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Negatively-stained polysomes on rough microsome vesicles viewed by electron microscopy: further evidence regarding the orientation of attached ribosomes

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Abstract. Rough microsomes, derived from rough endoplasmic reticulum of rat liver, were studied by electron microscopy after negative staining, to seek further information about the orientation of ribosomal small and large subunits in bound polysomes. Rough microsomal vesicles were fixed with 2% formaldehyde, centrifuged onto electron-microscopic grid membranes, and were then negatively-stained with 2% phosphotungstic acid. In these preparations, viewed with the electron microscope, flattened rough microsomal vesicles with bound polysomes were sometimes discernible, and the individual ribosomes in the polysomes occasionally showed small and large subunits. The small subunits were uniformly oriented toward the inside of the polysomal curve. The large and small subunits appeared to be alongside one another on the membrane, consistent with the orientation that has been described by Unwin and his co-workers. The boundary between the small and large subunits occurred at approximately the same level in the ribosome where inter-ribosomal strands have been described previously in surface views of bound polysomes in positively-stained electron-microscopic tissue sections. This further confirms the identity of the strands as messenger RNA.

Key words: Polysomes – Ribosomes – Subunits – Liver – Electron microscopy – Negative stain – Rat (Sprague-Dawley)

Introduction

Polysomes that are bound to membranes of the rough endoplasmic reticulum (RER) produce most secretory, membrane, and lysosomal proteins. The detailed orientation of the individual ribosomes in bound polysomes has been of interest over the years. More recent findings are those of Unwin and his coworkers (Unwin and Taddei 1977; Unwin 1977, 1979; Kühlbrandt and Unwin 1982; Milligan and Unwin 1986), who studied 2-dimensional crystals of ribosomes bound to RER membranes in oocytes of hibernating lizards. Electron micrographs of the crystals were analyzed by optical diffraction and Fourier synthesis to yield 3-dimensional reconstructions of the ribosomes. These showed that the large and small ribosomal subunits were arranged alongside one another on the RER membrane. This model differed markedly from the traditional view that the small subunit was situated on top of the large subunit, away from the membrane (see Results and Discussion). These studies also showed that the long axis of the small subunit was approximately parallel to the RER membrane. Tests on the binding characteristics of the crystalline ribosomes gave essentially the same results as had been obtained for normal bound ribosomes from secretory cells, suggesting that the binding of the crystalline ribosomes to the RER membrane, and of the ribosomal subunits to each other, was essentially normal.

Since the crystalline ribosomes of the Unwin studies were not involved in translation, they were not organized into polysomes. It was not possible, therefore, to relate the orientation of the ribosomes to the overall organization of bound polysomes. In the present study, an in vitro approach has been used to examine surface views of polysomes on flattened, negatively-stained rough microsome vesicles viewed by electron microscopy (EM). The findings indicate that the ribosomal subunits are arranged side-by-side on the RER membrane, consistent with the results of Unwin's group (described above), and that the small ribosomal subunits are oriented toward the inside of the polysomal curve.

Materials and methods

Animals

Mature Sprague-Dawley rats (Charles River Breeding Laboratories, branch office in West Portage, Mich.) were maintained on a

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13 h light, 11 h dark regimen, and fed water and rat chow ad libitum.

