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# INTERACTION OF THE 5′-ENDS OF 28S RNA IN DIMERIZATION OF HAMSTER RIBOSOMES

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Free ribosomes extracted from hamster cells and 28S RNA purified from these ribosomes are known to form dimers. We find that spleen phosphodiesterase inhibits ribosomal dimer formation, but only when a free 5′-hydroxyl end group, produced by the action of alkaline phosphatase, is present. Hence, formation of dimer ribosomes probably involves interaction at or near the phosphorylated 5′-ends of 28S RNA.

Dimer RNA molecules show a modal length, when measured on electron micrographs, of 2.1 μm, which is about double the length of 28S RNA. Electron micrographs of 115S dimer ribosomes often show profiles consistent with our interpretation that in dimers the 28S RNA chains are loosely linked by their 5′-ends.

## INTRODUCTION

Free ribosomes isolated from rats and Syrian hamsters, unlike those of mice and men (and groundhogs), dimerize when placed at low temperatures and in specific ionic conditions (1, 2). Dimers of ribosomes have also been found in hypothermic chick cells and in extracts of Escherichia coli (3, 4).

A large component of the RNA molecules purified from dimers of hamster ribosomes sediments at about 40S and is derived from two 28S molecules; hence the association between two monomeric ribosomes leading to dimer formation is presumably through RNA-RNA interaction in the large ribosomal subunits (5). 28S RNA molecules derived from mouse ribosomes do not form dimers (5).

We examined dimers of hamster ribosomes by electron microscopy (6–10) to see if dimerization imposed constraints on the orientation of the two parts of the dimer. We also used phosphodiesterases, specific for either the 5′- or 3′- end of RNA polynucleotides, to find out which end of the RNA chain is involved in forming dimers.

## MATERIALS AND METHODS

### Cell Cultures and Sources of Materials

Baby (Syrian) hamster kidney (BHK21) cells, provided by Dr. John Littlefield, were grown as monolayers in Dulbecco's Modified Eagle Medium (Gibco) supplemented

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with 15% fetal calf serum. Alkaline phosphatase (E. coli, code BAPF, chromatographically purified, free of RNase), Phosphodiesterase I (snake venom phosphodiesterase), and Phosphodiesterase II (spleen phosphodiesterase) were purchased from Worthington Biochemical Corp.; Enzite-RNase from Miles-Seravac; Brij 36-T from Canamex (Mexico); sucrose (free of ribonuclease activity) from Schwarz-Mann; and Cytochrome C (equine heart), Aquacide II-A, and pronase (free of nucleases) from Calbiochem.

### Preparation of Buffers and Cell Extracts

For isolation of ribosomal dimers, we used TKM buffer (H buffer of Reader and Stanners [2]) of the following composition: 10 mM Tris-HCl, pH 7.4; 100 mM KCl; and 20 mM $MgCl_2$, or TNM buffer (L buffer of Stanners et al. [5]), which is similar to TKM buffer except for an equimolar substitution of NaCl for KCl and a lower $Mg^{++}$ content (2 mM). Purified RNA was extracted in TNM buffer.

Growing cell cultures were washed twice with phosphate-buffered saline and removed from the plastic substrate by incubation with 0.05% trypsin-0.02% EDTA in Puck's saline A (Gibco) at 37°C for 5 min. The cells were then washed in TKM buffer to remove trypsin. The following procedures were all carried out at 0–4°C: cells were centrifuged at 2,000 g for 5 min, resuspended in TKM buffer, and allowed to swell for 20 min in an ice bath. Brij 36-T was added to a final concentration of 0.5%. Cells were broken with 10 strokes in a Dounce homogenizer. Whole cells and debris were removed by centrifugation at 5,000 g for 5 min. Sodium deoxycholate (DOC) was added to the supernatant fraction to a final concentration of 0.5%. The suspension was then centrifuged at 12,000 g for 10 min and the supernatant used for preparation of dimer ribosomes on sucrose gradients.

### Sucrose Gradient Centrifugation

38 ml of 15–30% w/w sucrose gradients was prepared in TKM buffer, and 30 absorbance units (260 nm) of cell extract in 1.5 ml were layered at the top. The gradients were spun at 4°C in an SW 27 rotor at 27,000 rpm for 4.5 hr in a Spinco Model L2-65 ultracentrifuge and analyzed at 254 nm on an Isco Model 640 Density Gradient Fractionator and Model UA-4 Ultraviolet Analyzer. S values were measured both by comparing rates of sedimentation with those of the subunits of Escherichia coli ribosomes (taken as 30S and 50S) and by direct calculation based on centrifugal forces and the physical properties of sucrose solutions (11). Both methods gave the same results.

### Preparation of Dimer Ribosomes

Cell extracts were run on sucrose gradients and the 115S region containing dimer ribosomes was collected by pooling all fractions in the peak with an absorbance greater than one-half of the maximum. The ribosomes were diluted with TKM buffer and pelleted by centrifugation at 4°C in an SW 41 rotor at 37,000 rpm for 6 hr. The pellet was resuspended by gentle stirring overnight in TKM buffer at 0°C. The resulting suspension was used for electron microscopy of dimers or for extraction of RNA.

