# MOBILITY RESTRICTION IN VIVO OF THE HEAVY

# RIBOSOMAL SUBUNIT IN A SECRETORY CELL

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ABSTRACT

Analysis in insect (*Chironomus tentans*) salivary gland cells of labeled RNA as a function of time after precursor injection and its distance to the nuclear membrane, cytoplasmic zone analysis, could previously be used to demonstrate the presence of short-lasting gradients in newly labeled ribosomal RNA. Since the gradients were sensitive to puromycin, they are likely to be a result of diffusion restriction due to an engagement of the subunits into polysomes. In the present paper the possibility was explored of recording gradients that were caused by labeled subunits in puromycin-resistant associations, which, in all probability, involve the endoplasmic reticulum. It was found that labeled 28 S and 5 S RNA but not 18 S RNA were present in radioactivity gradients lasting for at least 2 days but less than 6 days. The gradients also remained during inhibition of RNA synthesis by actinomycin, and they were completely resistant to puromycin whether given in vivo or in vitro. The semipermanent gradients formed here offer a unique parameter for the in vivo study of conditions for formation and maintenance of heavy subunits in puromycin-resistant bonds.

An explanation for these and previous results is that the light subunit, although restricted in movement by engagement to polysomes, is nevertheless free to exchange and spread between rounds of translation, whereas at least part of the heavy subunit population is bound to the endoplasmic reticulum and less free to spread. These results offer a good in vivo correlate to previous results on in vitro exchangeability of subunits.

Polysomes have mainly been studied with homogenization and subfractionation techniques. For several reasons, it would be desirable to supplement such techniques with procedures whereby the results can be more directly related to the in vivo situation. One such possibility is afforded by cytoplasmic zone analysis, a technique described in a preceding paper (7). Here, the distribution of RNA components is recorded as a function of time after RNA precursor administration and the distance of the labeled RNA to the nuclear membrane. The ribosomal RNA components, 18 S, 28 S and 5 S RNA, appear in steep specific activity gradients for a few hours after precursor administration (exit gradients). The fact that these gradients are leveled out after administration of puromycin, completely for 18 S RNA and largely for 28 S RNA, suggests that they are caused by ribosomal subunits (RSU) located in polysomes. Thus, these gradients may constitute a parameter for polysomes in the living cell, permitting the study of polysome formation and maintenance without homogenization and subfractionation. The RSU can, however, be present either in free or in bound

polysomes. In the case of the heavy RSU, this could imply that they might exist either attached to endoplasmic reticulum (ER) membranes or without such attachment, and it has been shown that this subunit either can have puromycin-resistant attachments or be dissociated from polysomes with puromycin (13), a difference which is likely to be related to the mode of attachment of the heavy RSU to ER membranes.

We feel that the usefulness of cytoplasmic zone analysis would be greatly increased if it could distinguish between heavy RSU present in puromycin-sensitive and -insensitive associations as a parameter probably related to their mode of association to the ER membranes. If newly synthesized heavy RSU become attached to ER membranes, this might result in puromycin-resistant gradients which last considerably longer than gradients for puromycin-sensitive components. Such gradients should exist for 28 S and 5 S RNA but not for 18 S RNA which, as a component of the light RSU, is entirely sensitive to puromycin (3, 13). The object of the present work was to test for the presence and duration of such gradients as a possible parameter for ER-bound polysomes.

## MATERIALS AND METHODS

Late fourth instar larvae of the dipteran *Chironomus tentans* were used. Animals were given tritiated precursors, and salivary glands were prepared and dissected as described in the previous paper (7) which also gives the analytical procedures for RNA. The cytoplasm of the cells was divided into three zones, an inner, middle, and outer zone. The zones are defined by their distance from the nuclear membrane.

