

# MOVEMENTS AND ASSOCIATIONS OF RIBOSOMAL SUBUNITS IN A SECRETORY CELL DURING GROWTH INHIBITION BY STARVATION

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## ABSTRACT

In *Chironomus tentans* salivary gland cells, the cytoplasm can be dissected into concentric zones situated at increasing distances from the nuclear envelope. After RNA labeling, the newly made ribosomal subunits are found in the cytoplasm mainly in the neighborhood of the nucleus with a gradient of decreasing abundance towards the periphery of the cell. The gradient for the small subunit lasts for a few hours and disappears entirely after treatment with puromycin. The large subunit also forms a gradient but one which is only partially abolished by puromycin. The residual gradient which is resistant to the addition of the drug is probably due to the binding of some large ribosomal subunits to the membranes of the endoplasmic reticulum (J.-E. Edström and U. Lönn. 1976. *J. Cell Biol.* 70:562-572, and U. Lönn and J.-E. Edström. 1976. *J. Cell Biol.* 70:573-580).

If growth is inhibited by starvation, only the puromycin-sensitive type of gradient is observed for the large subunit, suggesting that the attachment of these newly made subunits to the endoplasmic reticulum membranes will not occur. If, on the other hand, the drug-resistant gradient is allowed to form in feeding animals, it is conserved during a subsequent starvation for longer periods than in control feeding animals. This observation provides a further support for an effect of starvation on the normal turnover of the large subunits associated with the endoplasmic reticulum. These results also indicate a considerable structural stability in the cytoplasm of these cells with little or no gross redistribution of cytoplasmic structures over a period of at least 6 days.

Studies on the ribosome cycle and the interactions of polysome components with the endoplasmic reticulum (ER) have been carried out mainly with the aid of homogenization and subfractionation. The application of such techniques has recently led to several similar models for the ribosome cycle in membrane polysomes (2, 10, 13). We have found it possible to supplement this approach with a procedure, cytoplasmic zone analysis, which measures the distribution of ribosomal subunits (RSU) in the cytoplasm as related to their

distance from the nuclear envelope (7). After their release from the nucleus, labeled RSU are found mainly in the vicinity of the nucleus, with decreasing amounts towards the periphery of the cell. The duration and pharmacological properties of these gradients are influenced by associations formed by the RSU. Since the procedure is applied to fixed cells, it is independent of artefacts that can arise as a result of conventional cell fractionation procedures.

In growing salivary gland cells from *Chirono-*

*mus*, both the light and the heavy RSU appear in steep gradients during the first hours after their appearance in the cytoplasm (7). The gradients disappear during the administration of puromycin. Our interpretation of these results is that the gradients are due to binding of RSU to polysomes. Thus, RSU appear to form polysomes in the central part of the cytoplasm when leaving the nucleus. The 18S RNA (marker for the light RSU) is close to distributional equilibrium 6 h after labeled RNA precursor injection, but parts of the 28S and 5S RNA (marker for the heavy RSU) remain in gradients for at least 2 days (11). Furthermore, this residual gradient is resistant to the addition of puromycin. These drug-resistant gradients of long duration are likely to be due to heavy RSU binding to the ER.

We wished to obtain further information on the relation between RSU and the ER in these cells in which at least 80–90% of the protein synthesis is accounted for by secretory proteins (5, 14). Therefore, we explore in the present communication the possibility of analyzing gradient formation and maintenance during growth inhibition caused by starvation. This situation is of interest insofar as it would be expected to prevent the normal expansion of the ER during growth. It will be shown that whereas puromycin-sensitive gradients form much as they do in growing cells there are distinct effects on the drug-resistant gradients for the heavy RSU.

## MATERIALS AND METHODS

### *Biological Material*

Larvae were used from a stock of *Chironomus tentans* originally collected at Drottningholm outside Stockholm and were kept in the laboratory for several generations. They were cultured in deionized water containing NaCl (0.4 g/liter) and cellulose tissue. Fermented nettle powder was given twice weekly. Cultures were aerated and kept at +18°C with a 16/8 h day/night rhythm. Animals were starved under the same conditions but in the absence of cellulose and nettle powder.