### Electron Microscopy of Dimer Ribosomes

Dimer ribosomes were taken directly from gradients by catching drops on grids (200 or 300 mesh) coated with formvar and carbon. The ribosomes on the grids were washed with several drops of TKM buffer to remove sucrose and negatively stained with 2% aqueous uranyl acetate, pH 4.0. The preparations were examined in a Siemens 101 electron microscope operating at 80 kV with an instrumental magnification of 80,000. Grids were placed in the microscope with the specimen side away from the electron beam, and resulting micrographs were printed with the emulsion side of the negative facing the emulsion side of the photographic paper.

### Extraction of RNA from Dimer Ribosomes

The method of Stanners et al. (5) was used for extraction. Dimers were collected from sucrose gradients and diluted 1:1 in TNM buffer. DOC was added to a final concentration of 0.5%, and the mixture was incubated for 20 min at 10°C. After cooling for 30 min, a precipitate of DOC-protein was formed which was then sedimented by low-speed centrifugation. The supernatant fraction was carefully removed and treated with pronase at a concentration of 250 μg/ml for 30 min at 10°C. This suspension was layered over an 11.5 ml, 15–30% w/w sucrose gradient prepared with TNM buffer and 100 μg/ml pronase. The gradients were spun at 24,000 rpm in a Spinco SW 41 rotor for 12 hr at 4°C and fractionated and analyzed on the Isco apparatus. RNA, sedimenting at 40–42 S, was collected and dialyzed overnight in 2 changes of 10 volumes of TNM buffer and concentrated to 1/50 volume in Aquacide II-A at 4°C.

### Electron Microscopy of Dimer RNA

After concentration the RNA was prepared for electron microscopy by the technique of Lang and Mitani (12). The following stock solutions were used: cytochrome C, 0.01% in distilled water; 2 M ammonium acetate in 1 mM disodium EDTA, pH 7.0; 37% formaldehyde diluted 1:10 in distilled water; and 0.5–1.0 μg/ml of 40–42S RNA. All solutions except the RNA were filtered through a Millipore filter (0.22 μm pore size) prior to use. A mixture was prepared from the above stocks in this order: 0.02 ml RNA, 0.3 ml ammonium acetate with EDTA, 2.5 ml distilled water, 0.08 ml cytochrome C, and 0.15 ml formaldehyde. Droplets, 40 μl in size, were deposited onto a covered teflon dish and placed in the refrigerator. After 2 hr, grids (coated with formvar and carbon) were gently touched to the surface of the droplet, removed, stained for 30 sec in a solution containing 20 ml absolute alcohol and 0.2 ml of 5 mM uranyl acetate in 50 mM HCl, and finally dried by dipping for 15 sec in isopentane. The specimens were rotary shadowed by use of an 8 mil platinum-palladium wire (80:20) at a distance of 5 cm and a height of 5 mm from the specimen. The grids were examined in the electron microscope with an instrumental magnification of 16,000. The lengths of the molecules on photographic prints were determined with a map measurer.

## RESULTS

### Sucrose Gradient Centrifugation of Ribosomes

When ribosomes are extracted in the cold from cultured BHK21 cells, most of the free ribosomes exist as dimers and sediment in sucrose gradients at 115S (2). A typical profile shows particles sedimenting at 40S, 80S, and 115S (Fig. 1). A small 90S peak was also seen, probably consisting of an 80S ribosome with an additional attached subunit, as suggested by Stanners et al. (5). A few small classes of polyribosomes could be resolved in most gradients.

If the preparation was not carefully maintained in the cold, either during the initial disruption of the cells or during gradient isolation, the number of dimers isolated decreased and the number of monomers increased. Free ribosomes, prepared from mouse L-cell fibroblasts under conditions of extraction in the cold similar to those described above, did not dimerize. As also found by Reader and Stanners (2), they were predominantly in the 80S form.

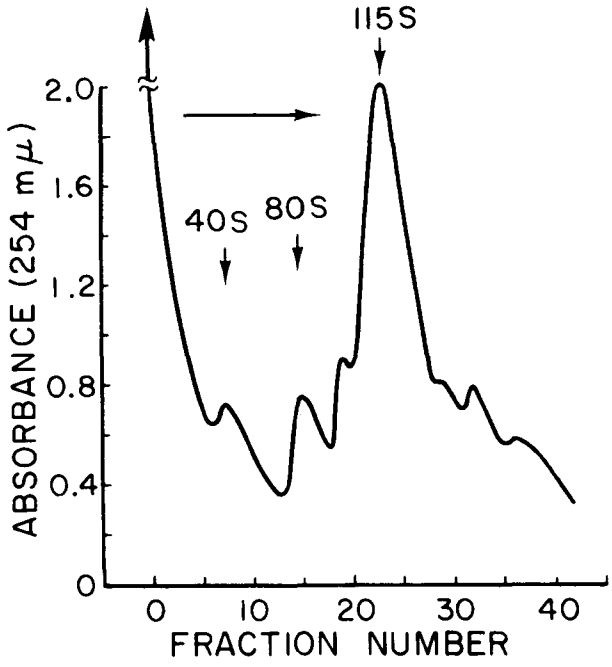


Fig. 1. Sucrose gradient analysis of ribosomes from cultured BHK21 cells. Cell extracts were layered onto 38 ml, 15–30% (w/w) sucrose gradients in TKM buffer and centrifuged at 4°C for 4.5 hr at 27,000 rpm in an SW 27 rotor. Arrow indicates direction of sedimentation.

