

## CYTOPLASMIC ZONE ANALYSIS

### RNA Flow Studied by Micromanipulation

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#### ABSTRACT

A technique is described, cytoplasmic zone analysis, by which it is possible to study the flow of different RNA-containing components in the cytoplasm after their release from the nucleus. After a pulse of RNA precursors, the salivary glands of the insect *Chironomus tentans* are fixed and microdissected for the isolation of three zones of cytoplasm situated at increasing distances from the nucleus. The RNA from each zone is isolated and analyzed by gel electrophoresis. The three ribosomal RNA components, 18 S, 28 S and 5 S RNA, appear in steep, specific radioactivity gradients (exit gradients) during the time interval 2–3 h after a precursor injection, the 18 S RNA gradient lying 30–50  $\mu\text{m}$  peripheral to that of the 28 S or 5 S RNA gradient. Administration of puromycin led to the complete disappearance of the 18 S RNA and most of the 28 S RNA gradient within 45 min, suggesting that the gradients are caused by an engagement of the ribosomal subunits into polysomes close to the nucleus immediately or soon after their exit from the nucleus. The gradients may offer a unique model for the study of polysome formation and maintenance in vivo.

Studies on the interaction of the endoplasmic reticulum (ER), ribosomal subunits (RSU) and messenger ribonucleoprotein (RNP) have mainly been based on subcellular fractionation. This approach, in spite of its power, has the disadvantage of disturbing normal structure with limited lifetime of the preparations. A new technique which allows the study of component interactions in the translatory system in the living undisturbed cell will be described here. After a pulse of RNA precursors to the living animal, cells are fixed and microdissected into nuclei and cytoplasmic zones situated at increasing distances from the nucleus. The RNA from each cellular fraction is isolated and separated into its main classes by gel electrophoresis. The pattern of appearance of each class of RNA can be followed as a function of time and the distance from the nucleus. This pattern is influ-

enced by the associations in which the RNA-containing components, e.g. RSU, engage in vivo during their spread in the cytoplasm. This permits conclusions to be drawn regarding the nature of these associations.

The salivary gland cell of the dipteran *Chironomus tentans* has many advantages for this work. It is a fully differentiated cell, specialized for production of a few secretory proteins (8). It does not divide but grows by continuous increase in size. It is so large that at least three concentric cytoplasmic zones can be isolated. It has an RNA metabolism intense enough that sufficient label in RNA can be obtained from a limited number of cells.

In this paper the main methodological procedures and the early events are described. It will be shown that both labeled light and heavy RSU appear in steep gradients which can be eliminated

with puromycin, in the former case completely, in the latter case largely. The gradients are most easily understood as a result of an early or immediate engagement of the RSU into polysomes. They may thus provide a convenient model for the study of factors influencing polysome formation *in vivo*.

## MATERIALS AND METHODS

### *Animals and Labeling Conditions*

Late fourth *C. tentans* instar larvae, 7- to 9-wk old and weighing around 25 mg, were injected with 25  $\mu$ Ci [ $^3$ H]uridine (50–58 Ci/mmol), when not otherwise specified, with a micropipette 10- $\mu$ m wide at the tip. During the injection the pipette was held by a de Fonbrune micromanipulator under the control of a stereomicroscope.

The isotopes were evaporated to dryness *in vacuo* in two steps to make possible the dissolution of the isotope for each animal in 1  $\mu$ l of 0.67% NaCl, 0.04% KCl. Pipettes were made and filled in an oil chamber arrangement as previously described (4).

### *Preparative Procedure*

The salivary glands were prepared as described by Pelling (12) with slight modifications, i.e. fixed at +4°C for 5 min in freshly prepared ethanol/glacial acetic acid, 3:1 by volume, followed by 2  $\times$  15-min rinses in cold 70% ethanol and finally transferred to ethanol:glycerol mixture, 1:1 by volume, in which dissection takes place with the de Fonbrune micromanipulator in an oil chamber arrangement under control in a phase-contrast microscope (4). The geometry of the cells is complicated and pockets penetrate deep into the cytoplasm from the gland lumen. Furthermore, cells differ in size and shape within and between glands. The zones are defined only by their distance from the nucleus and not from the plasma membrane. Flat cells with centrally situated nuclei are selected. The secretion can be removed from the fixed cells and pulled out of the pockets during the dissection. The outer parts of the cytoplasm from a cell were first peeled off and placed separately in the oil chamber, then the intermediate parts and finally the remainders surrounding the nucleus (Fig. 1). Parts from 12 cells were pooled for analysis when not otherwise specified. In certain cases, the cytoplasm was divided into only an outer and an inner zone. The cells used had a diameter of 150–200  $\mu$ m. The cytoplasmic zones had a width of 20–30  $\mu$ m.