## RESULTS

The newly labeled light RSU, measured as 18 S RNA, is at distributional equilibrium 6 h after precursor administration as measured by the 18 S/ 4 S RNA label ratio. The 4 S RNA label is likely to reflect the volume distribution since it is distributed like the glycerol in the dissection medium in which the cells are soaked during the dissection (7). The 18 S/4 S RNA label ratios which remain similar in the three zones after this time are not given here but only data for 28 S and 5 S RNA.

### Gradients for 28 S RNA

Cytoplasm analyzed 18 h after precursor injection shows a relatively even distribution of labeled ribosomal RNA between the inner, middle, and outer cytoplasmic zones (Fig. 1). This contrasts
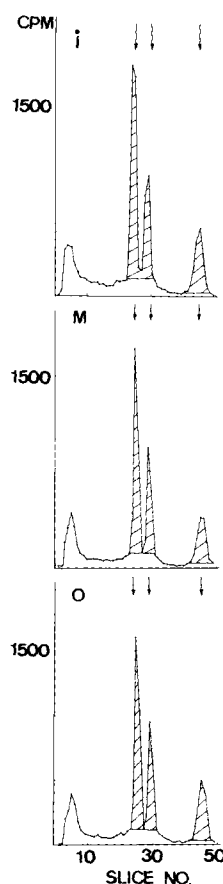


FIGURE 1 Electrophoretic separations in 1.5% agarose of the RNA from cytoplasmic zones of 12 cells isolated from the salivary gland of an animal that was injected with 25 μCi of tritiated uridine 18 h before sacrifice. Arrows denote, from left to right, the positions of 23 S, 16 S, and 4 S RNA, added markers of RNA from *E. coli*. The letters *i*, *m*, *o* stand for inner, middle, and outer cytoplasmic zones.

with the steep gradients which can be recorded shortly after the entrance of ribosomal RNA into the cytoplasm (7). Close inspections or measurements show, however, that 28 S RNA is still in a central excess relative to 4 S RNA after 18 h (Fig. 2 A). This difference applies to the inner zone compared to the middle zone as well as to the middle zone compared to the outer zone. The nucleus contains only 2-4% of the total labeled RNA after these times and even less at later times (reference 8 and unpublished observations), and an admixture of a minor part of the nuclear sap can therefore not contaminate the inner zone to any measurable extent.
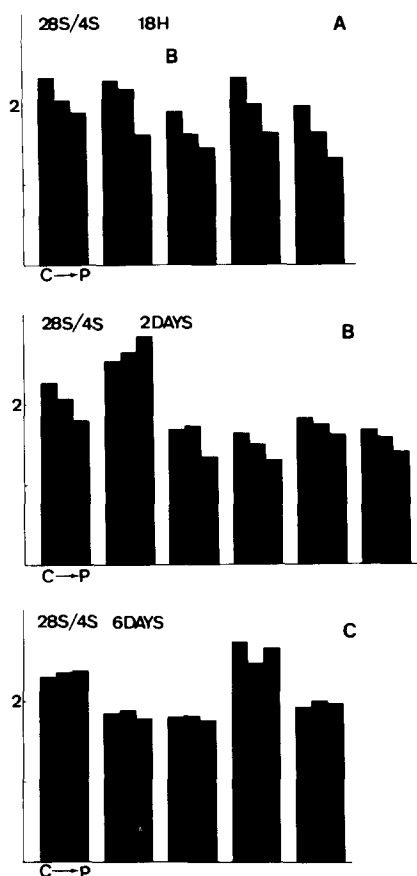
FIGURE 2 Staple diagrams from five different animals of the 28 S/4 S RNA label ratio 18 h (A), 2 days (B) and 6 days (C) after injections of 25 μCi of tritiated uridine. The letters c and p stand for central and peripheral parts of the cytoplasm, respectively.

2 days after precursor injection the activity gradient for 28 S RNA, expressed relative to 4 S RNA is, with one exception, still present but less pronounced (Fig. 2 B). Gradients were not detectable 6 days after precursor injection (Fig. 2 C). In Fig. 3, the 28 S/4 S labeled RNA ratios have been summarized as a function of time after precursor injection.