### Electron Microscopy of Dimer Ribosomes

In the sections that follow, we will use the terminology of Nonomura et al. (10) in describing ribosomal features. Figure 2 shows a representative field of ribosome dimers taken directly from the 115S region of a sucrose gradient. Generally, 90% of the ribo-

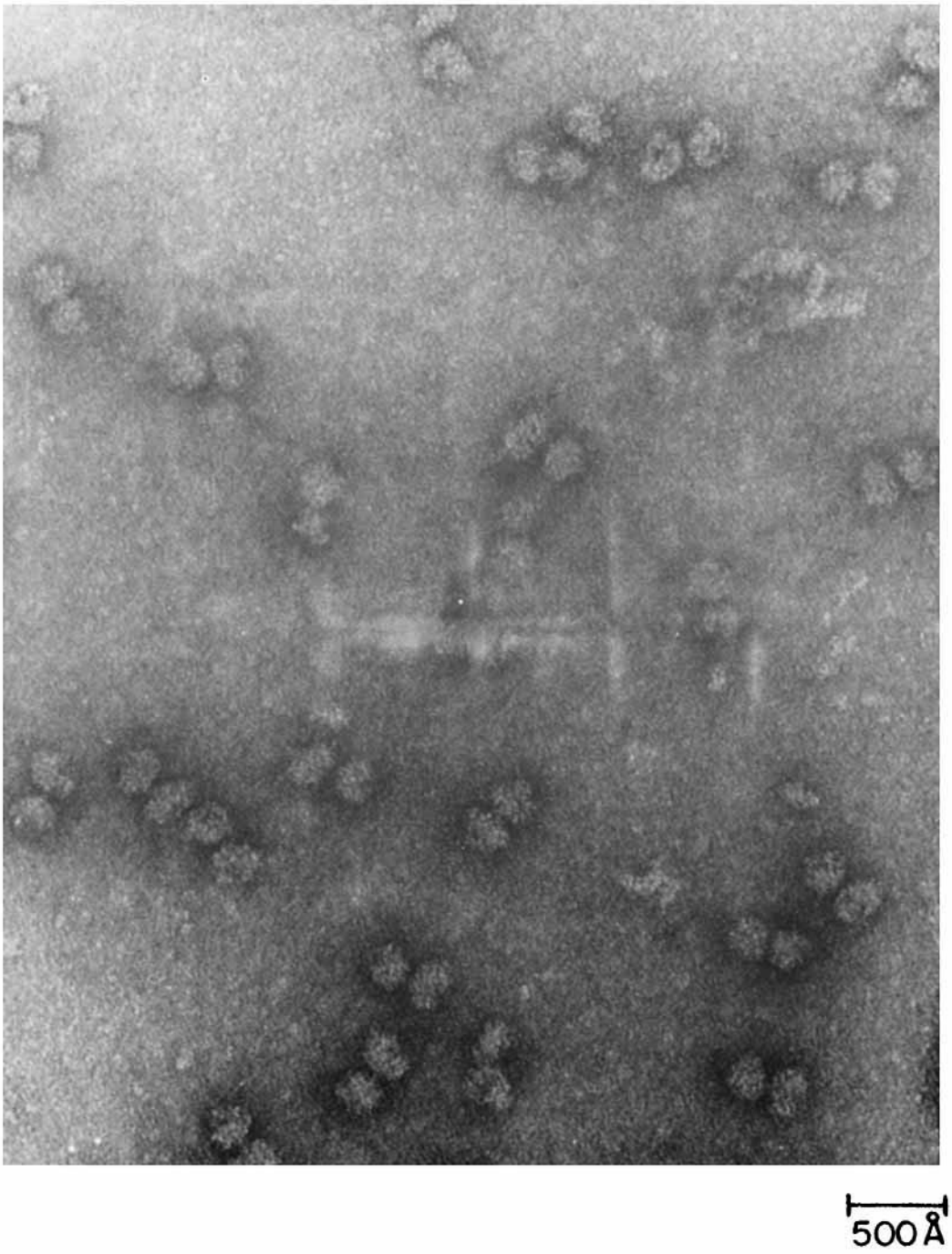


Fig. 2. Dimer ribosomes from BHK21 cells. (× 280,000)

somes (at least 300 were counted per sample) were seen as dimers, but a few monomers or small clumps of three or four ribosomes were also present.