### *Labeling Conditions, Preparative and Analytical Procedures*

Late fourth instar larvae (7–9 wk old), weighing about 25 mg and starved for 3 days, were injected with tritiated uridine (42–50 Ci/mmol) and cytidine (26 Ci/mmol), 20  $\mu$ Ci of each, dissolved in 1  $\mu$ l of 0.67% NaCl, 0.04% KCl. Since there is less RNA labeling in starving than in feeding animals, two radioactive nucleoside precursors were required for sufficient incorporation. Although both cytidine and uridine are potential DNA precursors, the relative incorporation into DNA is insignificant when

both precursors are given and allowed to metabolize for several days (8). Feeding animals were injected with tritiated uridine only, in a dose of 25  $\mu$ Ci, as in previous investigations.

The salivary glands were prepared and dissected into cytoplasmic zones with a de Fonbrune micromanipulator as previously described (7). Flat cells of relatively uniform size with central nuclei were selected. The cytoplasmic zones were defined by their distance from the nucleus and not from the plasma membrane. The outer parts of the cytoplasm were first peeled off, then the intermediate parts and finally the remainder surrounding the nucleus. 12 cells were used for each analysis. The resulting three samples were always analyzed in parallel.

Agarose gel electrophoresis and RNA extractions were done as previously described (7).

### *Chemical Determinations*

Protein was determined according to Lowry et al. (12) with bovine serum albumin as standard. RNA was determined according to Fleck and Munro (9).

### *Materials*

Commercial sources of chemicals were as follows: The Radiochemical Centre (Amersham, England) for [5,6-<sup>3</sup>H]uridine (42–50 Ci/mmol) and [5-<sup>3</sup>H]cytidine (26 Ci/mmol), Calbiochem (San Diego, Calif.) for actinomycin D, Bausch & Lomb, Inc., (Consumer Products Div., Rochester, N.Y.) for agarose, Serva (Heidelberg, Germany) for sodium dodecyl sulphate, and Sigma Chemical Co. (St. Louis, Mo.) for puromycin.

## RESULTS

### *Metabolic Parameters during Starvation and Feeding*

**GROWTH OF ANIMALS:** 7-wk-old larvae, weighing 20 mg, grow continuously, adding about 0.4 mg body weight per day under our culturing conditions. As shown in Fig. 1, the protein and RNA contents in the salivary glands increase with the weight of animals. Larvae kept in the absence of food lose weight during the first 2 days. From day 3 and onwards, there is no change in body weight. When feeding is reinstated, the animals resume growth. On the average, about two-thirds of the larvae survived starvation for 6 days. The remainder either died or pupated. In animals starved for 6 days and weighing 24.0–25.9 mg, the protein and RNA values (means  $\pm$  SEM) were  $35.0 \pm 2.38 \mu\text{g}$  ( $n = 11$ ) and  $4.52 \pm 0.661 \mu\text{g}$  ( $n = 12$ ) per pair of glands, respectively. Although slightly lower, these values are not significantly different from values for controls, the protein and RNA values of which were  $36.2 \pm 1.81 \mu\text{g}$  ( $n = 14$ ) and  $4.81 \pm 0.540 \mu\text{g}$  ( $n = 14$ ), respectively.