Since 4 S RNA is likely to be a suitable volume marker as has previously been demonstrated, the data in Figs. 2-3 show the presence of a gradient in labeled 28 S RNA lasting for at least 2 days. We are not able, due to the small amounts of material available, to determine the optical density amounts of 28 S RNA in the different zones. The fact that after longer times, 6 days (present paper) and 5 wk (7), there are no indications of a gradient

in radioactivity suggests that the data reflect the presence of a specific radioactivity gradient, i.e. the 28 S RNA synthesized during the last 2 days is located relatively closer to the nuclear envelope than older RNA, suggesting a delay in its peripheral spread.

Since the light RSU equilibrates relatively rapidly in the cytoplasm, within about 6 h, the gradient in 28 S/4 S RNA label should also be displayed in the 28 S/18 S RNA ratio. Fig. 4 A shows the gradient to be consistently present after 18 h, and Fig. 4 B with one exception also in all the animals investigated after 2 days. Fig. 4 C shows that after 6 days there is no longer any evidence of a gradient in labeled 28 S/18 S RNA ratios. Consequently, the 28 S/18 S RNA label gradients mirror 28 S/4 S RNA label gradients.

## Gradients for 5 S RNA

If the semipermanent gradients which we have recorded for 28 S RNA are due to a specific activity gradient for the heavy RSU, it should be possible to record a similar gradient for 5 S RNA provided 5 S RNA is irreversibly bound to the heavy RSU. From an analytical point of view, 5 S RNA has the advantage that it is not only a different parameter than 28 S RNA but it is measured in an area of the electrophoretic separation essentially free from other RNA and the base line is close to the background levels. Because the radio-
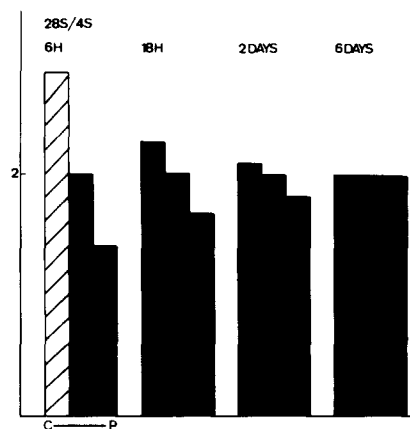


FIGURE 3 Summary diagram of the 28 S/4 S label ratio in the cytoplasmic zones at different times after precursor injection. The middle zone has been given the arbitrary value of 2.0; the inner and outer zones have been normalized accordingly. The shaded staple for the inner zone at 6 h indicates that this fraction may be contaminated by nuclear RNA. For symbols, see Fig. 2.
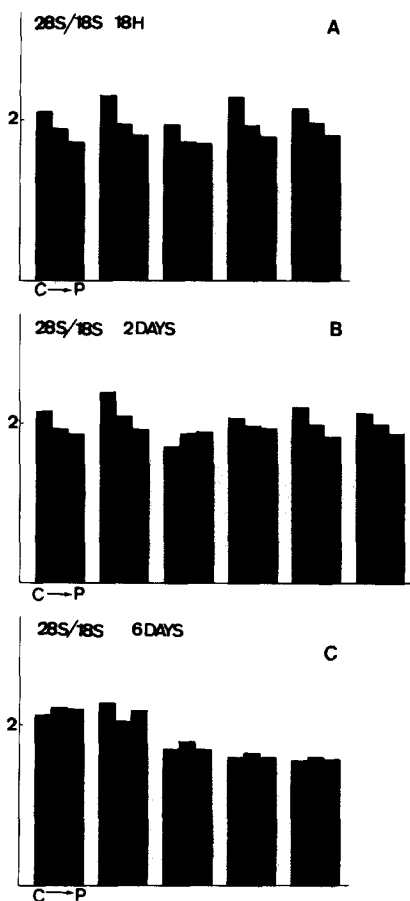
FIGURE 4 Staple diagrams of the 28 S/18 S RNA label ratio 18 h (A), 2 days (B) and 6 days (C) after precursor injection, for five different animals. For symbols, see Fig. 2.

