, mainly during the third period (Figure 5a).

When the salivary glands are close to hystolysis, all of the synthesized RNA is very unstable (Figure 5c) and sedimented heterogeneously in a broad range of sedimentation coefficient from 4 to 60 S (Figure 6a). During cellular growth, although a large amount of relatively stable RNA is synthesized (0° extractions, Figure 5a; see also Figure 10), the hot extracted RNA is still unstable (Figure 5a) and also sedimented heterogeneously (Figure 6b).

Intracellular Distribution of Rapidly Labeled RNA. We fractionated cells into nuclear and cytoplasmic fractions, using the procedure detailed in Methods. The cytoplasmic fraction contains 100% of the cytoplasmic material and routinely provides 90–95% of cellular RNA. In Table II, we present some information about the chemical composition of the nuclear fraction: the RNA:DNA ratio is very reproducible, and the protein content variation is probably due to gland envelope insoluble fragments sometimes passing through the nylon net.

The kinetics of uridine-*5-t* incorporation into nuclear and cytoplasmic fractions was studied; curves of specific activity versus time are presented in Figure 7. The curves indicate clear separation of the two cellular compartments. In the nuclear fraction an apparent steady state is already reached at 30 min and the amount of radioactivity transfered to cytoplasm RNA increases linearly during the first 4 hr of incorporation, which means that the bulk of this RNA population is fairly stable. The kinetics of uridine-*t* incorporation in hot extracted RNA (Figure 5) probably gives a measure of the overall turnover of nuclear RNA. The rate of increase of cytoplasmic RNA specific activity and the nuclear RNA overall turnover can be explained if only about 10% of RNA synthesized per 10 min in the nucleus goes to the cytoplasm. In this estimate it was assumed: (1) that the nuclear RNA represents 5% and the cytoplasmic RNA 95% of total cellular RNA, which agrees with data from Table II; (2) the pool of labeled ribonucleotide precursors is in excess at least during the first 4 hr of incorporation, which is a reasonable assumption according to

TABLE II: Chemical Composition of Nuclear Fraction.[a]

No. of Glands	RNA:DNA	Acid-Insoluble Protein/DNA	Acid-Soluble Protein/DNA	Yield (%)
440	0.70	8.0	1.8	33
260	0.76	12.8	3.4	29

[a] Nuclear fractions prepared as in Methods and homogenized in 50 mM NaOH. Ten per cent trichloroacetic acid soluble and insoluble protein measured by the method of Lowry *et al.* (1951). RNA alkaline hydrolysate assayed by the orcinol method (Mejbaum, 1939); DNA acid hydrolysate assayed by the diphenylamine method (Giles and Myers, 1965). Total nucleic acid content per salivary gland (determined independently): 5.5 μg of DNA and 132 μg of RNA per 10 glands. The nuclear fraction yield was given by the ratio between the amount of DNA finally obtained in the nuclear fraction and amount of input DNA. The ratio RNA:DNA in nuclear fractions (0.70 and 0.76) and in the intact tissue (132:5.5) shows that the nuclear fraction RNA represented 5.6% of total cell RNA. Ninety-two per cent of total cell RNA was found in cytoplasmic fraction. This nuclear preparation showed DNA-dependent RNA-polymerase activity both in presence of Mg^{2+} or Mn^{2+} (R. V. Santelli and H. A. Armelin, unpublished results).

known data of uridine-*t* incorporation in total salivary gland RNA (Meneghini *et al.*, 1968). Certainly 10% is a rough figure. However the accuracy of these values should not be the main concern at this point, but the important conclusion is that the majority of synthesized RNA never leaves the nucleus.