### *Extraction and Agarose Gel Electrophoresis of RNA*

The extraction technique, originated by Pelling (12), was used as described by Daneholt (3) with minor modifications. The samples were transferred to 200  $\mu$ l of 1

mg Pronase/ml 0.02 M Tris buffer, pH 7.4 with 0.5% sodium dodecyl sulfate (SDS) and with 30  $\mu$ g of *Escherichia coli* carrier RNA, preincubated for 30 min at +37°C. The isolated samples were dissolved for 5 min under shaking at +25°C, after which 5  $\mu$ l of 4 M NaCl was added, followed immediately by 500  $\mu$ l of cold ethanol, and the mixtures were shaken and stored at –20°C overnight. They were then spun for 10 min at 10,000 rpm in the cold, the supernate was removed and the precipitate dissolved in 20  $\mu$ l of 0.02 M Tris buffer, pH 7.4 containing 0.5% SDS. The dissolved RNA was separated in a 1.5% agarose gel slab.

Agarose gels were made by swelling the agarose for 15 min in electrophoretic buffer before dissolution for 5–10 min in a boiling water bath. The solution was poured into a slab mould of the dimension 50 mm  $\times$  90 mm  $\times$  3 mm provided with two or three application grooves, 10 mm  $\times$  2 mm  $\times$  1 mm. The gel was ready for use after 45 min at +4°C. It was placed in an electrophoretic chamber according to Wieme (16), which was placed in the cold. The separation time was about 2.5 h at 40V/12 cm which allowed 4 S RNA to move about 60 mm. The carrier *E. coli* RNA (23 S, 16 S, 4 S RNA) was localized with a Minuvis lamp at 254 nm and registered with india ink. The slab was washed for 2  $\times$  50 min in 5% trichloroacetic acid at +4°C, followed by two rinses in distilled water, each for 50 min, after which the gel was sliced into 1.1-mm wide slices. The slices were each placed in a disposable vial for scintillation counting (Packard Instrument Co., Downers Grove, Ill.) and 10 ml of scintillation cocktail was added (30 ml Soluene-100, 20 ml methoxyethanol, 5.5 g Permablend III (Packard) and toluene to 1,000 ml). The vials were left at +37°C overnight, after which they were ready for counting in a Packard scintillation counter.

### *Acrylamide Gel Electrophoresis in the Presence of 8 M urea*

A 7.5% acrylamide gel containing 8 M urea was made in the following way. 19.2 g of urea (Schwarz/Mann Ultra Pure, Schwarz/Mann Div. of Becton, Dickinson & Co., Orangeburg, N. Y.) were dissolved in a solution made up of 7.5 ml of 40% acrylamide in distilled water, 13.4 ml of (1%) bis-acrylamide in distilled water and 4 ml of buffer (0.5 M Tris, pH 7.8, 0.02 M EDTA). After the addition of 50  $\mu$ l of TEMED and 350  $\mu$ l of freshly prepared 10% ammonium persulfate, the gel was allowed to polymerize for 60 min at room temperature. The gel was prepared in a vertically placed form which allows the circulation of cooling tap water on both sides. Application pockets were made by the insertion of a piece of teflon before polymerization. Electrophoresis was performed for 3 h at 300 V/15 cm and 42 mA. 0.05 M Tris buffer, pH 7.8, containing 0.002 M EDTA and 0.2% SDS was used as circulating buffer.

The RNA sample to be analyzed by electrophoresis was dissolved in 50  $\mu$ l of application buffer, freshly