pronounced (Fig. 6 B), again a parallel to the 28 S RNA results. The gradient has leveled out after 6 days as expected from previous zone analyses of 28 S RNA (Fig. 6 C). In every respect the 5 S RNA analyses thus confirm the results obtained for 28 S RNA, suggesting strongly that they reflect the behavior of the heavy RSU.

## Gradients in Actinomycin-Treated Animals

The circumstance that the injection procedure has much the character of a pulse label (10) and that, after 18 h, only 2-4% of the radioactivity is localized in the nucleus speaks against any interpretation that the semipermanent gradient is simply an exit gradient as previously observed after short labeling times, superimposed upon evenly distributed RNA. This would occur if there were still a considerable export of RNA 1-2 days after precursor injection. This alternative is unlikely not only because of the low nuclear labeling and the
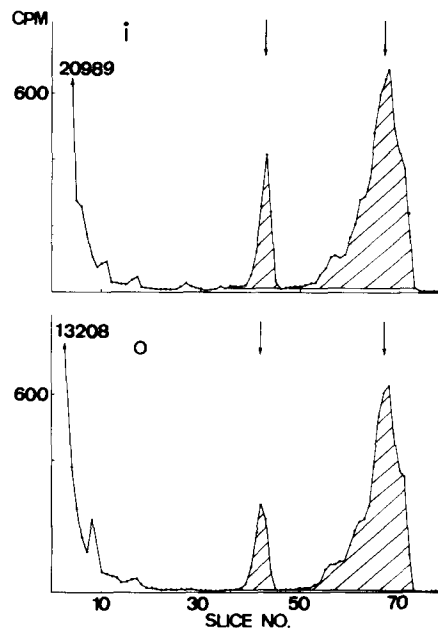


FIGURE 5 Electrophoretic separations of low molecular weight RNA in 7.5% polyacrylamide gels containing 8 M urea. The RNA was isolated from microdissected cytoplasmic zones of fixed salivary gland cells injected with 25 $\mu$Ci of tritiated uridine 18 h before sacrifice. The arrows indicate, from left to right, 5 S and 4 S RNA positions of added E. coli RNA markers. The letters i and o denote the inner half and the outer half of the cytoplasm, respectively.

activity amounts are lower in 5 S than in other studied components, it was advantageous to split the cytoplasmic material into only two parts, an inner and an outer zone. This can be done since the nuclear contribution is small (18 h or more after precursor injection) and the possible contamination in the inner zone is therefore minimal. Steep 5 S gradients mimicking those of 28 S RNA have previously been recorded 3 h and 6 h after precursor injection (7).

18 h after precursor injection, there is a gradient in the 5 S/4 S RNA label ratio with the slope as usual in a peripheral direction (Figs. 5 and 6 A). It is considerably less steep than previously found for shorter times but similar to the 28 S gradient after the same time. The gradient in labeled 5 S/4 S RNA still remains after 2 days although less
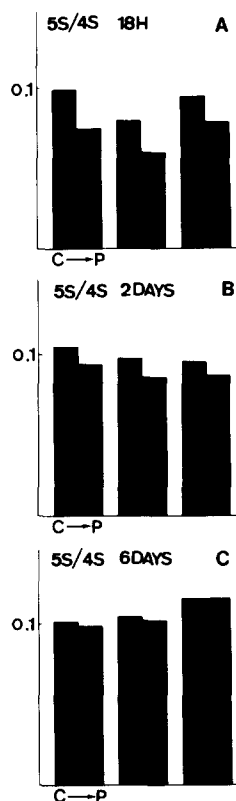
FIGURE 6  Staple diagrams of the 5 S/4 S RNA label ratio 18 h (A), 2 days (B) and 6 days (C) after precursor injection (25 μCi of tritiated uridine) for three different animals. For symbols, see Fig. 2.