A gallery of dimers, showing profiles that correspond to frontal or lateral views, was selected from a large number of such micrographs. In Fig. 3, Rows A and B show dimers in which both monomers are in the left-featured frontal view; the monomers appear to be related by a twofold axis of rotation (seen most clearly in A1, A5, B1, B6). In Row C, one ribosome of the dimer is in lateral profile and the other is in frontal view. The lateral views are either right-lateral (C1, C4, C5, C6) or left-lateral (C3 and probably C2). Row D shows dimers in which only one of the ribosomes is in lateral view.

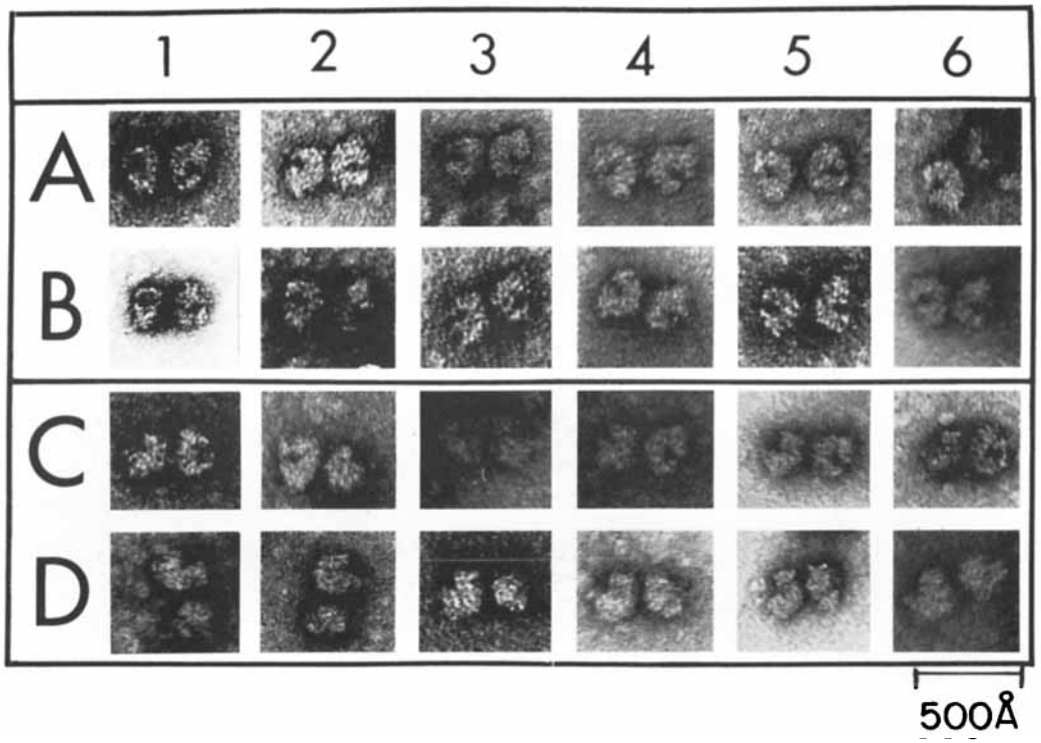


Fig. 3. Selected electron micrographs of dimer ribosomes. A and B: both monomers are oriented in frontal view. C and D: one or both monomers of the dimer appear in either right or left lateral profile (× 280,000)

### Ribonucleic Acid Extracted from Dimer Ribosomes

Stanners et al. (5) found three distinct species of RNA (18S, 28S, and about 40S) on sucrose gradients of extracts prepared from purified dimer ribosomes. We confirm their result (Fig. 4). In our experiments the sedimentation constant of the heaviest fraction varied between 40S and 42S; we will refer to this as 41S RNA. The ratio of the combined absorbance of the 28S and 41S peaks to the 18S peak was about 3, which is slightly greater than that found by Stanners et al. (5). The presence of a significant 28S peak was probably due to partial dissociation of dimer ribosomes before extraction of RNA.