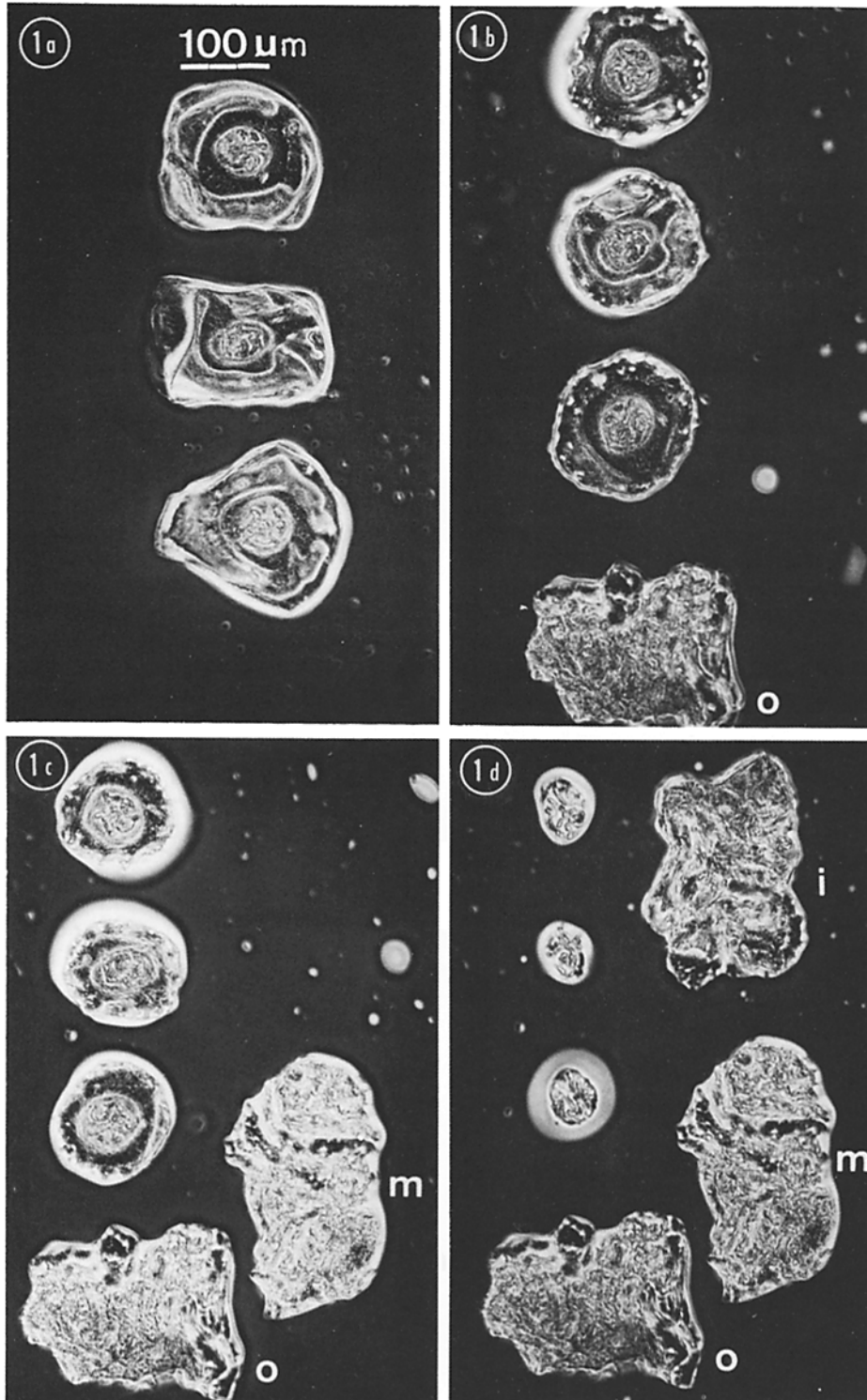


FIGURE 1 Three cells isolated by micromanipulation (*a*), after removal of the outer zone (*b*), after removal of the middle zone (*c*), and after removal of the inner zone (*d*). The bar in (*a*) is 100  $\mu\text{m}$  long; *i*, *m*, and *o* denote material from the inner, middle and outer zones, respectively.

prepared by making 8 M urea in circulating buffer. The sample was heated for 1 min at 50°C in this solution, after which 10  $\mu$ l of glycerol-bromophenol blue mixture was added and the solution applied on top of the gel under a layer of application buffer. Slicing and counting was performed as for agarose gels, except that 0.4% water was added to the scintillation cocktail.

## RESULTS

### Control Experiments

**FIXATION SHRINKAGE:** The preparative procedure, i.e. fixation, ethanol treatment and storage in ethanol-glycerol, resulted in some shrinkage. Fresh glands were placed in a physiological solution (13), and seven cells were selected for measurement of the largest cell diameter. They were then fixed according to the routine procedure and the same cells measured again in the ethanol:glycerol solution. After fixation, the diameters of the cells were  $81 \pm 2\%$  (mean  $\pm$  SEM) of those of the same cells in the fresh state.

**DISSECTION ACCURACY:** It is not imperative that the cytoplasm be dissected into three portions of absolutely equal volume since, in this and the following work, conclusions are based on relations between RNA fractions within one and the same separation and on the way these relations change. Nevertheless, the volumes of the different zones should be roughly similar to make compari-

son possible between different experiments. This is probably the case. The distribution of 4 S RNA which is likely to represent the volume distribution was investigated in analyses of zones from animals which had received precursors 18 h or more before precursor injection. It was found that the average recovery of 4 S RNA was within a few percent of the 33%, representing one-third of the total amount, and that the values showed a standard of deviation of  $\pm 2.7\%$  (Table I). A test of the justification of using 4 S RNA as a volume marker was done with the aid of  $^{14}\text{C}$ -labeled glycerol added to the medium in which dissection normally takes place. The medium penetrates the fixed tissue like an embedding medium. If its concentration in different parts of the cytoplasm is similar, wt/vol, the  $^{14}\text{C}$  label will constitute a volume index. We have no guarantee that this is the case, although there is no reason to see why it should be unevenly distributed. If, however, the tritium-labeled 4 S RNA distributes in proportion to  $^{14}\text{C}$ -labeled glycerol, it would considerably strengthen the basis for using 4 S RNA as a volume marker. The peripheral zone was isolated in six different glands and compared with the remaining cellular parts after the glands had been blotted with Millipore filter pieces to remove excess moisture. The ratios between 4 S RNA-tritium and  $^{14}\text{C}$  are given in Table II. The glycerol data suggest that 4 S

TABLE I  
*Distribution of Radioactive 4 S RNA in Percent of the Total Cytoplasmic Content in Cytoplasmic Zones 18 h and 2 Days after Precursor Injection*

	Inner zone	Middle zone	Outer zone
Mean $\pm$ SEM	34.4 $\pm$ 0.7 (2.6)	31.9 $\pm$ 0.7 (2.7)	33.5 $\pm$ 0.5 (2.1)

Each mean value is based on 16 determinations. Values for standard deviation are within parentheses.