the nucleus over a period of more than 20 h. Protein synthesis was not measurably affected by the actinomycin treatment. Incorporation of tritiated leucine into hot trichloroacetic acid-precipitable material was measured in vivo over a period of 3 h in glands from animals kept for 24 h in actinomycin D medium and was, on the average, 108% of the controls. The results of the action of the drug are in agreement with previous data for *C. tentans* (4).

## Gradients in Puromycin-Treated Animals

The exit gradients recorded in our previous work 2-3 h after precursor injection (7) were entirely sensitive to puromycin given in vitro within 45 min in the case of the light RSU and to about two-thirds in the case of the heavy RSU. Puromycin sensitivity is indicative of polysome binding, but heavy RSU attached directly to ER membranes in bound polysomes may not show such a sensitivity.

The circumstance that gradients for the light RSU level out within 6 h whereas a gradient remains for the heavy RSU for at least 2 days suggests that at least part of this population is bound to a structure, like the ER, that would retard its mobility. In view of the properties of puromycin referred to above, the drug was used to test whether the semipermanent gradient had the properties expected if due to heavy RSU bound to ER membranes, i.e. was resistant to puromycin.

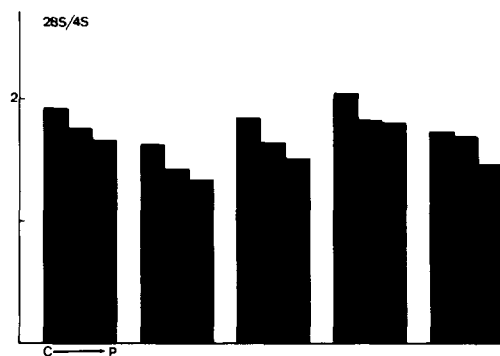In one set of experiments, animals were sacri-

fact that only 28 S RNA label appears in a gradient after 1-2 days whereas both 28 S and 18 S RNA appear in exit gradients, but it could also be more directly excluded with the aid of actinomycin experiments.

Animals were given tritiated uridine in injections and were then kept for 24 h in normal medium, after which they were transferred to the same medium containing 10 μg of actinomycin D/ ml and kept for another 24 h before sacrifice. The results showed that gradients that exist after 1 day are maintained for at least another day although the RNA synthesis was blocked (Fig. 7). Control experiments showed that the synthesis of RNA as measured by the incorporation of tritiated uridine in vivo during 15 min was low after 3 h in actinomycin D medium (22% of the controls) and negligible after 24 h in the same solution (5% of the controls). Thus, the semipermanent gradients are not dependent on the supply of new RNA from



FIGURE 7  Staple diagram of the 28 S/4 S RNA label ratio during actinomycin D treatment. Five different animals were kept for 24 h in normal culture medium after injection of 25 μCi of tritiated uridine and were sacrificed after another 24 h in medium supplemented with actinomycin D (10 μg/ml). For symbols, see Fig. 2.

ficed 18 h after injection of tritiated uridine. One gland from each animal was placed in Cannon's modified medium (12) with puromycin (100 $\mu$g/ml), the control sister gland in medium without the drug. This treatment has previously been shown to inhibit protein synthesis by 89% (7). The glands were fixed after incubation for 45 min. Fig. 8 shows that the heavy RSU gradient is similar in both drug-treated and control sister glands. It does, consequently, not have the character expected for exit gradients, most of which show sensitivity to puromycin.