Isolation of rough microsomes

Rough microsomes (RM) were prepared from rat liver as described by Gaetani et al. (1983), using a triethanolamine (TEA) homogenization buffer (Walter and Blobel 1983), consisting of 250 mM sucrose, 50 mM TEA, 50 mM potassium acetate, 6 mM magnesium acetate, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.5 mM PMSF, with a final pH of 7.6. Briefly, 1–2 g of tissue was diced in 4X cold homogenization buffer (wt/vol), and was then homogenized by about 5 passes in a Potter-Elvejhem tissue homogenizer. Partial protection from ribonuclease digestion was provided by care with glassware and handling, as well as by the endogenous ribonuclease inhibitor present in rat liver (Gaetani et al. 1983). The homogenate was centrifuged for 10 min at 8000 RPM in a Sorvall RC2-B refrigerated centrifuge, using an SS-34 rotor. The supernatant was decanted and centrifuged again for 15 min. The resulting postmitochondrial supernatant contained predominantly rough and smooth microsomes derived from fragmented rough and smooth endoplasmic reticulum. Rough microsomes were then isolated (Gaetani et al. 1983) on a 3-layer discontinuous sucrose gradient (1.35, 1.55, 1.8 M sucrose) by centrifugation with a Beckman L8–70 ultracentrifuge (SW-40TI rotor) for 12 h at 36 000 RPM and 4°C. The "heavy" rough microsomal fraction was mixed 1:1 with cold glycerol (Aldrich, Milwaukee, Wis., spectrophotometric grade, No. 19 161.2), and 200 µl aliquots were stored in cryogenic tubes in a liquid nitrogen refrigerator (–196°C). This storage allowed subsequent processing to be carried out more conveniently. In previous biochemical work (Kreibich et al. 1978, 1983; Gaetani et al. 1983; Walter and Blobel 1983), RM and stripped RM have been stored routinely in cryoprotectant (50-66% glycerol or 250 mM sucrose) at −70°C or −80°C, with little or no loss of activity for later in vitro protein translation. Glycerol (up to 40%) is sometimes used to stabilize native protein structure (Gekko and Timasheff 1981), and has been utilized for this purpose in biochemical studies involving RM (Yu et al. 1989).

Application to EM grid membranes, negative staining, and electron microscopy

An aliquot of RM stock was brought to −20°C, and a 5 µl sample was taken up with a Pipetman and added to 1 ml of cold TEA buffer (same as the homogenizing buffer described above, but lacking EDTA, DTT, and PMSF), with brief manual mixing. The material was then fixed in 2% paraformaldehyde by mixing equal parts of the above solution with cold 4% paraformaldehyde (in the same TEA buffer), mixing manually. After 0.5–1 h of fixation at 4°C, 100 µl of the solution was added to each of several cold "platform microtubes", which were 1.8 ml microtubes containing a plug of polymerized epoxy resin (Spurr 1969) at their tips. An EM grid was inserted into the solution in each tube and was left face-up on the surface of the plug. The grids had been coated previously with a Formvar membrane and carbon, and were glow-discharged just before use to make the membrane surface more hydrophilic. The tubes were centrifuged for 10 min at 4°C in a Microfuge B (Beckman), at about 8700 × g, to aid in bringing RM vesicles down on the EM grid membrane.

The grids were then washed 1 min in double-distilled water at room temperature. Since subsequent steps would involve floating the grids face down on drops of fluid, it was necessary at this point to dry the back of each grid, without drying the face. This was accomplished by placing a grid face down in a 10 µl droplet of distilled water on parafilm, and gently applying a piece of Whatman No. 2 filter paper to the grid, absorbing the water from the back of the grid, but leaving fluid between the grid and the parafilm. The grid was then grasped with jeweler's forceps and quickly floated face down on another drop of distilled water, remaining there for 1 min. For negative staining (Shelton and Kuff 1966), the grids were floated for 2 × 1 min at room temperature on drops of 2% phosphotungstic acid (in double-distilled water, pH 5.8, containing 5 mM $MgCl_2$). Finally, each grid was picked up with jeweler's forceps and the edge was brought obliquely against a piece of filter paper for a moment, to draw off excess staining solution, after which the grid was air-dried. Grids were viewed with a Philips 201 electron-microscope operated at 60 kV, and most micrographs were taken at 20000 × initial magnification (checked with a carbon grating replica).

Conventional EM of rat pituitary

Pituitary tissue from rats was fixed, embedded, sectioned, and positively stained for electron microscopy by conventional methods, as described previously (Christensen et al. 1987).