TABLE II  
*Distribution of [ $^3\text{H}$ ]4 S RNA and [ $^{14}\text{C}$ ]Glycerol*

	Outer zone			Inner + middle zone		
	$^3\text{H}$ -cpm	$^{14}\text{C}$ -cpm	$^3\text{H}/^{14}\text{C}$	$^3\text{H}$ -cpm	$^{14}\text{C}$ -cpm	$^3\text{H}/^{14}\text{C}$
Animal 1	1,373	657	2.09	2,997	1,344	2.23
	1,889	872	2.15	3,958	1,721	2.30
Animal 2	2,118	921	2.30	4,143	1,936	2.14
	1,953	876	2.23	4,000	1,688	2.37
Animal 3	1,729	910	1.90	3,786	1,893	2.00
	1,655	836	1.98	3,338	1,795	1.86

Animals were sacrificed 2 days after injection of 25  $\mu\text{Ci}$  of uridine. After fixation the glands were stored in ethanol:glycerol, 1:1 by volume, for 72 h at +4°C with  $^{14}\text{C}$ -labeled glycerol added. After ethanol-precipitation of the dissected sample, the  $^{14}\text{C}$ -glycerol in the supernate was counted in Instagel while the [ $^3\text{H}$ ]4 S RNA in the pellet was measured after gel electrophoresis.

RNA is a suitable volume marker which would *a priori* be expected in view of the diffusible nature of this component.

The spread in values for 4 S RNA within zones is most probably due to the subjective element in the zone divisions rather than to a variable recovery of RNA. The former error is reduced by the use of at least 12 cells per analysis. In the analytical procedure, tissue is dissolved in detergent solution and analyzed after a single precipitation of RNA, without any other treatments, reducing the possibilities of irregular losses. Analyses of different zones from the same cells were always run in parallel to facilitate comparison and reduce errors.

#### Types of RNA in the Cytoplasm

At least five different types of RNA with a nuclear origin are present in the cytoplasm of *C. tentans* salivary gland cells. The temporal pattern of their appearance has previously been described (5) and will be briefly summarized here. There is an early appearing messenger-like RNA, later appearing Balbiani ring transcripts, RNA in the two ribosomal components (28 S and 18 S RNA), and 4-5 S RNA. The work reported here was focused on the ribosomal RNA components.

Fig. 2 shows the labeled RNA in the three cytoplasmic zones 5 wk after precursor injection. There is, from the left to the right, Balbiani ring RNA close to the origin, followed by 28 S, 18 S and 4 S RNA, obviously in similar amounts in the three zones. This should reflect the distribution of stable RNA and/or the steady-state labeling picture, depending on the degree of reutilization of label in the animal. Determination of the 28 S/4 S and 18 S/4 S RNA ratios shows no evidence for the presence of gradients (Fig. 3). Consequently, the 28 S/18 S RNA ratio is similar in the three zones.

There is a great deal of variation between different animals in the amount of label incorporated. This can be of the order of 10 times per cell. There is also some variation in the temporal pattern of appearance of the different RNA species in the cytoplasm, although this variable is rather moderate if animals of similar size and age are used.

#### Spread of Ribosomal RNA

Ribosomal RNA begins to appear in the cytoplasm 90-120 min after precursor injection, and the early distribution after 120 min is shown in Fig. 4. Label in the two ribosomal components

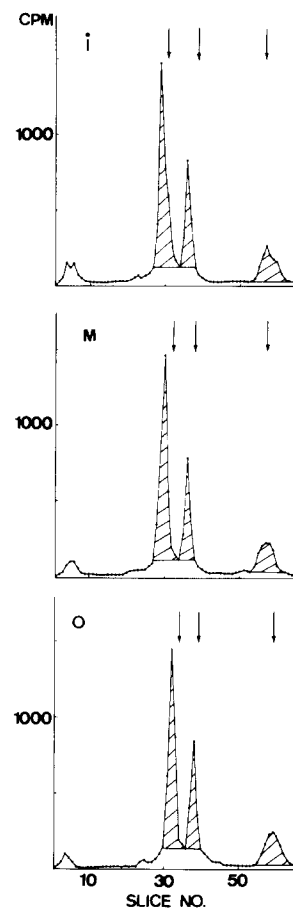


FIGURE 2 Electrophoretic separations in 1.5% agarose of the RNA from cytoplasmic zones of 12 cells isolated from the salivary gland of an animal that was injected with 10  $\mu$ Ci of tritiated uridine 5 wk before sacrifice. The arrows indicate, from left to right, the positions of 23 S, 16 S and 4 S RNA added markers from *E. coli*. The letters *i*, *m*, and *o* stand for inner, middle and outer cytoplasmic zones. The components shown in the separations are the Balbiani ring transcripts (slice 3-5), 28 S, 18 S, and 4 S RNA.

decreases with increasing distance from the nucleus forming exit gradients (Fig. 5). The gradient for the light RSU (18 S RNA) lies peripheral to that of the heavy RSU (28 S RNA). We find it difficult to obtain the inner cytoplasmic zone free from contamination by nuclear sap. This must be kept in mind in the evaluations of the ratios for the inner zone, because it can lead to artefactual values. All conclusions are, however, based on results of analyses of the middle and outer zones.