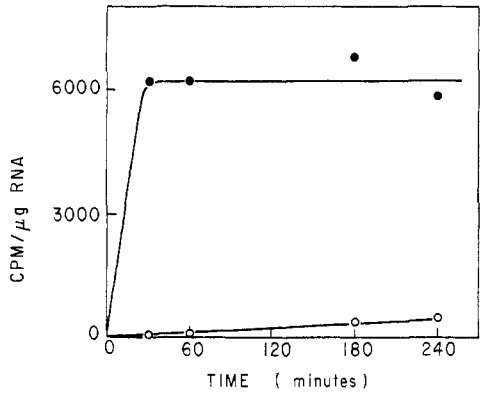

FIGURE 7: Incorporation kinetics of uridine-*5-t* into nuclear and cytoplasmic fractions. Groups of 25 third-period larva per each point; uridine-*5-t* (19 Ci/mmole, 1 mCi/ml). Nucleus–cytoplasm fractionation as in Methods. Nuclear fractions and aliquots of cytoplasmic fractions resuspended in 50 mM NaOH and then precipited with 200 mM $HClO_4$; RNA and radioactivity content in precipitates determined through alkaline hydrolysis. The nuclear fractions yielded 0.15–0.20 μg of RNA/gland and cytoplasmic fractions yielded 7.3–7.6 μg. The total radioactivity in counts per minute per fraction at 30, 60, 180, and 240 min were respectively 35,000, 44,000, 180,00, and 280,000 (for cytoplasmic) and (estimates assuming a 35% yield in nuclei preparation; highest yield observed in this kind of preparation) 123,000, 137,000, 123,000, and 115,000 (for nuclear). (●) Nuclear fraction; (○) cytoplasmic fraction.

TABLE III: Labeled RNA Distribution among Cellular Fractions.

Cellular Fraction	Temp of Extraction (°C)	Total of Cpm/Fraction × 10^{-2}	% of Radioactivity Incorpd/Fraction
Experiment 1:[a] Chromatin–Supernatant Fractionation			
Chromatin	0 + 65	30	5
Supernatant	0	75	13
	65	450	82
Experiment 2:[b] Nucleus–Cytoplasm Fractionation			
Chromosomal	0 + 65	1000	59
Cytoplasmic	0	450	26
	65	6	0.3
Nucleoplasmic	0	114	6.6
	65	51	3.0

[a] Experiment 1: Fifty third-period larvae; 30-min uridine-*t* (7.2 Ci/mmole; 1 mCi/ml) incorporation. Chromatin–supernatant fraction as in Methods; phenol-extracted RNA precipitated with 5% trichloroacetic acid to determine the counts per minute incorporated in RNA per fraction. An aliquot of supernatant was taken for centrifugation in magnesium–sucrose gradient and 95% of total radioactivity in this sample sedimented between 180 and 260 S; another aliquot was treated with RNase (10 μg/ml, 10 min, 0°) and the labeled heavy particles were completely eliminated. [b] Experiment 2: Seventy-five third-period larvae; 30-min uridine-*5-t* (19 Ci/mmole; 1 mCi/ml) incorporation. Nuclear–cytoplasm fractionation as in Methods; nuclei fractionated in chromosomal pellet and postchromosomal supernatant. Phenol-extracted RNA precipitated with 5% trichloroacetic acid to determine the counts per minute incorporated in RNA per fraction. The total radioactivity incorporated in RNA was determined through phenol extractions of total salivary glands.

Some experiments were designed to investigate the distribution of rapidly labeled RNA among nuclear compartments. In expt 1 of Table III, the salivary glands were fractionated into chromatin and supernatant. Ninety-five per cent of labeled RNA was found in the supernatant, 82% of which was extracted only at 65° (this agrees with our first results, Armelin, 1969; Armelin *et al.*, 1970), and the majority was contained in RNase-sensitive particles sedimenting between 180 and 260 S. In expt 2 of Table III the cells were first fractionated into nucleus and cytoplasm as described in Methods. The nuclei were then disrupted by passing repeatedly up and down a thin Pasteur pipet which breaks nuclear membranes, liberating chromosomes with well-preserved structure. After centrifugation we obtained two fractions: the chromosomal fraction in the pellet and the nucleoplasmic fraction in the supernatant. The radioactivity showed the following distribution among cellular compartments: cytoplasm 26% (which is in good agreement with the 30 min point in the kinetics experiment of Figure 7); chromosomes 60% and nucleoplasm 10%. These results indicated clearly that the nucleoplasm contains a small pool of labeled RNA and the chromosomes are the main nuclear compartment of rapidly labeled RNA, which is only liberated with severe homogenization.