Results and discussion

In these RM preparations, viewed with the electron microscope, many of the vesicles were flattened, and polysomes were often discernible.

Fig. 1 a and b show negatively-stained polysomes on flattened RM vesicles, from material prepared as described above. The individual ribosomes, which were white because of the negative staining, generally consisted of 2 subunits, one larger than the other. Labeled drawings above the 2 figures may aid in their interpretation. In most of the ribosomes, the smaller subunit was oriented toward the inside of the polysomal curve.

As a frame of reference, these negatively-stained images of bound polysomes prepared in vitro may be compared with the well-known positively-stained polysome surface views that are seen occasionally in conventional EM tissue sections when the RER happens to be cut in grazing section (Palade 1955). Fig. 1 c and d show examples of positively-stained polysome surface views from tissue sections of rat pituitary, similar to those described in our previous study (Christensen et al. 1987). Labeled drawings below the positively-stained figures may aid in their interpretation. In prior detailed descriptions of positively-stained polysome surface views (see particularly Bonnett and Newcomb 1965; David and Metzler 1967; Christensen et al. 1987), the individual ribosomes usually appeared oblong, with the long axis oriented perpendicular to the presumed path of the mRNA. The end of the ribosome that was oriented toward the inside of the polysomal curve was usually wider than the outer end. These features can be observed in Fig. 1c and d.

Ribosomal subunits were not visible in the positively-stained surface views of the present study (Fig. 1 c and d), consistent with most past literature. In general, polysomal appearance and the spacing of the ribosomes were similar in the negatively-stained and positively-stained polysomes, which are shown here at the same magnification. However, the negatively-stained ribosomes (Fig. 1 a and b) appeared somewhat smaller than those that were positively stained (Fig. 1c and d). This is to be expected in negative staining, since the stain may overlap boundaries,