3 h after precursor injection the picture is simi-

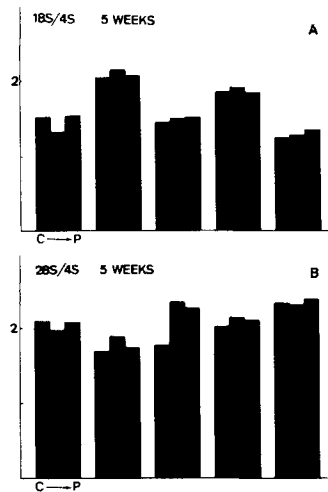


FIGURE 3 Staple diagrams of the 18 S/4 S RNA label ratio (A) and 28 S/4 S RNA label ratio (B) in 5 animals injected with 10  $\mu$ Ci of tritiated uridine 5 wk before sacrifice. The letters *c* and *p* stand for central and peripheral part of the cytoplasm, respectively.

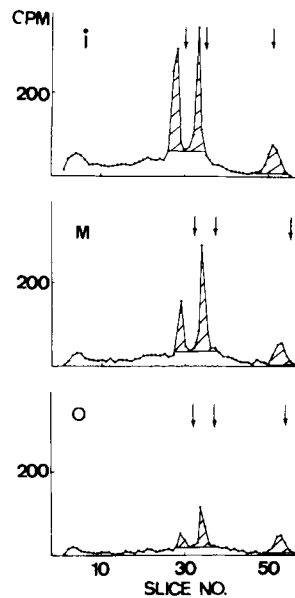


FIGURE 4 Electrophoretic separations of the RNA extracted from cytoplasmic zones from 12 salivary gland cells from an animal injected with 25  $\mu$ Ci of tritiated uridine 2 h before sacrifice. For other details, see Fig. 2.

lar although 28 S RNA has regularly reached the most peripheral zone of the cytoplasm, which is not always the case after 2 h. The 28 S RNA gradient is well pronounced (Fig. 6). The 18 S RNA gradient is, however, not quite so distinct as

after 120 min. From the data of Fig. 5 and Fig. 6, one can calculate that the relative specific activity gradient of 18 S RNA is about 1.5 zone widths more peripheral than the 28 S RNA gradient, corresponding to 30–50  $\mu$ m. To estimate relative specific activity, the 28 S RNA values were divided by two to become comparable with the 18 S RNA data. Since the time difference in the exit from the nucleus to the cytoplasm between the heavy and light RSU is of the order of 30 min (1, 2, 5), the initial net peripheral migration rate for the light RSU containing 18 S RNA is of the order of 1  $\mu$ m/min. 6 h after injection there is still a distinct 28 S RNA gradient, while at this time the 18 S RNA gradient has disappeared (Fig. 7).

#### Puromycin Treatments

Puromycin is known to release both RSU from free polysomes and the light one from bound poly-

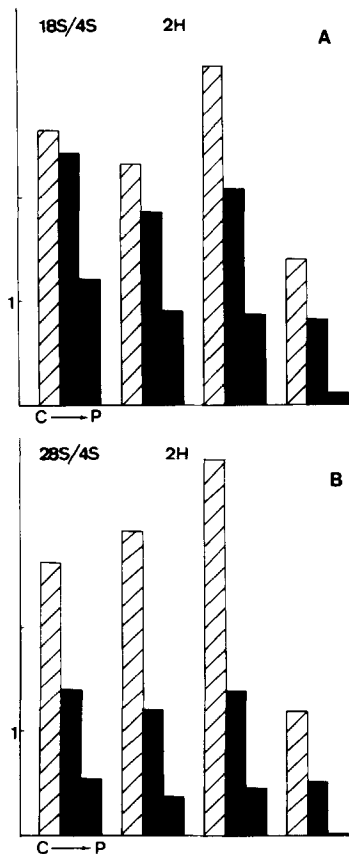


FIGURE 5 Staple diagrams of the label ratios in 18 S/4 S (A) and 28 S/4 S RNA (B) in the three cytoplasmic zones in four different experiments 2 h after precursor injection.

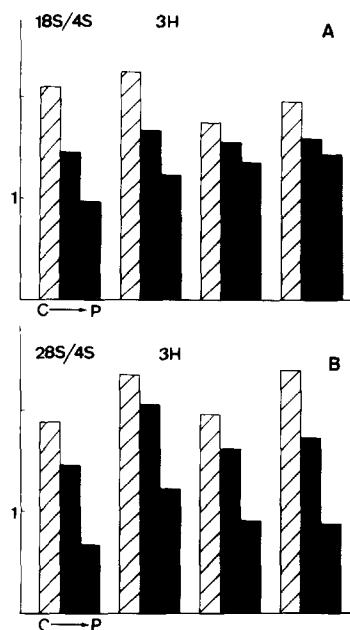


FIGURE 6 Diagrams of the label ratios in 18 S/4 S (A) and 28 S/4 S RNA (B) 3 h after precursor injection.