The nuclear fraction was homogenized; an aliquot of the

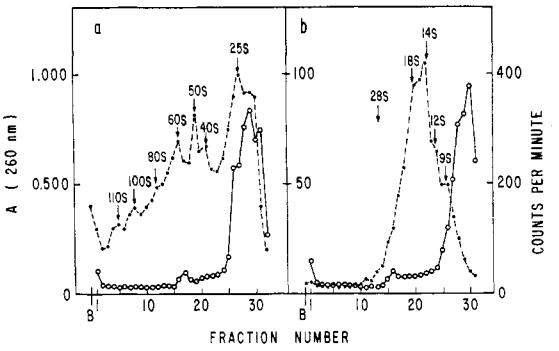

FIGURE 8: Labeled nuclear particles after 30-min uridine-*t* incorporation. Two-hundred third-period larvae; uridine-*5-t* (24.5 Ci/mmole, 0.5 Ci/ml). Nuclear fraction prepared as in Methods; then homogenized in 10 mM Tris-HCl–100 mM NaCl 1 mM $MgCl_2$–200 mM sucrose (pH 7.4) buffer with five strokes in a Dounce homogenizer (loose fit); chromosomal fraction collected by centrifugation 10,000*g*, 10 min). From the supernatant one aliquot was placed on a 15–30% sucrose gradient (50 mM Tris-HCl–100 mM NaCl–1 mM EDTA (pH 7.4)) (80 min, 45,000 rpm) (8a); another aliquot made 1% sodium dodecyl sulfate and 3% diethyl pyrocarbonate, placed on identical gradient (3.5 hr, 45,000 rpm) (b). (○) Absorbance; (●) cpm.

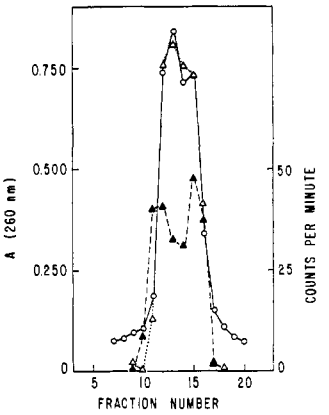

FIGURE 9: Hybridization of chromosomal RNA and nuclear particle RNA with DNA fractionated in a CsCl gradient. Nuclear fraction fractionated in chromosomal fraction and post-chromosomal supernatant as in Figure 8. Postchromosomal particles pelleted by centrifugation (2 hr, 200,000*g* through 1 ml of 500 mM sucrose). RNAs from chromosomal fraction and pelleted particles were purified as in Methods. Filters with immobilized gradient fractions DNA were cut in two halves, one for each RNA solution. Annealing reaction: 20 hr, 6 × SSC, 65°; chromosomal RNA 48 μg/ml; particle RNA 80 μg/ml; DNA retention in the filters between 90 and 100%.

supernatant was analyzed in a EDTA–sucrose gradient and the profile is shown in Figure 8a, to give the same distribution of particles as described above (Figures 2 and 4), with several components with sedimentation coefficients from 25 to 120 S. In order to analyze the RNA present, another aliquot of the supernatant was centrifuged in a sodium dodecyl sulfate–diethyl pyrocarbonate–EDTA sucrose gradient (Figure 8b); the RNA population is composed of several species with sedimentation coefficients from 9 to 30 S. The number of components in the RNA profile approximates the number of components in the particles profile. These nuclear particles were isolated by centrifugation and their RNA was purified by the phenol procedure; the chromosomal pellet RNA was also extracted. The chromosomal and particle RNA were hybridized with the fractions of a CsCl gradient of salivary gland nuclear DNA; the results are shown in Figure 9. The hybridization due to particle RNA gave a radioactivity profile that follows perfectly the main band of DNA; the same results observed with cytoplasmic RNA (Armelin and Marques, 1972). In the case of chromosomal RNA we observed a relatively higher hybridization in the heavier fractions of the gradient, which was also observed with hybridization of total nuclear RNA.