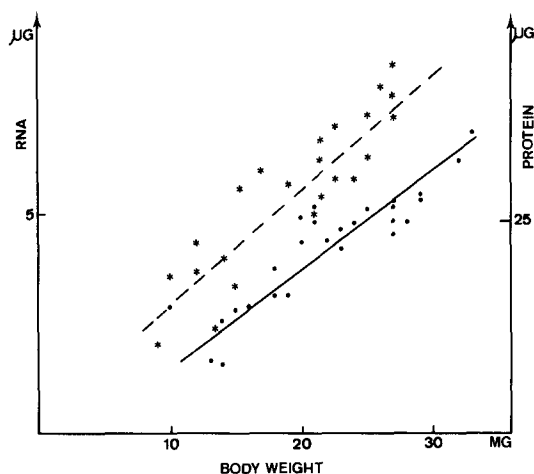


FIGURE 1 Relation between salivary gland protein, RNA content, and body weight in *Chironomus* larvae. Protein was determined after animals of different weights had been kept for 24 h in a solution of 0.1 mg/ml pilocarpine to eliminate secretion from the glands (1). Protein was determined according to Lowry et al. (12) after dissolution of one pair of glands in 1 N NaOH for 60 min at 45°C. Broken line and stars refer to protein measurements ( $r = 0.91$ ,  $n = 22$ ,  $P < 0.01$  according to statistical tables for Pearson's product-moment correlation). RNA was determined in feeding, untreated animals according to Fleck and Munro (9), but the procedure was slightly modified and scaled down 10 times. This procedure is particularly favorable since the DNA/RNA ratio is about 0.05 in salivary gland cells (6). Unbroken line and dots refer to RNA values per pair of glands ( $r = 0.92$ ,  $n = 26$ ,  $P < 0.01$ ). The data for protein and RNA are in good agreement with earlier published values (4, 17).

The animals in the two groups had the same weight when starvation was started and the controls were sacrificed.

**PROTEIN SYNTHESIS:** After 3 days of fasting, the glands of fasted animals incorporated 74% as much tritiated leucine into proteins as the glands of their fed littermates, and after 6 days, 36%. There was a rapid increase in leucine incorporation when animals were refed at day 6. After 150 min of feeding, the leucine incorporation was 78% of the value for fed animals, and after 5 h, 111%. The glands were incubated for 5 min in an *in vitro* medium (16) containing tritiated leucine, and the hot trichloroacetic acid (TCA)-precipitable activity was determined (mean of 10–12 determinations).

**HALF-LIFE OF "STABLE" RNA (RIBOSOMAL RNA AND 4S RNA):** Stable RNA decays with an

apparent half-life of 16 days in glands from growing animals. The effect of starvation was tested on the half-life of RNA labeled 6 days before the onset of starvation as described previously (8). In such animals starved for 6 days, the radioactivity in RNA, i.e., mainly ribosomal RNA (rRNA) and 4S RNA, was not measurably different from the values for feeding controls. The values per gland were (mean  $\pm$  SEM)  $7,170 \pm 403$  cpm ( $n = 24$ ) for controls as compared to  $6,825 \pm 499$  cpm ( $n = 22$ ) for starved animals. It cannot be excluded, however, that there is a difference for RNA formed during starvation.

**AVAILABILITY OF RNA PRECURSORS:** The radioactivities in hemolymph, cold TCA-soluble extract and -insoluble residue of glands were followed as a function of time after precursor injection in both feeding and starving animals (Fig. 2). Since the feeding animals were routinely given tritiated uridine only whereas it was necessary to inject both tritiated cytidine and uridine into the starving animals, the two sets of analyses are not strictly comparable. The purpose is rather to provide a basis for interpreting the experiments in which isotopes were given for zone analysis. The radioactivity in hemolymph decreases with rather similar kinetics in the two kinds of animals and in agreement with previous data for feeding animals (15). The curves for the TCA-soluble activity show, in both cases, a peak after about 6 h and are similar in their general course. The nonsoluble residue approaches a plateau after about 18 h, but the level of activity in relation to the TCA-soluble activity is considerably lower during starvation than during feeding.

It was found in electrophoretic analyses that the decreased RNA labeling during starvation is most pronounced for rRNA, with low rRNA/4S RNA ratios. There is also a tendency for increased 28S/18S RNA ratios during starvation.

The relative amount of labeled nuclear RNA is strikingly increased in starving as compared to feeding animals. This effect is most clearly seen after longer times. Thus, at 18 h after precursor injection, up to 25% of the total cellular label in RNA is in the nucleus as compared to 5% or less for feeding animals. After 2 days, the corresponding values are 6% and 2%, respectively.

#### *Distribution of Labeled RNA during Starvation*

Animals were fasted for 3 days before injection of tritiated nucleosides. They were then sacrificed