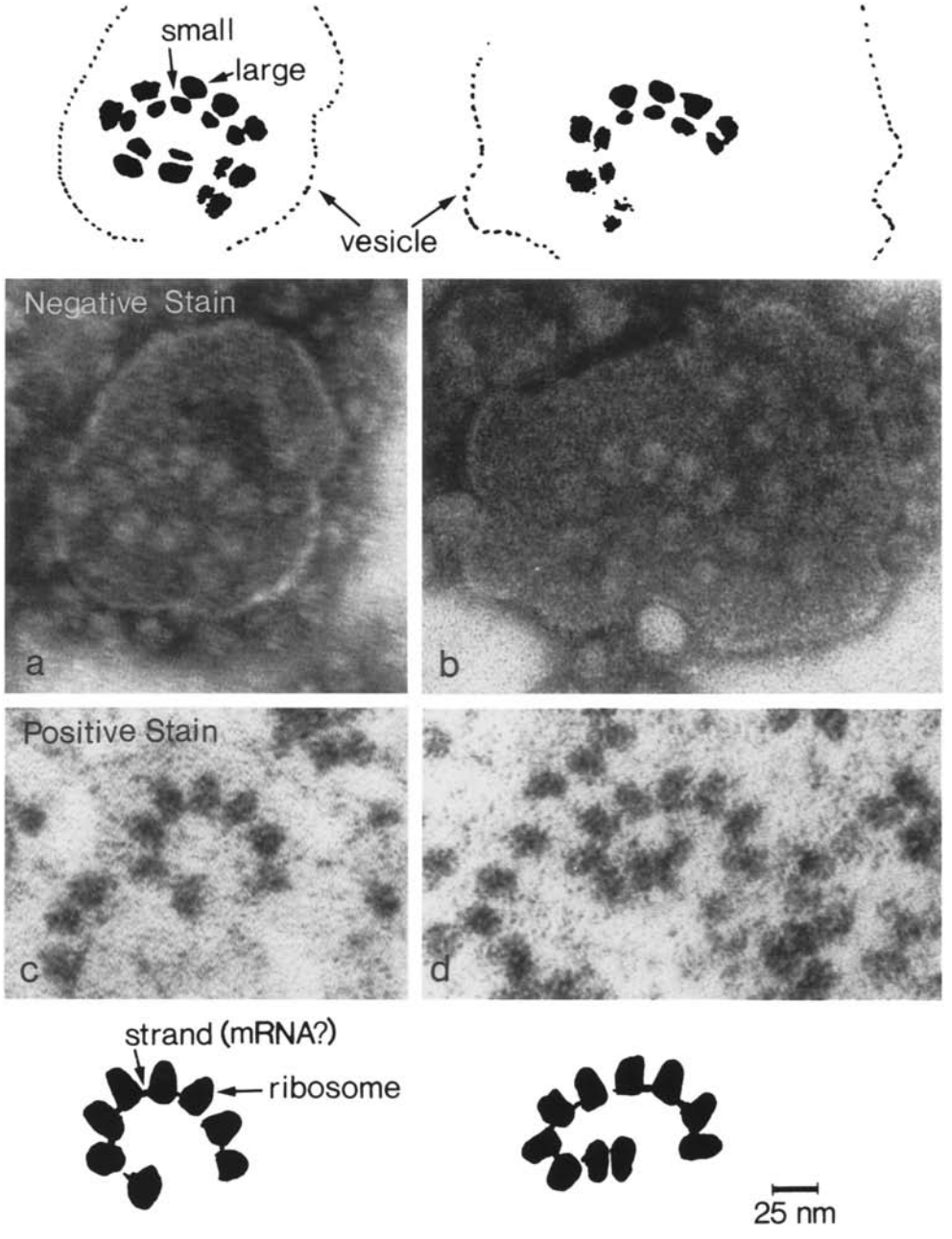

Fig. 1a, b. Negatively-stained bound polysomes on flattened rough microsome vesicles from rat liver. Diagrams above figures show pertinent structures and labels. Note large and small ribosomal subunits (appearing white as a result of negative staining). The small subunit is consistently oriented toward the inside of the polysomal curve. × 230000. **c, d** Examples of positively-stained bound polysomes in conventional tissue sections of a somatotrope from rat pituitary, provided to allow comparison with above negative-stain results (see text). Diagrams below figures show pertinent structures and labels. Note appearance of ribosomes and the strand that sometimes passes between them. Ribosomal subunits cannot be distinguished in these positively-stained preparations. × 230000

thus making the ribosome and its subunits appear smaller.

In the literature on surface views of positively-stained polysomes in tissue sections, a thin strand has occasionally been described extending between ribosomes (Watson 1959; Ross and Benditt 1964; David and Metzler 1967; Bielka 1982 [his Fig. 4 b, EM by David]; Christensen et al. 1987). The strand usually was seen to contact the ribosomes near their inner ends. Examples of this strand are shown in Fig. 1 c and d. It has seemed reasonable in the past to suggest that the strand represented the mRNA, perhaps thickened by secondary RNA structure or adsorbed protein, but no particular evidence for this interpretation has been available previously. The strands were not visible in the negatively-stained polysomes of the present study (Fig. 1 a and b). However, the interface between small and large subunits occurred at approximately the same position on the negatively-stained ribosome where the strands were found in the positively-stained preparation. This would support the view that the strands represent mRNA, which is known to pass between the small and large ribosomal subunits.

The negative-stain images of the present study seem consistent with the side-by-side model for subunit arrangement in bound ribosomes, but not with the traditional model. In negative staining, the stain accumulates around structures of interest, which are then seen in negative image. Fig. 2 shows how the stain would presumably be distributed on a ribosome oriented according to the traditional model (upper left) or to the side-by-side model (upper right). Below each diagram is the appearance that would be expected if the negatively-stained ri-