Entrance of Labeled RNA into the Cytoplasm. The cytoplasmic RNA labeling was analyzed by sedimentation, by direct centrifugation of the cytoplasmic fraction in sodium dodecyl sulfate–diethyl pyrocarbonate–EDTA sucrose gradients, as described in Methods. Profiles of sedimentation of RNA are presented in Figure 10. The labeling sequence in the main bands of these gradients is shown in Figure 11. In order to follow particles labeling aliquots of the cytoplasmic fraction were placed on the top of magnesium- or EDTA–sucrose gradients. Sedimentation profiles of cytoplasmic particles are presented in Figures 12 and 13; the labeling sequence in the main bands of these gradients is summarized in Table IV.

No significant background of heterogeneous RNA was found under the 28S uv peak (Figure 10a); probably the labeled RNA is pure 28 S, being the RNA of largest size found in cytoplasmic fractions. The curves of radioactivity increase in Figure 11 show that 28S labeled rRNA appears in cytoplasm at 35 min, whereas 18S labeled rRNA appears at 25 min. The labeling sequence of 60S and 40S uv peaks in EDTA gradients (Figure 12), is in accord with this. The profile of Figure 10c shows that after 180 min of incorporation only about 25% of labeled RNA that went to the cytoplasm is rRNA. We estimated by sedimentation analysis that during the 10-min incorporation, in third-period larvae, only 5% of labeled RNA extracted at 0° is rRNA (Armelin, 1969). However, after 10 min of incorporation at least 50% of labeled RNA is only extracted at 65° and probably it does not contain rRNA. It turned out that rRNA might represent only about 2% of RNA synthesized per 10 min. Although this value seems too

TABLE IV: Entrance of Radioactivity in Polysomes.[a]

Time of Incorp (min)	Type of Sucrose Gradient	Distribution (%) of Radioactivity among Bands			Ribosomes Found in Polysome[b]
		<20 S	20–60 S	>60 S	
30	Mg	53	31	16	72
	EDTA	56	42	2	
180	Mg	22	26	51	68
	EDTA	26	70	4	

[a] Third-period larvae; uridine-*5-t* (19 Ci/mmole; 1 mCi/ml). Cytoplasmic fractions (prepared as in Methods) centrifuged in Mg– and EDTA–sucrose gradients (45,000 rpm, 2 hr, 2–5°). The radioactivity in each band was expressed in per cent of the total. The total radioactivity in each profile per equivalent aliquots of cytoplasmic fractions were: 12,000 cpm for 30-min incorporation and 130,000 cpm for 180-min incorporation. Polysome-bound ribosomes were estimated through the ratio between the sum of areas under 60S and 40S uv peaks in EDTA–gradient and the area under the 75S uv peak in Mg gradient. [b] Estimated as per cent of total.

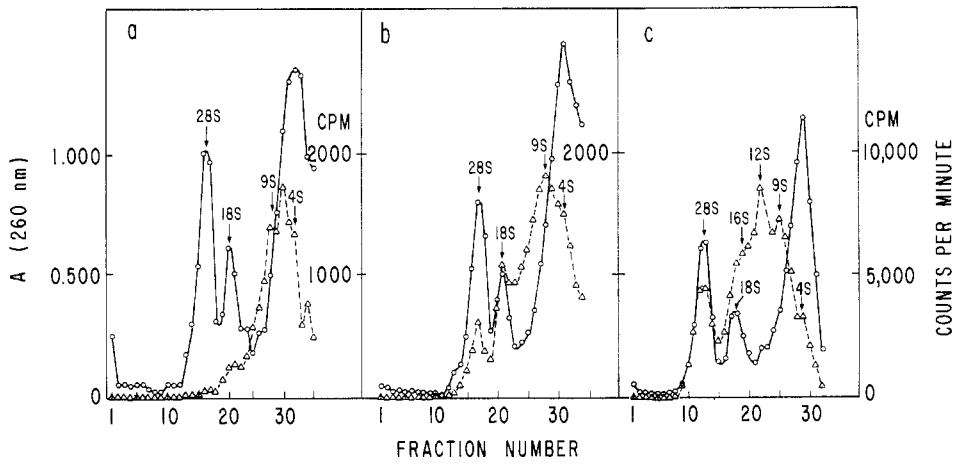

FIGURE 10: Entrance of labeled RNA into the cytoplasm. Groups of 25 third-period larva; uridine-5-*t* (19 Ci/mmole, 1 mCi/ml). Incorporation times: 30 min (a), 60 min (b), and 180 min (c). Before fractionation the salivary glands were mixed with an equal number of "cold" glands from animals of the same batch. Aliquots of cytoplasmic fractions placed on 15–30% sucrose gradients (50 mM Tris-HCl–100 mM NaCl–1 mM EDTA (pH 7.4)), just after addition of sodium dodecyl sulfate and diethyl pyrocarbonate (see Methods); centrifugation: 3.5 hr, 45,000 rpm. Absorbance at 260 nm (○); radioactivity (△). Radioactivity was not detected in the bottom of the tubes.