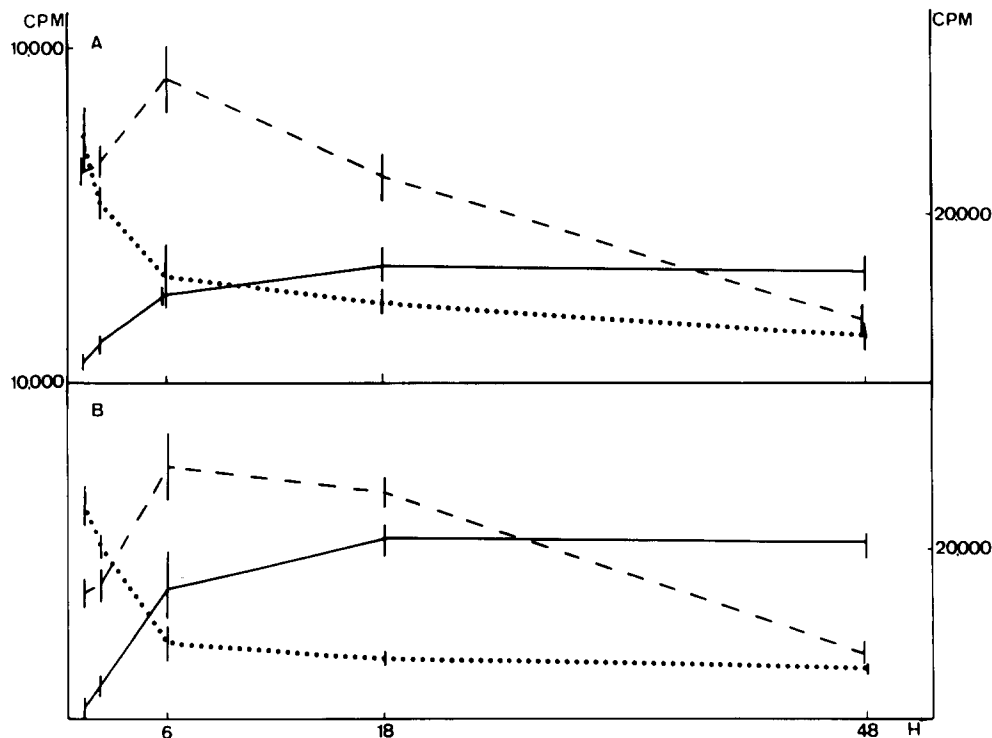


FIGURE 2 Time-course of radioactivity distribution between hemolymph, acid-soluble and -insoluble gland extracts after injection of tritiated nucleosides to starving (*A*) and feeding (*B*) animals. Feeding animals were given 1  $\mu$ Ci of tritiated uridine at 42 Ci/mmol supplemented with a 24-fold amount of unlabeled uridine, corresponding to the physical amounts of 25  $\mu$ Ci of undiluted tritiated uridine. Nongrowing animals were given 0.5  $\mu$ Ci of tritiated uridine (42 Ci/mmol) and 0.5  $\mu$ Ci of tritiated cytidine (26 Ci/mmol), and the solutions were supplemented with 19-fold amounts of unlabeled nucleosides so as to correspond to the physical amounts given in the zone analyses on starving animals. At each time-point, four or five animals were analyzed. Hemolymph in a quantity of 1  $\mu$ l was withdrawn and measured in standard toluene scintillator, supplemented with Soluene (Packard Instrument Co., Inc., Downers Grove, Ill.). Each pair of glands was dissolved, after a brief washing in physiological salt solution, in 0.5% sodium dodecyl sulfate, 0.02 M Tris buffer (pH 7.4). After precipitation with ice-cold 10% TCA for 30 min, the supernate (1 ml) was counted in 4 ml of Instagel (Packard) and the pellet measured in toluene scintillator after solubilization with Soluene. Bars give SEM. The solid line represents the acid-insoluble material, the broken line the supernate, and the dotted line the hemolymph activity. The scale to the right refers to the hemolymph, the scale to the left to the acid-insoluble and -soluble radioactivities.

after different times between 2 h and 2 days of further starvation. 12 cells were isolated from each pair of glands and dissected into nuclei and three concentric cytoplasmic zones and analyzed electrophoretically. The radioactivities under the 28S and 18S RNA peaks were divided by the radioactivity under the 4S RNA peak. The 4S RNA serves as a volume marker and has been found to distribute evenly in the cytoplasm of fixed cells (7). Since the inner cytoplasmic zone might have been contaminated with some nuclear sap, values for this zone have not been used for any conclusions for labeling times of 18 h or less when there

are still appreciable amounts of labeled RNA in the nuclei. Since gradients in the amount of labeled RSU are best expressed as values for one zone with respect to another, the middle zone has been given an arbitrary value. In this way, the variability among animals with regard to the time it takes the RSU release from the nucleus is not demonstrated. This variability, however, has been shown for feeding animals in earlier papers (7, 11), and is of the same order in the present material.