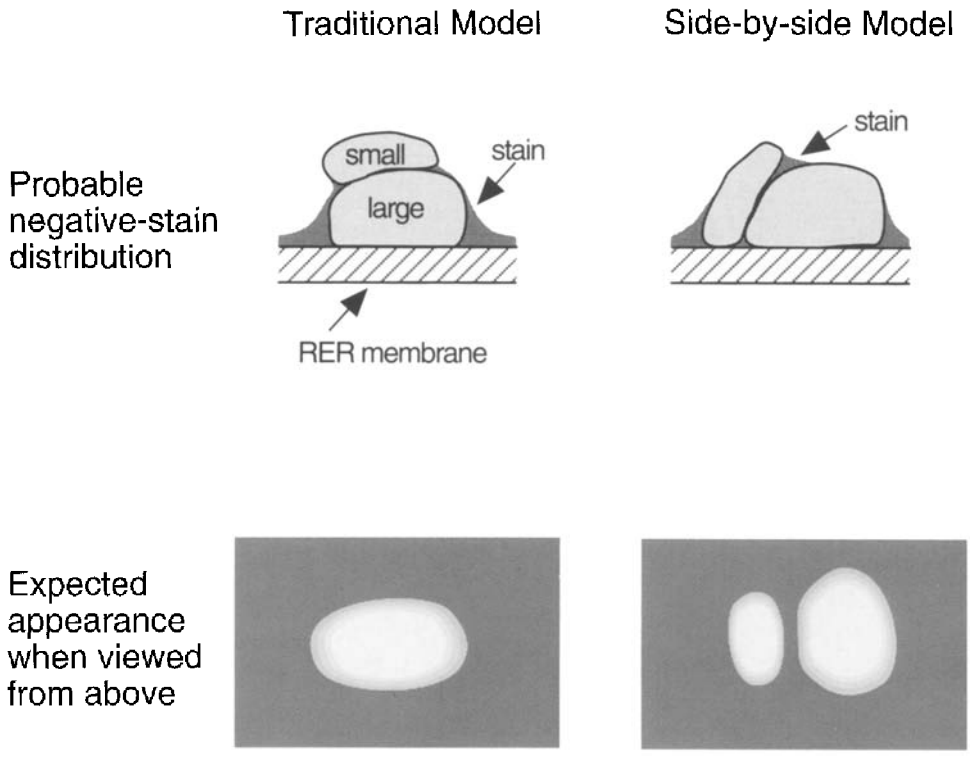

Fig. 2. Diagrams illustrating 2 main models for ribosomal orientation on the membrane of rough ER, and how ribosomes might be expected to appear in negatively-stained preparations. In the traditional model (*upper left*), the large subunit is in contact with the RER membrane and the small subunit lies on top of the large subunit, away from the membrane. Negative stain (*dark shading*) would accumulate at the sides of the ribosome. In the side-by-side model (*upper right*), the 2 subunits occur alongside one another on the membrane, and negative stain may be visible between subunits. Below each diagram is a representation of how a negatively-stained ribosome arranged according to that model might be expected to appear in EM, when viewed from directly above, the vantage point in the present study. Negatively-stained ribosomes in Figures 1a and 1b appear as would be expected for the side-by-side model

bosome were viewed from directly above with the electron-microscope, as in the present study. In the traditional model, the large subunit is attached to the RER membrane and the small subunit is situated on top of the large subunit, away from the membrane. The negative stain would be gathered primarily around the sides of the ribosome. When viewed from above, one would expect the negative stain image to appear as a single oval. On the other hand, in the side-by-side model, the subunits are arranged alongside one another on the RER membrane, and stain may penetrate between the subunits. When viewed from above, the stain lying between the subunits would cause the small and large subunits to be seen individually. The negatively-stained ribosomes in Fig. 1 a and b have the appearance that would be expected for the side-by-side model.