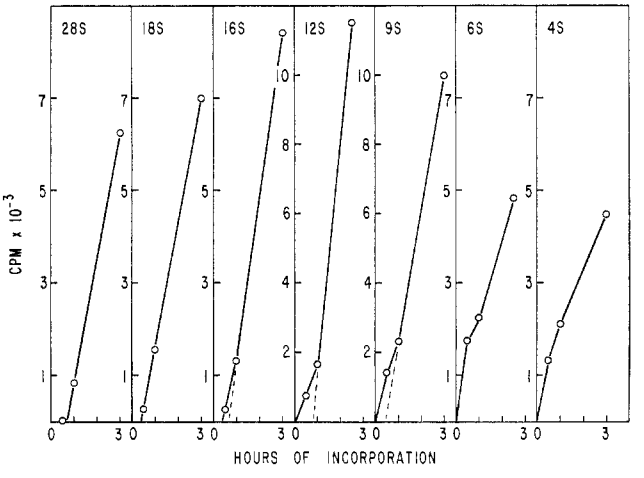

FIGURE 11: Kinetics of uridine-*t* incorporation into several fractions of cytoplasmic RNA. The counts per minute into fractions 28, 18, 16, 12, 9, 6, and 4 S in gradients of Figure 10 were taken and normalized using, as reference, the areas under 28S and 18S uv peaks. Normalized counts per minute plotted *vs.* time of incorporation in hours.

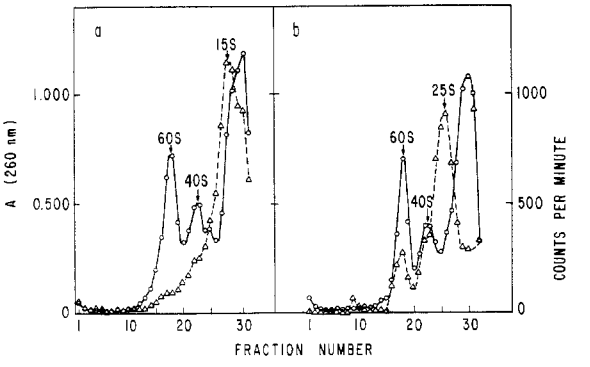

FIGURE 12: Distribution of labeled RNA among cytoplasmic particles in 1 mM EDTA. Aliquots of cytoplasmic fractions (prepared as in Figure 10) on EDTA gradients; centrifugation: 48,000 rpm, 80 min. Incorporation time: 30 min (a) and 60 min (b). Absorbance at 260 nm (○); radioactivity (△). It was not detected significant radioactivity amount in the bottom of tubes.

low, it can account for all observed results since this RNA class is very stable.

In third-period salivary gland 50–60% of labeled RNA in cytoplasm, after 3-hr incorporation, is in fractions of 9–16 S (Figure 10c). Throughout this and the accompanying paper, results are presented indicating that these 9S to 16S RNA fractions have several properties attributed to mRNA, for which we will call it messenger-like RNA (ml-RNA).[1] The sedimentation profiles (Figures 10 and 11) indicated that 9S ml-RNA shows up in cytoplasm at 25 min of incorporation and 12S and 16S ml-RNA at about 35 min. This labeling pattern suggest that 9S, 12S, and 16S ml-RNA are relatively stable and they accumulate in the cytoplasm being the most abundant mRNA in the cell at this stage of development. The data in Table III indicated that cytoplasmic labeled RNA, after 30 min of uridine-*t* incorporation, is completely extractable with phenol at pH 7.4 and 0°. But with 30-min incorporation the labeled ml-RNA has not reached the cytoplasm yet. However after 3-hr incorporation only 8% of total labeled cytoplasmic RNA was not extracted at 0°, which indicates that the majority of ml-RNA must be easily extractable with phenol at pH 7.4 and 0°.