Both rRNA components distribute to a large extent in the cytoplasm surrounding the nucleus,

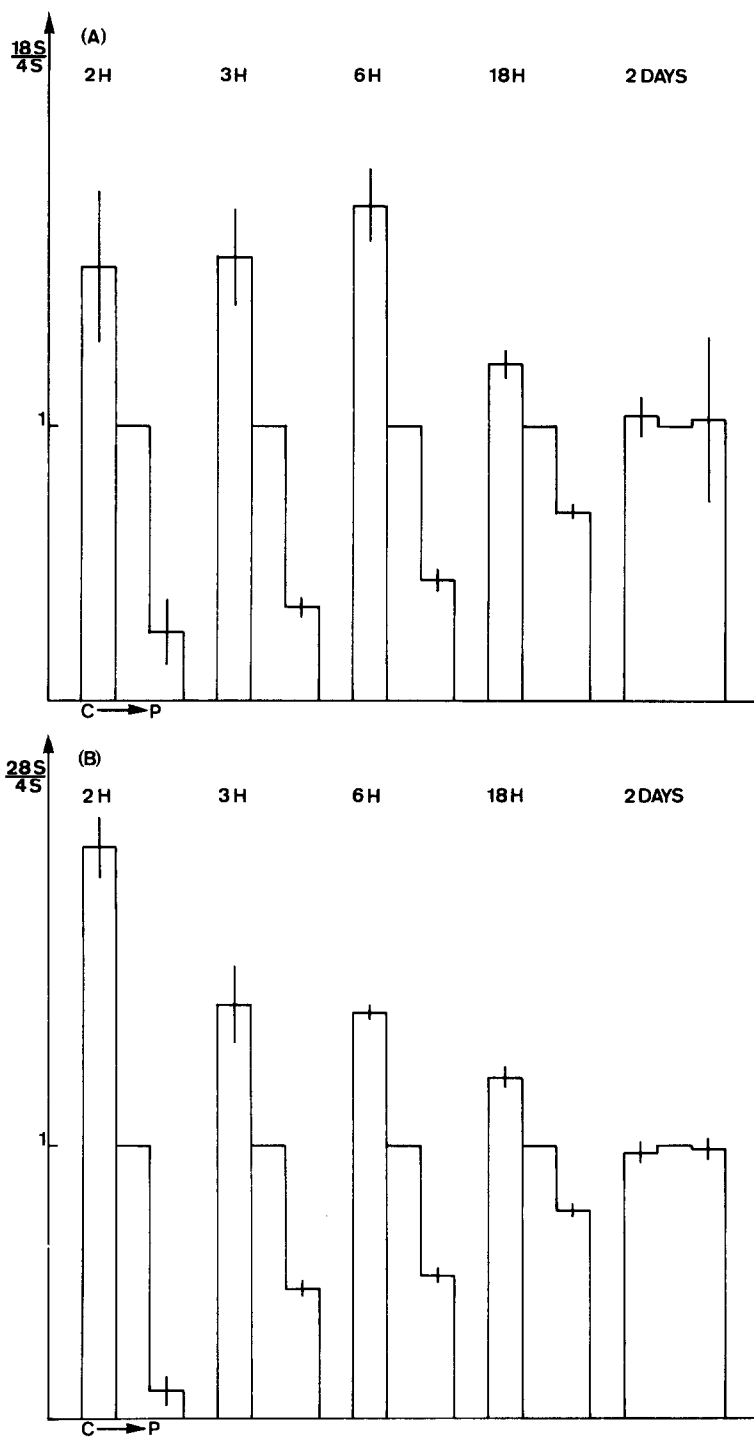


FIGURE 3 Summary diagrams of the distribution of labeled 18S RNA (A) and 28S RNA (B) in cytoplasmic zones in animals starved for 3 days before injection of tritiated nucleosides. The times give the interval between precursor injection and sacrifice. The middle zone has, in all cases, been given the arbitrary value of 1. The inner and outer zones have been normalized accordingly. The letters *C* and *P* stand for the center and periphery of the cytoplasm. Each column represents the mean of values for four animals sacrificed at the different times. Bars give the SEM in the inner and outer zones. The separation of columns for the inner zone in animals sacrificed after 18 h or earlier indicates that this fraction may be contaminated with labeled nuclear RNA.