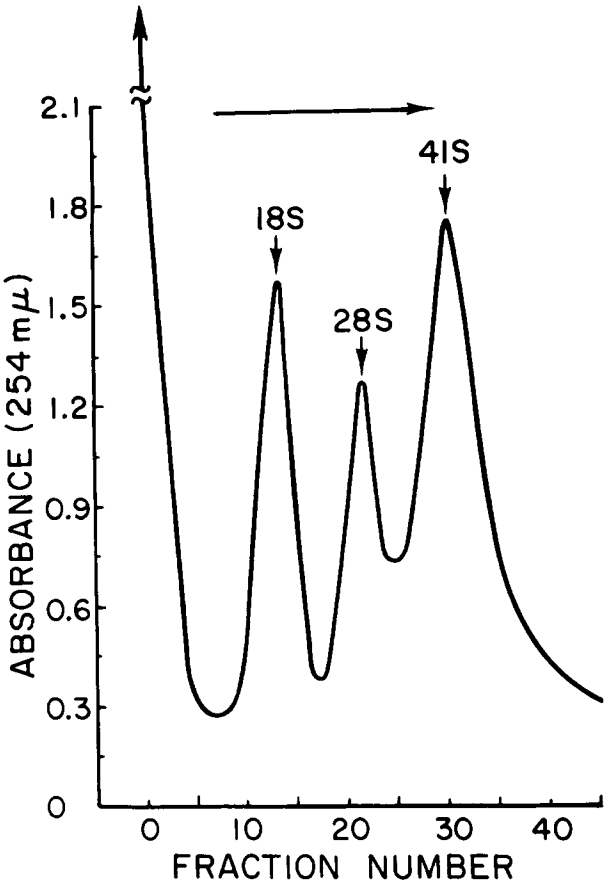


Fig. 4. Sucrose gradient analysis of ribosomal RNA extracted from dimer ribosomes. RNA was layered over 11.5 ml, 15–30% (w/w) sucrose gradients in TNM buffer containing 100 μg/ml pronase and centrifuged at 4°C for 12 hr at 24,000 rpm in an SW 41 rotor. Arrow indicates direction of sedimentation.

We examined a large number of electron micrographs of rotary-shadowed preparations taken from the 41S peak. The lengths of the RNA molecules in this peak ranged from 0.7 μm to 4.3 μm, with a modal length of 2.1 μm, a mean of 2.2 μm, and a standard sample deviation of 0.87 (Fig. 5). A representative molecule about 2 μm long, taken from the 41S region of a sucrose gradient, is shown in Fig. 6.

### Kinetics of Formation of Dimer Ribosomes and Effect of Enzite-RNase

Dimer ribosomes readily dissociate into monomers when warmed (2). We found that after only 1.5 hr at 37°C, 85–90% of the dimers had dissociated into 80S ribosomes.

To measure the kinetics of reassociation, dimers were isolated on sucrose gradients, dissociated by warming, and returned to 0°C. Samples were removed at 12-hr intervals and examined by electron microscopy to assess reassociation (Fig. 7). After 24 hr, only

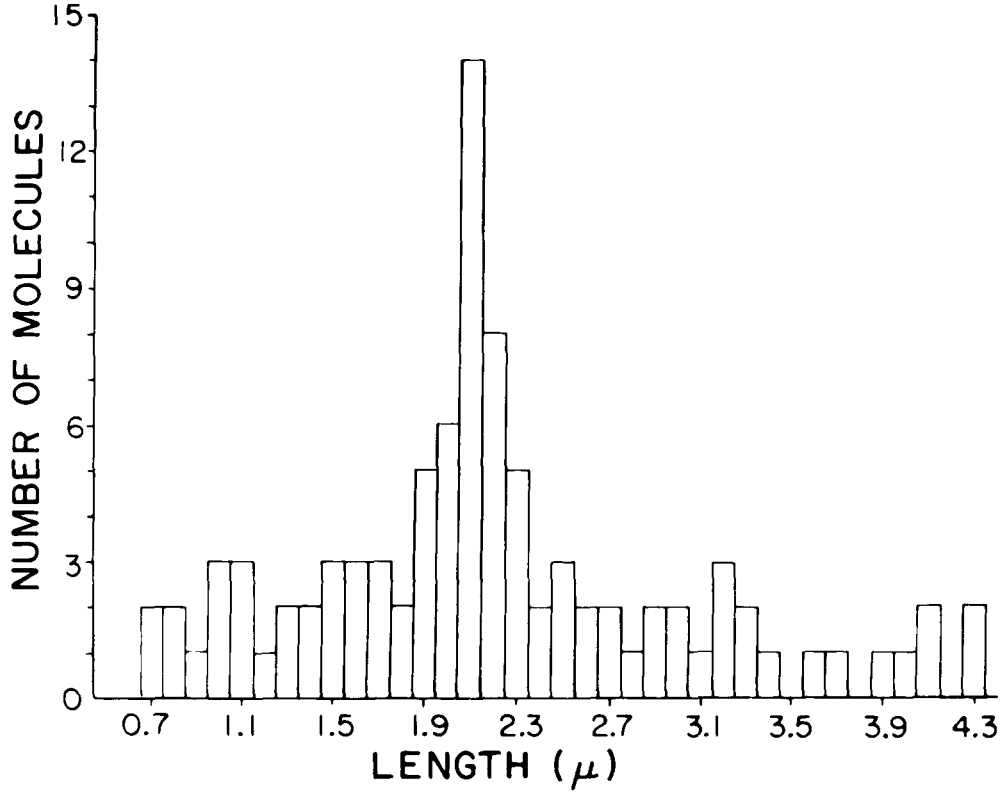


Fig. 5. Histogram showing length distribution of 41S RNA isolated from purified dimer ribosomes.

25% of the ribosomes were paired; after 60 hr, 80% were paired. Hence, many hours are needed, at 0°C, for a high degree of reassociation to occur. Ribosomes maintained at 37°C did not reassociate.