EDTA and Mg gradients (Figures 12 and 13; Table IV) showed that cytoplasmic fractions had no EDTA-insensitive particles with sedimentation coefficient higher than 60 S. 25S particles were very well defined in EDTA gradients (Figure 12b) and also detected in Mg gradients (Figure 13), presumably being the carriers of ml-RNA in the cytoplasm. All particles originated by EDTA dissociation of polysomes sedimented between 20 and 60 S, as indicated by data in Table IV, these particles must include ml-RNA carriers and ribosomal subunits. RNA of about 6 S (Figures 10a and 11 (6 S)) and particles of about 15 S (Figures 12a and 13) were readily labeled in cytoplasmic fractions, they do not seem to be in equilibrium with polysomes (see Table IV) and their function and origin cannot be decided with the available results.

[1] Abbreviation used is: ml-RNA, messenger-like RNA.

Discussion

The rapidly labeled RNA in *Rhynchosciara* salivary glands showed the following properties: (a) it has a half-life of a few minutes, in very distinct cellular developmental and physiological states; (b) most of it probably turns over at the chromosomal level; (c) its population involves species that range widely in molecular weight, giving sedimentation coefficients from higher than 60 S to as low as 6 S; (d) it is poorly extracted with phenol at pH 7.4 and 0°; (e) it can be released from chromosomes into heavy DNA-free particles, that are EDTA insensitive and have sedimentation coefficients up to 300 S; (f) it contains rapidly hybridizing sequences among which some are exclusively nuclear, and includes (G + C)-rich sequences (see Armelin and Marques, 1972); (g) the RNA class with these properties represents at least 90% of total synthesized RNA at any cellular state (since even in actively growing cells the ribosomal precursor represents less than 5% of total RNA synthesized). The RNA class that we have been calling rapidly labeled RNA, characterized by the properties described above, is also known as heterogeneous nuclear RNA or sometimes DNA-like RNA, and seems to be characteristic of every eucaryotic system.

It was verified in mammalian cells that the rapidly labeled RNA is bound to very large nuclear structures (Sarmarina *et al.*, 1968; Penman *et al.*, 1968), which can be found in cytoplasmic fractions due to nuclear leakage (Perry and Kelley, 1968; Plagemann, 1969; Kabat and Rich, 1969; Olnes, 1969). In the case of liver cells these structures were well defined as ribonucleoprotein with characteristic properties (Samarina *et al.*, 1968). The nuclear labeled particles we described here seem to have the same properties as liver cells ribonucleoprotein at least as far as sedimentation behavior, EDTA insensitivity, RNase sensitivity, and kind of RNA they contain. Probably these particles are a general feature of eucaryotic cells as exclusively nuclear structures, perhaps as the mRNA processing units localized at the chromosomal level.

Special ribonucleoproteic particles called informosomes have been detected in several systems, carrying ml-RNA (Spirin, 1966; Perry and Kelley, 1966, 1968; Infante and Nemer, 1968; Kafatos, 1968); the ml-RNA carrying particles (Figure 12b) found in salivary gland cells must be of the same type. The labeling sequence of ml-RNA into the cytoplasm (Figures 10 and 11), suggested that mRNA processing lasts about 30 min, a time period considerably higher than the half-life of the heterogeneous nuclear RNA, which usually is considered as precursor of mRNA. The slightly polydisperse particles extracted from nucleus (Figure 8a) have RNA of relatively small size (Figure 8b) and hybridization properties similar to cytoplasmic ml-RNA (Figure 9); they might be immediate precursors of cytoplasmic monodisperse ml-RNA carrying particles (Figure 12b). These considerations will be resumed in the Discussion of the accompanying paper (Armelin and Marques, 1972).