with a gradient of decreasing abundance towards the peripheral cytoplasm. The gradients are steep after short times but are no longer measurable after 2 days (Fig. 3). Furthermore, they are leveled out by the addition of puromycin (Table I). The gradients last longer than in feeding animals, possibly because the relations between precursor pools and labeled rRNA are different in the two types of animals. It was previously found that the 28S RNA forms a puromycin-resistant gradient lasting for at least 2 days. Starvation thus prevents the formation of this type of gradient.

#### Preformed Gradients

RNA precursors were injected into growing animals which were then placed in culture medium without food. Under these conditions, the 18S RNA gradient has all the characteristics previously recorded for feeding animals. On the other hand, one can now record a 28S RNA gradient after 6 days, which thus lasts even longer than under continuous feeding (Fig. 4). It is largely or entirely sensitive to puromycin immediately after it has become established in the cytoplasm (Table II). After 6 days it is, however, refractory to the drug whether administered *in vitro* or *in vivo*. At this time it is also resistant to actinomycin D, showing that it cannot have been formed as a result of

persisting export of RNA from the nucleus (Table II). It could, however, be eliminated by refeeding the animals for 2 days (Fig. 4).

#### DISCUSSION

In earlier work on growing cells we found that both the light and the heavy RSU appear, during the first few hours after precursor injection, mainly in the cytoplasm adjoining the nucleus, with a gradient of decreasing quantity towards the periphery of the cell (7). After the addition of puromycin, the gradient for the light RSU is entirely eliminated, and the one for the heavy RSU is eliminated to a large extent. The effect of the drug in animals labeled for 3 h (7) is to increase within 45 min the number of labeled RSU in the peripheral part of the cytoplasm as compared to parallel controls. This effect was confirmed in the present work in animals labeled for 3 h or more. This means that the RSU are made more mobile by the drug, suggesting in turn that gradients form because RSU become attached to polysomes close to the nuclear membrane when leaving the nucleus. The sensitive gradients disappear after 6–18 h, probably because all light RSU and heavy RSU which are not directly bound to the ER are able to diffuse between rounds of translation and distribute evenly in the cytoplasm. For the heavy RSU,

TABLE I  
Effect of Puromycin on 18S and 28S RNA Gradients in Starved Animals

Zone	Puromycin 45 min <i>in vitro</i> (control/treated)				Puromycin 45 min <i>in vivo</i> , 18 h
	3 h		18 h		
	1	2	1	2	
18S/4S RNA label ratio $\times 10^{-2}$					
Inner*	29/21	32/24	44/32	54/32	94 $\pm$ 3.7
Middle	19/18	20/19	29/28	35/28	100
Outer	09/21	08/18	19/28	19/26	107 $\pm$ 5.1
28S/4S RNA label ratio $\times 10^{-2}$					
Inner*	64/37	79/56	68/54	61/42	99 $\pm$ 3.3
Middle	36/34	51/48	49/48	46/39	100
Outer	17/33	25/53	30/53	29/41	104 $\pm$ 3.3

Distribution of labeled 18S RNA and 28S RNA in cytoplasmic zones after puromycin treatment in starved animals. Each numbered vertical column refers to cells from one animal. Asterisk (\*) for the inner zone values indicates that they may be affected by contaminating nuclear RNA. "Control" and "treated" refer to sister glands from one and the same animal. "Puromycin, 45 min *in vitro*": animals were sacrificed 3 or 18 h after precursor injection. One gland was placed in culture medium (16) (control) and the sister gland in medium supplemented with puromycin (100  $\mu\text{g/ml}$ ). After 45 min the glands were fixed and prepared for analysis. "Puromycin, 45 min *in vivo*": animals received 2.5  $\mu\text{g}$  of puromycin (over-all body concentration about 100  $\mu\text{g/ml}$ ) 18 h after precursor injection and were sacrificed after a further 45 min. Four animals were analyzed. The values given are mean  $\pm$  SEM after values for the middle zones have been given the arbitrary value of 1 and the inner and outer zones normalized accordingly.