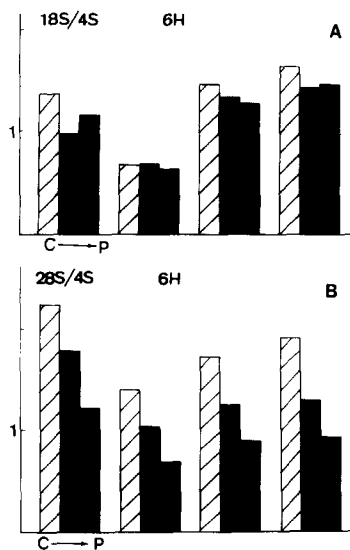


FIGURE 7 Staple diagrams of the label ratios in 18 S/4 S (A) and 28 S/4 S RNA (B) 6 h after precursor injection.

somes (9, 14). It should, therefore, offer a convenient means of deciding whether the gradients for the two RSU are caused by their engagement in polysomes. Thus, an increased tendency for a gradient to disappear due to puromycin would

indicate that it is caused by association of RSU into polysomes.

Animals were labeled for either 2 or 3 h and then sacrificed. One gland was placed in Cannon's modified culture medium (13) with puromycin (100  $\mu\text{g/ml}$ ), the sister gland in medium without puromycin, and both glands were fixed after incubation for 45 min. Control experiments showed that protein synthesis in the puromycin-treated gland was inhibited to 89% of the untreated sister gland during 30-min incorporation after preincubation of the gland for 15 min.

2 h after precursor injection, only labeled light RSU have appeared in sufficient quantities to allow an evaluation of the effect of the drug which is to permit the light RSU, measured as labeled 18 S RNA, to distribute evenly in the cytoplasm (Fig. 8). This is in striking contrast to the controls which show that the light RSU are present in steep specific activity gradients as previously observed.

After 3 h, the results for the light RSU are essentially the same as after 2 h (Fig. 9). At this time-point also the heavy RSU can be studied. It is present in the control cells, like the light RSU, in steep gradients in specific activity. Puromycin treatment eliminates most of the gradients for the heavy RSU but leaves a significant excess of labeled heavy RSU in the central parts of the cell

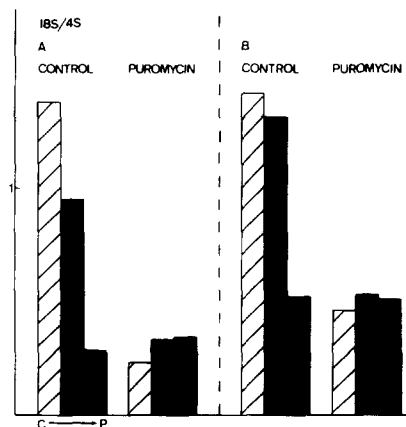


FIGURE 8 Staple diagrams of the 18 S/4 S RNA label ratio during puromycin treatment. Animals were given 25  $\mu\text{Ci}$  of tritiated uridine 2 h before sacrifice, after which they were sacrificed and one gland was taken to Cannon's modified culture medium (control) and the sister gland to the same medium supplemented with puromycin (100  $\mu\text{g/ml}$ ). After 45 min, both glands were fixed and prepared for analysis. (A) and (B) denote two different animals.

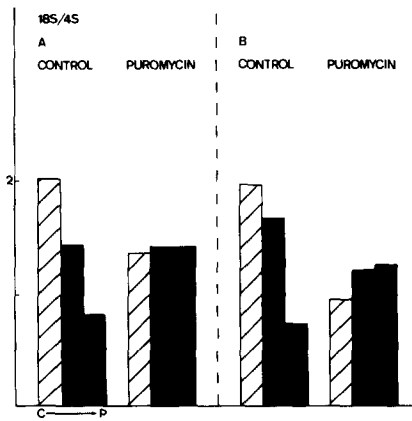


FIGURE 9 Staple diagrams of the 18 S/4 S RNA label ratio during puromycin treatment. Animals were sacrificed after 3 h, but other conditions were identical to those described in the text to Fig. 8.

(Fig. 10). Fig. 11 shows electrophoretic separations of controls and puromycin-treated glands 3 h after precursor injection and 45-min treatment *in vitro*.

#### Spread of 5 S RNA

The cytoplasmic 5 S RNA is localized in heavy RSU. An investigation of the distribution of newly labeled 5 S RNA would, therefore, provided 5 S RNA is irreversibly bound to the RSU, offer an opportunity of confirming the existence of exit gradients for the heavy RSU. The analytical situation is particularly favorable in this case, since it is measured together with the reference 4 S RNA in a range of the electrophoretic separation essentially free from messenger RNA.

The cells were dissected into three cytoplasmic zones but the inner zone was not analyzed. It was found that 5 S RNA appears 3 h after precursor injection in steep activity gradients similar to those of 28 S RNA (Figs. 12 and 13). Gradients still remain after 6 h, although less pronounced, again a parallel to the situation for 28 S RNA (Fig. 14). The 5 S data thus confirm earlier conclusions regarding a gradient in the cytoplasm for newly formed heavy RSU.