Since Stanners et al. (5) showed that purified 28S RNA forms dimers, we tested the ability of dissociated ribosomal dimers to reassociate after treatment with ribonuclease. Brief treatment with Enzite-RNase strongly inhibited reassociation (Fig. 7).

### Effects of Specific Phosphodiesterases on Reassociation of Monomeric Ribosomes

We also tested the effect of enzymes whose specificity would indicate whether a free end of an RNA molecule was exposed. Dimer ribosomes were isolated, dissociated to monomers by warming, and treated with spleen phosphodiesterase (which requires a free 5'-hydroxyl end group) or snake venom phosphodiesterase (which requires a free 3'-hydroxyl end group). Neither enzyme alone had any significant effect on dimer formation. In the presence of alkaline phosphatase, however, spleen phosphodiesterase prevented the reformation of dimers (Fig. 8). Attempts were also made to dissociate and reassociate purified dimer RNA, but very little reassociation occurred.

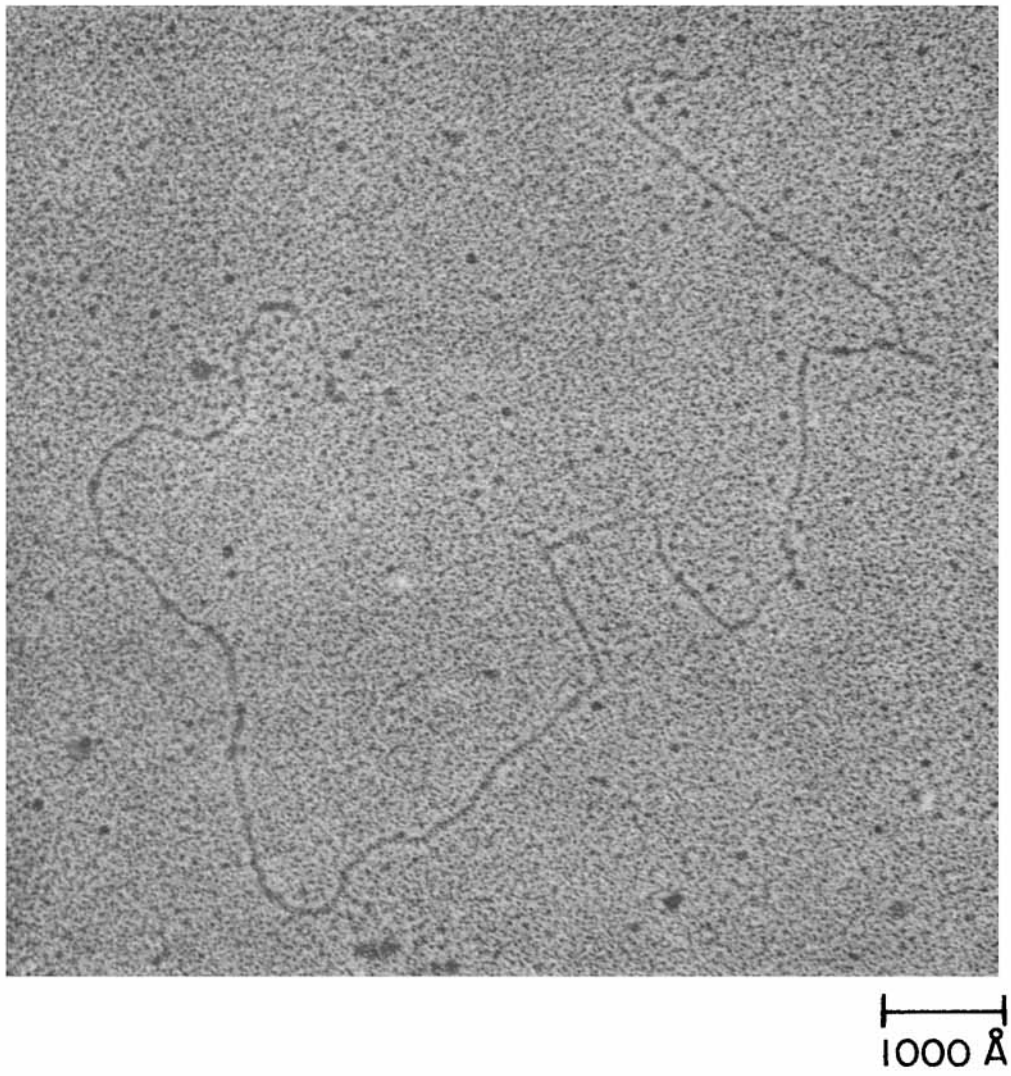


Fig. 6. Representative electron micrograph of 41S RNA. Length about 2 $\mu$m. (X 150,000)

## DISCUSSION

Our results confirm and extend those of Reader and Stanners (2) and of Stanners et al. (5): free ribosomes extracted from cultured hamster cells dimerize in the cold but dissociate again to form 80S monomers when warmed briefly to 37°C. Our sedimentation patterns both for ribosomes in cell extracts and for purified RNA extracted from ribosomal dimers are similar to those reported previously (2, 5).