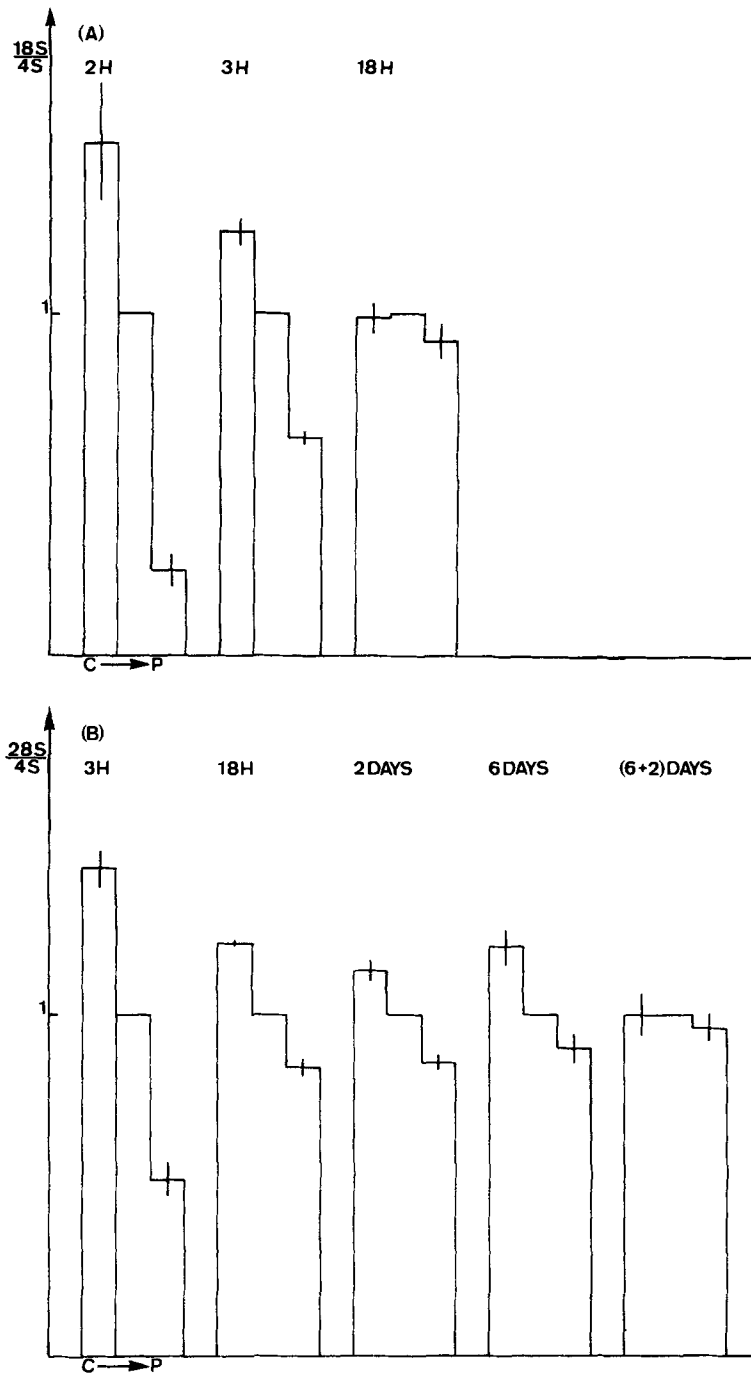


FIGURE 4 Summary diagram of the distribution of labeled 18S RNA (A) and 28S RNA (B) in cytoplasmic zones at different times after precursor injection in growing animals injected with tritiated RNA precursors and subsequently placed in culture medium without food. (6 + 2) Days refer to reinstated feeding for 2 days after 6 days of starvation. For symbols, see legend to Fig. 3.