#### DISCUSSION

The present approach requires that cytoplasm be isolated according to its position relative to the nuclear envelope. The dissection procedure might at first sight appear far from ideal for this purpose since the division is based on a subjective evalua-

tion of volumes; furthermore, the delimitations of the zone borders necessarily follow a somewhat irregular outline. In spite of these objections, the results indicated that the cytoplasm can be rather accurately divided into volumes of similar size. For establishing the presence of a gradient along the cell axis, a division of the cytoplasm into two zones might be considered sufficient. After short labeling times, 6 h and less, it is, however, not always possible to be certain that the cytoplasm adjoining the nucleus can be obtained free from labeled RNA present in the nucleus which might interfere with the cytoplasmic RNA analysis. Therefore, three zone analyses are desirable, permitting the study of the distribution of RNA along the radius in the outer two-thirds of the cytoplasm.

This investigation shows that newly synthesized heavy and light RSU both appear in steep specific activity gradients as soon as they appear in the cytoplasm. It furthermore shows that the gradient for the light RSU lies 30–50  $\mu\text{m}$  peripheral to that of the heavy RSU of the same age. This difference can most easily be understood as a result of the difference in processing times in the nucleus. The light RSU enters the cytoplasm about 30 min before the heavy one, and its initial spreading rate is therefore of the order of 1  $\mu\text{m}/\text{min}$ . There is another difference between the gradients; whereas the heavy RSU gradient is still present after 6 h, the one for the light RSU is more or less leveled out.

It might appear surprising that gradients form at all since particles of this size would be expected to

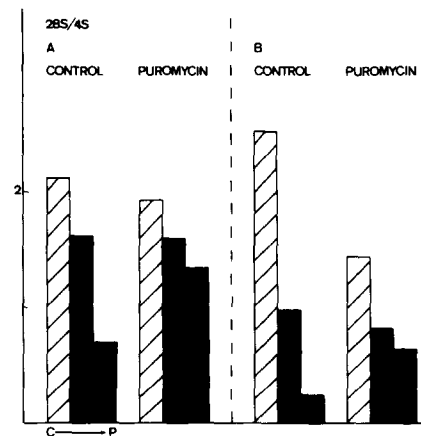


FIGURE 10 Staple diagrams of the 28 S/4 S RNA label ratio during puromycin treatment from animals recorded in Fig. 9.



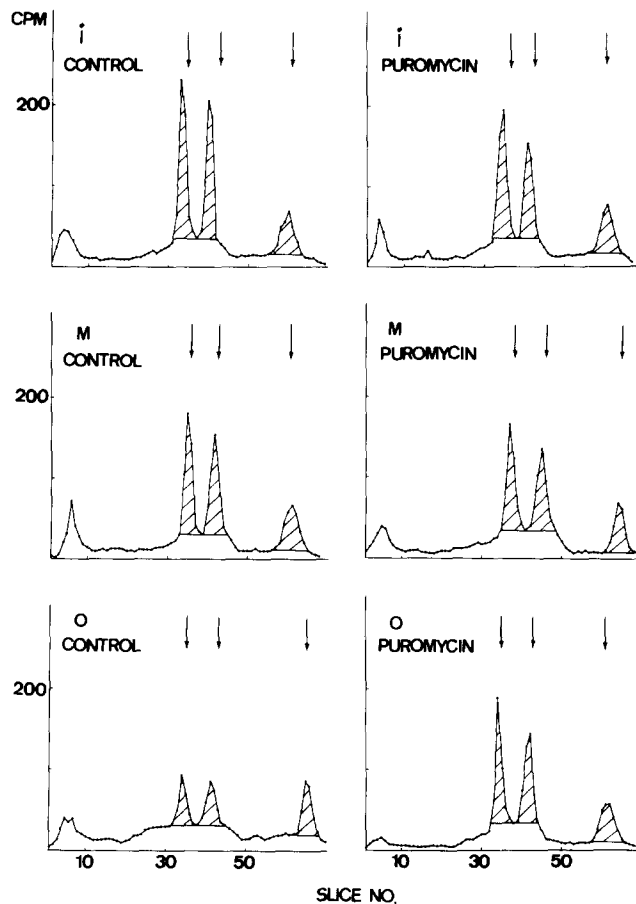


FIGURE 11 Electrophoretic separations in 1.5% agarose of RNA from cytoplasmic zones of controls and puromycin-treated glands. The conditions of the experiment are given in the text to Fig. 9; *i*, *m*, and *o* denote inner, middle and outer cytoplasmic zones, respectively.

spread rapidly through the cytoplasm. The puromycin experiments provide an explanation for this behavior since they show that puromycin causes a rapid leveling out of the gradients, for the light RSU a complete action, for the heavy one a partial but still considerable effect. In view of the well established effects of puromycin, there is consequently strong indication that the gradients form because of an early or immediate engagement of RSU into polysomes after their release from the nucleus. In such a case, the association must take place already in the vicinity of the nucleus. These results also show that at least the light RSU is able to reach distributional equilibrium within 45 min and that therefore the gradients that we observe cannot be explained as due to unspecific diffusion barriers in the cytoplasm, e.g. contorted diffusion

channels. This agrees with findings in other systems that RSU assemble to polysomes within a short time after their arrival in the cytoplasm (2, 7, 9).