Puromycin treatments were also carried out in vivo, in which case sister gland controls are not available. Animals weighing 25 mg were given 2.5 $\mu$g of puromycin (overall body concentration about 100 $\mu$g/ml) 18 h after isotope injection and were sacrificed 3 h later. The drug treatment was prolonged to 3 h to exclude any objection that the heavy RSU may not have time to equilibrate within 45 min. Although matched controls in the form of untreated sister glands are not available, one can see (Fig. 9) that the gradients do not differ significantly in size from those of animals not exposed to the drug. Injection of puromycin inhibits protein synthesis almost completely (97% inhibition) as measured by tritiated leucine incorporation into hot trichloroacetic acid-precipitable material over a period of 3 h.
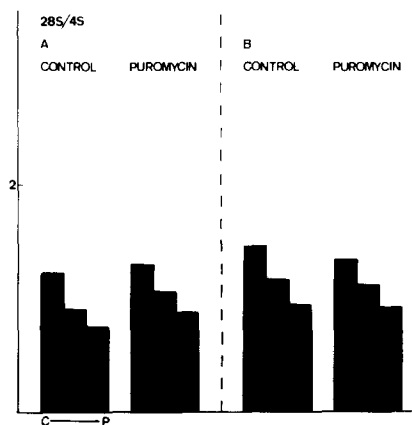


FIGURE 8 Staple diagram of the 28 S/4 S RNA label ratio during puromycin treatment. Animals were given 25 $\mu$Ci of tritiated uridine 18 h before sacrifice. One gland was taken to Cannon's modified culture medium (control) and the sister gland to the same medium supplemented with puromycin (100 $\mu$g/ml). After 45 min, both glands were prepared for analysis. (A) and (B) denote two different animals.
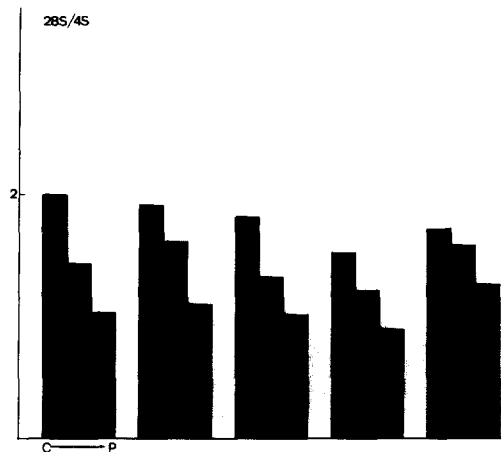


FIGURE 9 Staple diagram of the 28 S/4 S RNA label ratio during puromycin treatment in vivo. Five different animals were given 2.5 $\mu$g of puromycin 18 h after injection of 25 $\mu$Ci of tritiated uridine and were sacrificed 3 h later. For symbols, see Fig. 2.

The alternative was also made unlikely that the puromycin-resistant 28 S RNA gradients are caused by a nonribosomal component or contaminant. Cytoplasmic 28 S RNA in Diptera has a characteristic lability towards hydrogen-breaking agents and converts under such conditions to material comigrating with 18 S RNA (14). This property was used to eliminate such a possibility.

Animals were given puromycin for 3 h as described above after tritiated uridine had been given 18 h earlier. The extracts were treated with 8 M urea for 1 min at 50°C in 0.02 M Tris buffer, pH 7.4 with 0.5% sodium dodecyl sulfate. This urea treatment should more or less completely eliminate hydrogen bonds in adenine-uracil-rich RNA like *Chironomus* ribosomal RNA (6, 11). Zone analyses of the extracts showed that the gradient now lies in the dominating "18 S" ribosomal RNA peak (Fig. 10).