Because purified 28S RNA from hamster, but not mouse, ribosomes forms dimers of about 41S, it seems likely that dimerization of 80S ribosomes takes place through

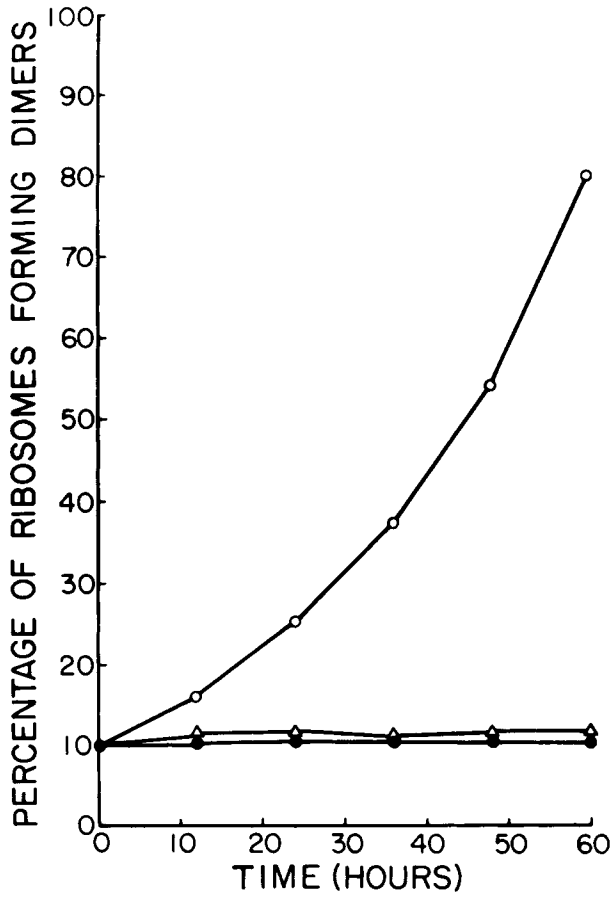


Fig. 7. Effect of Enzite-RNase on the reassociation of monomeric ribosomes. Purified dimer ribosomes (1 mg/ml in TNM buffer adjusted to pH 7.2) were dissociated to monomers by warming for 90 min and divided into three parts. One part was cooled and held at 0°C (○—○) and the second was kept at 37°C (●—●). The third part was treated at 37°C with 1 μg/ml Enzite-RNase in TNM buffer at pH 7.2; after 15 min of incubation, the sample was centrifuged at 39,000 g for 5 min to remove Enzite-RNase; the supernatant was then cooled and kept at 0°C (△—△). Samples were removed at intervals, and the percent of monomer ribosomes forming dimers was determined by electron microscopy.

interaction of regions of 28S RNA on the ribosomal surface (5).

We examined RNA-RNA 41S dimers in metal-shadowed specimens to determine the location and extent of joining of RNA molecules. We found a mean length for dimer RNA of 2.2 μm, which is about double the length (1.16 μm) of HeLa 28S RNA, as reported by Granboulan and Scherrer (13). Since the standard error of the mean value of the measured lengths of dimer RNA (and of 28S RNA, as reported by Granboulan and Scherrer) is about 0.09 μm, there is uncertainty in determining how much overlap of two 28S RNA molecules might occur in the dimer 41S RNA form. The region of joining might be close to the 5′-ends or as much as 300 nucleotides from these ends.