The side-by-side model (Unwin and Taddei 1977; Unwin 1977) arose from studies on 2-dimensional ribosomal crystals, in which the ribosomes were not involved in translation. The present findings offer evidence that the model is also valid for organized bound polysomes. When the 3-dimensional ribosomal reconstruction of Milligan and Unwin (1986), produced at 55 Å resolution, is viewed from above (their Fig. 3 b), it resembles the average appearance of positively-stained ribosomes in the surface views of polysomes from tissue sections (our Fig. 1c and d). The boundary between large and small subunits (arrowheads in their Fig. 3 a) is consistent with the boundary position in our negatively-stained ribosomes (our Fig. 1 a and b) and with the strand position in the positively-stained polysomes (our Fig. 1 c and d). Verschoor and Frank (1990) have produced a more detailed 3-dimensional reconstruction of free (unbound) 80 S ribosomes at 37 Å resolution. However, the orientation that those free ribosomes would have if they were bound to the RER membrane has not been established. Those authors showed considerable space between the large and small subunits, except near the base, where there is tight contact. The substantial negative stain between subunits in our micrographs would be consistent with that feature.

Our results do not appear to be consistent with the traditional model for the orientation of bound ribosomes. The development of the traditional model was based primarily on biochemical studies of subunit binding to the RER membrane, and on electron micrographs of ribosomes seen in side view. Sabatini et al. (1966) showed biochemically that the large ribosomal subunits were firmly attached to the RER membrane, while the small subunits were only weakly attached to the membrane, if at all. When rough microsome (RM) vesicles were negatively stained on EM grid membranes, and then viewed with the electron microscope (Sabatini et al. 1966; Shelton and Kuff 1966), no interpretable images of ribosomal subunits or of organized polysomes were distinguished on the exposed face of RM vesicles. However, ribosomes were observed in side view, attached to the edges of the flattened vesicles. These ribosomes had a bipartite appearance, and the larger subunit was attached to the edge of the vesicle, while the smaller subunit was on top of the larger one, away from the vesicle edge. The boundary between large and small subunits was approximately parallel with the edge of the vesicle. Although conventional electron micrographs of positively-stained ribosomes in tissue sections rarely showed distinct ribosomal subunits, occasional micrographs were published in which apparent large and small subunits could be distinguished in ribosomes bound to the RER,

when the RER membrane was observed edge-on, and the ribosomes were therefore seen in side view (Palade 1967, 1975; Florendo 1969; David and Uerlings 1970). In those instances, again, the large subunit was situated on the RER membrane, and the small subunit formed a roof over the large subunit and was not in contact with the membrane. However, the paper by David and Uerlings (1970) also included occasional ribosomal side-views in which both subunits appeared to be in contact with the membrane.

In the literature, positively-stained polysomes seen in surface views of the RER in tissue sections (similar to those shown here in Fig. 1 c and d) have almost never exhibited distinguishable ribosomal subunits. However, there are 2 exceptions. The paper by Florendo (1969) included a surface view of a partial polysome (his Fig. 2), with subunits visible in 3 ribosomes. The smaller subunits were oriented toward the inside of the polysomal curve. He commented that the micrograph suggested "that both large and small subunits are closely apposed to the underlying membrane." However, the appearance of these ribosomes, seen from above, was the same as that of ribosomes shown in side view elsewhere in the paper, which was not what would have been anticipated. To reconcile this apparent inconsistency, he postulated that the ribosomes were actually oriented obliquely, and that the "small subunit-large subunit axes of ribosomes arranged in whorls [polysomes] are disposed in three dimensions on the surface of a truncated cone." The surface view in his paper did not happen to show a strand. The other paper, by Bonnett and Newcomb (1965, their Fig. 10, inset), also included ribosomal subunits in surface views of bound polysomes. However, the subunits appeared to be about the same size, and so it was difficult to draw conclusions about the orientation of the small subunits. A freeze-fracture study on rough microsome vesicles, seen in surface view after deep etching (Ojakian et al. 1977, their Fig. 3), showed grooves across 2 of the bound ribosomes, defining subunits. Again, the subunits were of approximately equal size.