Salivary gland cells contain polysomes in free and bound form. Whereas light RSU in both types of polysomes should be sensitive to puromycin, ER-bound polysomes might not release their heavy RSU if these are directly connected to the ER. One possible explanation for the partial refractoriness of the heavy RSU gradient to puromycin would therefore be that some heavy RSU have become associated to bound polysomes. This would also explain why at 6 h this gradient still remains in contrast to the light RSU gradient. Investigations have been carried out after longer labeling times to clarify this question (10).

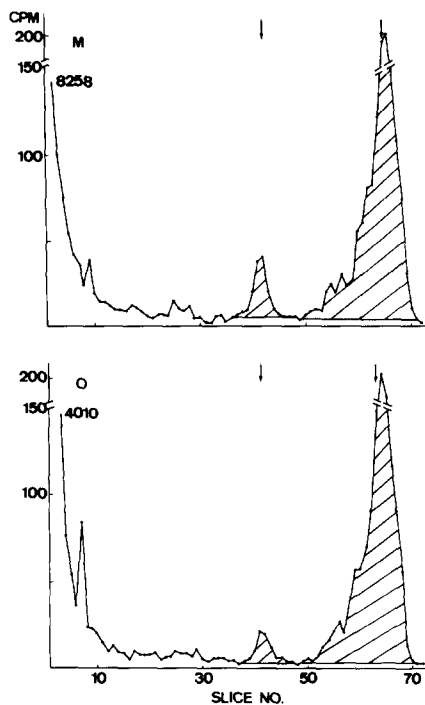


FIGURE 12 Electrophoretic separations of low molecular weight RNA in 7.5% polyacrylamide gels containing 8 M urea. The RNA was isolated from microdissected cytoplasmic zones from fixed salivary gland cells injected with tritiated uridine 3 h before sacrifice. The arrows indicate, from left to right, 5 S and 4 S RNA positions of added markers from *E. coli*. The letters *m* and *o* denote the middle third and the outer third of the cytoplasm, respectively.



FIGURE 13 Diagram of 5 S/4 S RNA label ratio in different cytoplasmic zones 3 h after precursor injection. The cytoplasm of the inner zone which was not analyzed is marked with broken lines.

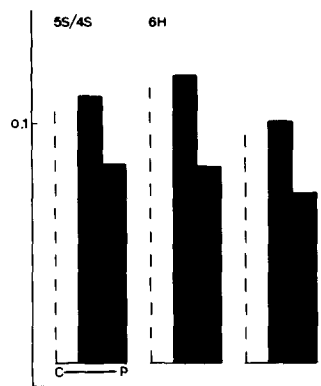


FIGURE 14 Diagram of 5 S/4 S RNA label ratio in different cytoplasmic zones 6 h after precursor injection.

Zone analyses of 5 S RNA shows that this RNA fraction, like the 28 S and 18 S RNA components, appears in steep radioactivity gradients. Since it is a constituent of the heavy RSU, this behavior is the expected one if it does not exchange with any pool of free 5 S RNA in the cytoplasm. Such an irreversibility of binding has previously been demonstrated with ribosomes containing oocyte type of 5 S RNA which was found not to exchange with somatic type of 5 S RNA (11). These 5 S RNAs differ in nucleotide composition (6). In the present case, the supplementary demonstration is made that there is no measurable exchange within a somatic 5 S RNA population. Our results, in confirmation of previous experience, also show that the 5 S RNA must be contained in the heavy RSU already when they enter the cytoplasm (15). If it were first released to the cytoplasm and only afterwards combined with the heavy RSU, it would not be expected to appear in gradients since RNA of this size should distribute evenly as soon as it appears.

The specific advantage of using 5 S RNA for an assay of exit gradients lies in the fact that its electrophoretic migration is such that it is essentially uncontaminated by other RNA, in contrast to the 28 S and 18 S RNA components which overlie a heterodisperse distribution of nonribosomal RNAs. Nevertheless, also in the latter two cases the differences in amounts between the zones are such that they cannot easily be explained by differences in underlying nonribosomal RNA.

The presence of exit gradients for the two RSU, probably caused by their engagement into polysomes, may provide a unique model for the study of factors influencing polysome formation. This is

likely because the studied sequence of events will occur in the intact cells in the living animal.

The authors are indebted to Elisabet Ericson and Kerstin Spetz for skillful technical assistance, to Chana Szpiro for culturing the animals, to Anne-Marie Makower for drawing the illustrations, and to Hannele Jansson for typing the manuscript.

The present work was supported by grants from the Swedish Cancer Society and Karolinska Institutet (Reservationsanslaget).

Received for publication 10 November 1975, and in revised form 7 May 1976.

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