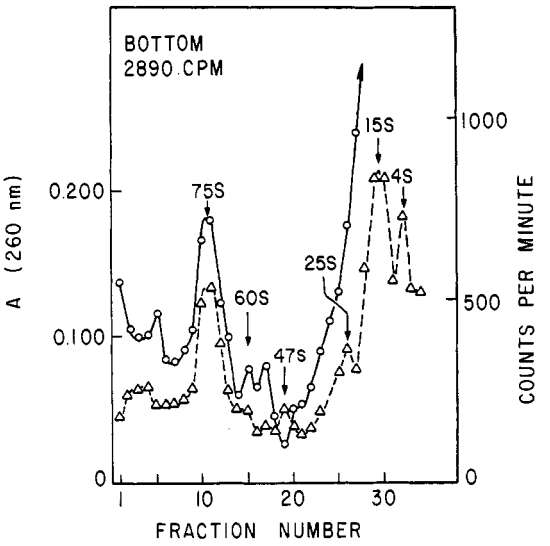

FIGURE 13: Distribution of labeled RNA among cytoplasmic particles in 1 mM Mg^{2+}. Two-hundred fourth-period larva 3-hr uridine-5-*t* (24.5 Ci/mmole, 1 mCi/ml) incorporation. An aliquot of cytoplasmic fraction (prepared as in Methods) placed on a Mg gradient. Centrifugation: 45,000 rpm, 2 hr. Absorbance at 260 nm (O); radioactivity (△).

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Transcription and Processing of Ribonucleic Acid in *Rhynchosciara* Salivary Glands. II. Hybridization of Nuclear and Cytoplasmic Ribonucleic Acid with Nuclear Deoxyribonucleic Acid. Indication of Deoxyribonucleic Acid Amplification†

Hugo A. Armelin* and Nelson Marques‡

ABSTRACT: Hybridization of nuclear and cytoplasmic RNA with nuclear DNA from *Rhynchosciara* salivary glands, were studied. The results suggested that the salivary gland cells have rapidly hybridizing RNA sequences restricted to the nucleus. Annealing reactions performed with DNA fractionated in CsCl gradients indicated that this exclusively nuclear RNA contains sequences rich in G + C. Apparently the hybridization observed with cytoplasmic RNA is due to reaction of messenger-like species. The results of hybridization reactions with both nuclear and cytoplasmic RNA suggested that there is gene amplification at the time of "DNA puff" development. Hybridization competition did not allow the detection of RNA sequences that are specific to the time of DNA puff development.

In mammalian cells RNA–DNA hybridization indicated that some transcribed RNA sequences are exclusively nuclear (Shearer and McCarthy, 1967; Melli and Bishop, 1969). In these studies for kinetics reasons, the experimental conditions limited the hybridization reaction to repetitive DNA (Britten and Kohne, 1968; Melli and Bishop, 1969), on one side, and to special classes of rapidly hybridizing RNA sequences, on the other (Darnell and Balint, 1970). The function of the repetitive DNA and of exclusively nRNA[1] sequences is very obscure. However, repetitive DNA is of general occurrence in eucaryotes and the same might be true for exclusively nRNA sequences. Consequently the elucidation of their function is very important to understand gene control mechanisms in higher organisms. Unfortunately the hybridization reaction has shown severe limitations to elucidate this question but it still remains as the most simple and powerful technique available to study nucleic acid sequence homology (McCarthy and Church, 1970). It would be desirable to examine in another system some aspects of nRNA that have been studied in mammalian cells. The availability of n- and cRNA from *Rhynchosciara* salivary glands (Armelin and Marques, 1972) opened the possibility of studying hybridization of those RNA fractions with nDNA. Our interest in studying these problems in this tissue comes from: (1) in different stages of larval development salivary glands show different sets of genes activated, at least as far as autoradiography of uridine-*t* incorporation and puff pattern in polytene chromosomes

† From the Departamento de Bioquimica, Instituto de Quimica, Universidade de São Paulo, São Paulo, Brazil. *Received October 12, 1972.*

* Corresponding address: University of California, San Diego, Department of Biology, La Jolla, Calif. 92037.

‡ Predoctoral fellow from Fundacão de Amparo á Pesquisa do Estado de São Paulo.

[1] Abbreviations used are: nRNA, cRNA, and ml-RNA, nuclear, cytoplasmic, and messenger-like RNAs; SCC, standard saline citrate.