It may be of interest to note that the appearance of ribosomes in our negatively-stained preparations of bound polysomes is similar to results obtained by Shelton and Kuff (1966), in which small, free (unbound) polysomes were brought down on EM grid membranes and negatively stained. The intrinsic geometry of these free polysomes caused them consistently to assume a round or C-shaped configuration on the EM grid membrane, and the small ribosomal subunits were uniformly visible and were oriented toward the inside of the polysomal curve. The bound polysomes of the present study have a similar appearance on the membranes of flattened RM vesicles. This implies that the shape of small, bound polysomes, and the orientation of their ribosomes, may reflect inherent characteristics of polysomal organization.

Some possible concerns need to be considered in the interpretation of the negatively-stained images in the present study. One concern is that the side-by-side arrangement of subunits might have been produced as a drying artifact during negative staining, if a small subunit that was originally on top of the large subunit would be drawn down to the membrane by surface tension or other forces during drying. Air-drying during negative staining tends to flatten structures to some degree, although the distortion is not nearly as severe as that which occurs during simple air drying, since the negative stain gathers around small structures, forming a coat that partially protects them from the damaging effects of aqueous surface tension. Lake (1978) used tilting experiments to compare the extent of flattening in 3 different negative-staining approaches (double carbon, single carbon, droplet), and found that the longest dimension of the bacterial ribosomes varied from 180 Å to 250 Å in the 3 methods, indicating a considerable difference in flattening. However, the flattening did not seem to have an appreciable effect on structural detail seen in the negatively-stained ribosomes, since similar features were observed by all 3 methods. According to Hayat and Miller (1990), flattening of negatively-stained structures usually makes them appear somewhat larger than the corresponding fresh or positively-stained structures. Comparing the negatively-stained ribosomes in Fig. 1 a and b with the positively-stained ribosomes in Fig. 1 c and d shows that this is not the case in the present material, suggesting that flattening was not excessive.

If the binding between small and large subunits were unstable, then one could imagine an artifactual shift in the position of the small subunit during negative staining. However, this binding appears generally to be quite stable during negative staining, judging by the small number of detached subunits seen in fields of negatively-stained free ribosomes (monosomes) in bacteria (Lake 1981) and in eukaryotes (Verschoor and Frank 1990). If the binding between subunits in monosomes is relatively stable, then subunit binding should be even more stable in ribosomes that are organized into bound polysomes, where the small subunit would be further stabilized by the anchoring effect of the mRNA and of the nascent polypeptide. The small subunit binds the mRNA and tRNAs, and it might be expected, therefore, that formaldehyde fixation of a bound polysome would stabilize the mRNA and polypeptidyl-tRNA in the ribosomes. This should limit considerably the mobility of the small subunit, and thus make it even less likely to shift its position relative to the large subunit during negative staining.

In view of the above considerations, it seems unlikely in the present study that the side-by-side appearance of subunits in the ribosomes of Fig. 1 a and b would have resulted from detachment of small subunits from the top of large subunits during negative staining, and their movement down to the membrane, all on the same (inward) side of the ribosomes. It would seem more likely that the subunits were side-by-side before negative staining was carried out.

Another possible concern is that the side-by-side appearance might be a tilting artifact. If a ribosome whose small subunit was actually on top of the large subunit happened to be viewed from a tilted perspective, then the subunits might appear, mistakenly, to be side-by-side on the membrane. This is unlikely in the present study, since the ribosomes are all oriented with the same side up on

the RM vesicle (RER) membrane, and are being viewed from directly above, without any tilt. The ribosomes should have a consistent orientation on the RM vesicle membrane because they are attached to it by their specific attachment sites (Unwin 1979; Savitz and Meyer 1993). The RM vesicle membrane is being viewed from directly above because the vesicles have been flattened down on the EM grid membrane, which is in a plane perpendicular to the viewing axis. It does not seem likely, therefore, that the appearance of these ribosomal images could be ascribed to a tilt.

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