TABLE II  
Effect on Preformed Gradients of RNA and Protein Synthesis Inhibitors

Zone	Actinomycin D	Puromycin 45 min in vitro (control/treated)				Puromycin 3 h in vivo, 6 days
		3 h		6 days		
		1	2	1	2	
28S/4S RNA label ratio $\times 10^{-2}$						
Inner	116 $\pm$ 2.2	184*/103*	199*/088*	196/194	221/212	110 $\pm$ 2.2
Middle	100	098/099	097/080	174/168	180/178	100
Outer	93 $\pm$ 1.2	030/110	044/086	142/145	157/152	86 $\pm$ 2.3
18S/4S RNA label ratio $\times 10^{-2}$						
Inner		139*/117*	130*/081*			
Middle		090/098	067/088			
Outer		048/110	036/087			

Distribution of labeled 18S and 28S RNA in different cytoplasmic zones during actinomycin D and puromycin treatments in animals with preformed gradients. Each numbered vertical column refers to cells from one animal. Asterisk (\*) for the inner zone values indicates that they may be affected by contaminating nuclear RNA. "Control" and "treated" refer to sister glands from one and the same animal. "Actinomycin D": animals were placed in drug-containing medium (100  $\mu\text{g/ml}$ ) 5 days after precursor injection and sacrificed after 24 h. Doses of actinomycin D higher than those required for feeding animals have to be used for RNA synthesis inhibition. RNA labeling is 16% of that of controls after 2 h and 8% after 24 h as measured by the incorporation of tritiated uridine in vivo during 15 min. Protein synthesis was not measurably affected. Incorporation of tritiated leucine into hot TCA-precipitable material was measured in vitro in explanted glands and was on the average 91% of the controls after 24 h in actinomycin D. Four animals were analyzed. The values given are mean  $\pm$  SEM after the middle zones have been given the arbitrary value of 1 and the inner and outer zones normalized accordingly. "Puromycin, 45 min in vitro": animals were sacrificed 3 h and 6 days after precursor injection and treated as described in the text to Table I. "Puromycin, 3 h in vivo": animals received 2.5  $\mu\text{g}$  of puromycin by injection (overall body concentration about 100  $\mu\text{g/ml}$ ) 6 days after precursor injection and were sacrificed after 3 h. Four animals were analyzed, and the values were treated as described for the animals given actinomycin D.

however, a component of the gradient which is resistant to the addition of puromycin remains for at least 2 days, representing, in all probability, subunits anchored to the ER (11).

Starvation and cessation of growth do not prevent the formation of puromycin-sensitive gradients typical also of growing cells. (The gradients last somewhat longer during starvation, but this could be an effect of the different relations between precursor pools and net rRNA labeling in feeding and starving animals, although other causes are not excluded such as the effects of starvation on a pool of free RSU). There is, on the other hand, no formation of puromycin-resistant gradients for the heavy RSU. If, however, such a resistant gradient is first allowed to form, it will become stabilized during subsequent starvation. Results paralleling those of the 28S RNA marker were obtained with another heavy RSU marker, labeled 5S RNA (our unpublished results), in

agreement with the findings in growing animals (7, 11).

It seems likely that radioactivity gradients which are stable for days and are resistant to puromycin and restricted to the heavy RSU are due to an association between this RSU and the ER. Although formation of free polysomes may occur during starvation much as in normal cells, there is no measurable evidence for formation of puromycin-resistant associations for the heavy RSU, i.e. in all probability involving the ER. This probably means that heavy RSU which are already ER-bound are not exchanged for or diluted by newly synthesized units, to any measurable extent. This is in good agreement with the findings that preformed gradients for the heavy RSU are stabilized during starvation.

Starvation brings cells from a growing to a non-growing state, and the normal increases in cellular protein and RNA content cease. Starvation proba-



bly also results in cessation of the normal expansion of the ER. It is consequently possible that new associations of heavy RSU are dependent on net ER synthesis which would, in turn, indicate that heavy RSU attach irreversibly to the ER. The suggestion advanced for *Drosophila* cells that the RSU flow from free to bound polysomes (3) would be in good agreement with this view and with the previous results that most of the initial heavy RSU association is in puromycin-sensitive associations, although the dominating part of the protein synthesis consists of secretory proteins (5, 14).

A prerequisite for the detection of long-lasting gradients is that there are no gross structural relocations of the ER within time periods of days. The stability of puromycin-resistant gradients, particularly pronounced for preformed gradients during starvation, suggests a considerable structural stability of the cytoplasm in these salivary gland cells.

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