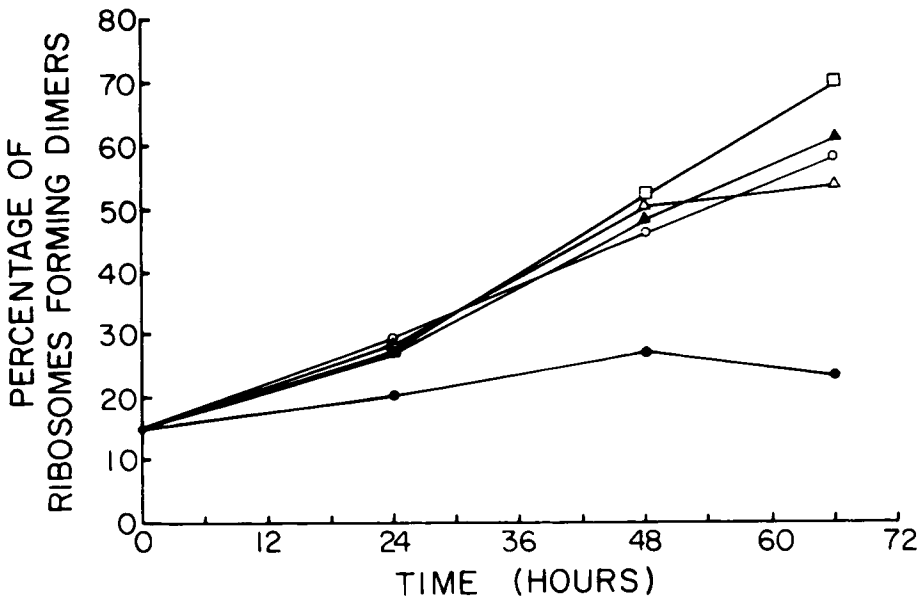


Fig. 8. Effects of phosphodiesterases on the reassociation of monomeric ribosomes. Purified dimer ribosomes were dissociated to monomer units as described in Fig. 7. Aliquots of the preparation of monomers (1 mg/ml in TNM buffer) were treated for 1 hr at 37°C, at pH values and enzyme concentrations indicated: alkaline phosphatase (200 μg/ml) and spleen phosphodiesterase (100 μg/ml), pH 7.2 (●——●); spleen phosphodiesterase (100 μg/ml), pH 7.2 (○–○); alkaline phosphatase (200 μg/ml) and snake venom phosphodiesterase (100 μg/ml), pH 8.1 (▲–▲); alkaline phosphatase (200 μg/ml), pH 8.1 (△–△); no enzyme, pH 7.2 (□–□). All samples were then placed at 0°C. Portions were removed after 24, 48, and 65 hr, and the percent of monomer ribosomes forming dimers was determined by electron microscopy.

The wide variation that we found in measured lengths of dimer RNA is unexplained. Granboulan and Scherrer (13) also found wide variation in lengths of RNA purified from bacteriophage, bacteria, and animal cells. We presume that RNA molecules randomly extend to different lengths during preparation for microscopy.

To find out whether dimerization of ribosomes involves the 5'- or 3'-ends of RNA molecules, we used phosphodiesterases to inhibit the reassociation of monomer ribosomes into dimers. Formation of dimers was strongly inhibited only when ribosomes were treated with both alkaline phosphatase and spleen phosphodiesterase.

We interpret the results as indicating that (a) dimerization involves the 5'-ends of the 28S RNA molecules, and (b) these 5'-ends are phosphorylated. The results also show that alkaline phosphatase alone did not inhibit dimerization. Hence formation of dimers, as expected, does not depend on the presence of a terminal phosphate group, since interaction between RNA chains probably involves base pairing.

Interaction of the 28S RNA molecules at their 5'-ends would be expected to favor formation of ribosomal dimers in which the monomers are related by a twofold rotational axis, and a number of such dimer profiles were seen in the electron micrographs (Fig. 3:

A1, A5, B1, B6). Since some dimers show a frontal view in one ribosome and either a frontal or a lateral view in the other, there probably is relatively free rotation around the junction.

Our results, in conjunction with those of Stanners et al. (5), suggest that in ribosomes, at least in those from Syrian hamsters and rats, the 5′-end of the 28S RNA molecules is exposed. Although the free ribosomes of mice and men (2), and the purified 28S RNA of mice (5), do not form dimers, this does not preclude the possibility that in these species the 5′-ends of 28S RNA are exposed and may simply lack the base sequences that are necessary for joining.

The primary sequence of the first 20 nucleotides of the 5′-end of 28S RNA from Novikoff (rat) hepatoma cells has been reported (14). As many as eight base pairs, not all adjacent, can be brought into coincidence by overlapping the sequences; whether pairing of these eight would be sufficient to hold two 28S RNA molecules in dimer form is uncertain. No palindromic regions are present in this nucleotide sequence of rat 28S RNA, and no analysis is available for Syrian hamster 28S RNA.

Other reports also indicate that ribosomal RNA is not entirely covered by protein: in the large subunit of Escherichia coli, the 23S molecule is known to be asymmetrically distributed (15), and measurements of X-ray scattering suggest that the large ribosomal subunit of Escherichia coli, strain MRE 600, has a tail that can be removed by pancreatic RNase (16). Hence it is possible that 28S (or 23S in bacteria) RNA may be partly exposed in ribosomes of cells from many species.

Although dimerization does not occur at the normal body temperature of hamsters, its occurrence at 0°C reveals the existence of free 5′-ends of 28S RNA, which might have some function in protein synthesis or in attachment of the large subunit to the endoplasmic reticulum (17). With bacterial ribosomes, extensive cleavage of ribosomal RNA with RNase does not impair their ability to synthesize polyphenylalanine in a polyuridylic-acid–stimulated system (18, 19, 20). Initiation of protein synthesis, however, has not been tested with ribosomes treated with specific exonucleases. The activity of treated ribosomes should be examined further by use of natural messenger RNA.

Reader and Stanners (2) reported that ribosomes derived from treatment of polyribosomes with RNase did not dimerize. Our results show that when free ribosomes are treated with RNase, dimer formation is prevented, which suggests that residual RNase on the preparations of ribosomes used by Reader and Stanners might have caused the inhibition of dimer formation. The question of whether dimers can be formed from ribosomes derived from polyribosomes without RNase treatment is still open.

## ACKNOWLEDGMENTS

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