DISCUSSION

It was previously shown that the two RSU measured as newly synthesized 28 S RNA, 5 S RNA and 18 S RNA are present in steep specific activity gradients with a slope towards the periphery of the cell. Puromycin experiments indicated that the gradients are largely or entirely the result of an engagement of the RSU into polysomes immediately or shortly after their release from the nucleus (7).
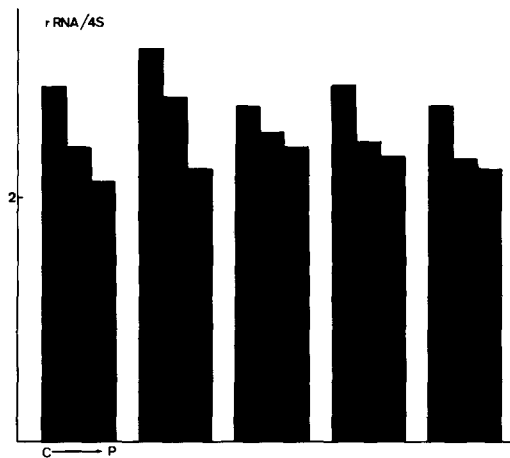
The slopes of the gradients for the two RSU are

FIGURE 10 Staple diagram of the rRNA/4 S RNA label ratio after puromycin treatment. Animals were given 25 µCi of tritiated uridine by injection and 18 h later 2.5 µg of puromycin and sacrificed 3 h later. The RNA extracts were heated for 1 min at 50°C in 8 M urea in 0.02 M Tris buffer with 0.5% sodium dodecyl sulfate. For symbols, see Fig. 2.

similar during their early presence in the cytoplasm. Later on, 6 h after precursor injection, they differed, however, markedly. The light RSU gradient was practically leveled out whereas the heavy RSU gradient was still prominent. From these data alone, the difference could have had trivial causes and be related to diffusion rates of particles of different size and/or nuclear processing times. Another possibility was, however, that the difference reflects the types of engagements in cytoplasmic structures in the cell.

Light polysome-bound RSU exchange with free subunits whether present in ER-bound or unbound polysomes. Heavy RSU present in unbound polysomes also show a similar exchange, whereas it is a disputed question how firmly heavy RSU are bound to the ER in bound polysomes. If such RSU are not free to exchange, one would expect their gradients to be more stable than if the RSU were exchangeable. Furthermore, such gradients should not be affected by puromycin.

In the present work, clear differences in spreading behavior were found between the two labeled RSU. At 18 h and 2 days after injection of tritiated uridine, there was an excess of labeled heavy RSU in the vicinity of the nucleus as measured by two independent markers, labeled 28 S RNA and 5 S RNA. The light RSU, measured as labeled 18 S RNA, was shown earlier to level out

already at 6 h. The 28 S/18 S RNA label ratios give a direct demonstration of the difference in distribution of the two RSU. The semipermanent gradient for the heavy RSU is not a small exit gradient due to persisting export of labeled RNA from the nucleus, shown most directly by the actinomycin experiments.

The heavy RSU gradient observed here should correspond to ribosomes in liver cells and HeLa cells that do not detach from membranes during puromycin treatment (1, 13). The 28 S RNA label gradient showed drug resistance whether treatment was performed for 45 min or 3 h, in vitro or in vivo. As shown earlier, a 45-min treatment is enough to annihilate the light RSU gradient.

The difference in spreading behavior between the two RSU is likely to be due to their different engagement to the ER. The salivary gland cells are studded with rough ER (9), and the production of secretory proteins may correspond to at least 80% of the total protein synthesis (5). It is known that the heavy but not the light RSU may be anchored to the ER (15). Furthermore, work in vitro (3) has shown that the light but not the heavy RSU when attached to membranes will exchange with added free RSU of the corresponding kind. The cytoplasmic zone analyses therefore provide a good in vivo correlate to these findings.

Labeled 28 S RNA when initially entering the cytoplasm (3 h after injection of uridine) was, to a high degree, sensitive to puromycin (7). This indicates that most of the newly synthesized heavy RSU first enter free polysomes. In view of the extensive secretory function of these cells, it seems unlikely that the majority of the polysomes are free. Heavy subunits may, therefore, as suggested for another Dipteran (2), go from free to bound polysomes unless they can exist in loosely membrane-bound polysomes, the heavy RSU of which would be sensitive to puromycin (13).

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