# EVIDENCE FOR SPECIFIC ASSOCIATION OF PROTEIN WITH NEWLY FORMED RIBOSOMAL SUBUNITS

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In the cytoplasm of mammalian cells a certain proportion of the ribosome subunits are present as particles which sediment in sucrose gradients at approximately 40S and 60S and contain 18S and 28S RNA respectively. It is in these particles that ribosomal RNA first appears in the cytoplasm (1-3). The newly synthesized 40S particles exhibit a sedimentation rate which is normally not distinguishable from that of the mature 40S particles (1-4). However they possess buoyant densities in CsCl which are markedly lower (4). This distinctive buoyant density behavior is not a procedural artifact, since it can be shown that both mature and newly formed particles sediment essentially the same before and after banding on CsCl (5,6). Moreover the property of lower buoyant density is also exhibited by the newly formed 60S particles (6). Both new and mature particles contain the same RNA components, so that the cause of the lower buoyant density must be either that the newly formed structures are more hydrated, or that they have a greater content of protein or other low density material. Preliminary experiments have shown that the same buoyant density differences are manifested in $CsSO_4$ density gradients where an amplification of hydration affects might be anticipated. Therefore we decided to explore how the difference in buoyant density between new and mature 40S particles is affected when preparations are submitted to various treatments designed to remove any additional components which might be complexed with the particles. Our results indicate that the difference in buoyant density is most

likely due to certain protein-containing components which are specifically associated with the new particles.

## MATERIALS AND METHODS

Suspension cultures of L cells pulsed for 30 minutes with 5 μc/ml $^{3}$H-uridine were used as the source of 40S particles. Conditions of labeling, preparation of homogenates, and fractionation of cytoplasmic particles by zonal centrifugation on sucrose gradients have been described previously (2,4). Fractions from the sucrose gradients corresponding to the 40S peak were pooled, diluted with TEA buffer (0.02 triethanolamine-HCl, pH 7.8, containing 0.05 M KCl and 0.001 M $MgCl_2$), and either recentrifuged on a second sucrose gradient or submitted to the enzymatic digestions and varied ionic conditions listed in Table I. Unless otherwise noted, enzyme incubations were in 0.5 ml volumes at 28°C for 10 minutes (trypsin and phospholipase) or for 2 minutes (ribonuclease). The reactions were terminated by lowering the temperature to 1°C and adding formaldehyde to a final concentration of 6%. After 24 hr fixation in formaldehyde at 3-5°C the preparations were rapidly dialyzed and banded on preformed CsCl density gradients as described previously (4).

Enzymes used were Worthington products: trypsin, 2x cryst.; ribonuclease A (boiled for 10 min, pH 7); and phospholipase C.

## RESULTS

In our previous experiments (4) the mature 40S particles, observed by their absorbance at 260 mμ, formed a relatively sharp major band with a buoyant density of 1.49, whereas the newly synthesized particles, observed by their content of radioactive nucleoside, formed a rather broad band about 0.04-0.05 density units lower. Now it can be seen (Fig. 1a, Table I) that the broad radioactive peak was a composite of two species, one banding about $0.03_5$ density units lower, the other about $0.05_5$ units lower.

Various treatments, e.g. rerunning of 40S particle preparation on sucrose

before the formaldehyde fixation, incubation in buffer at elevated temperatures, or incubation in ionic environments which favor dissociation of non-specifically absorbed protein, resulted in density gradient profiles in which the major radioactive peak was comparatively sharp and displaced from the absorbance peak

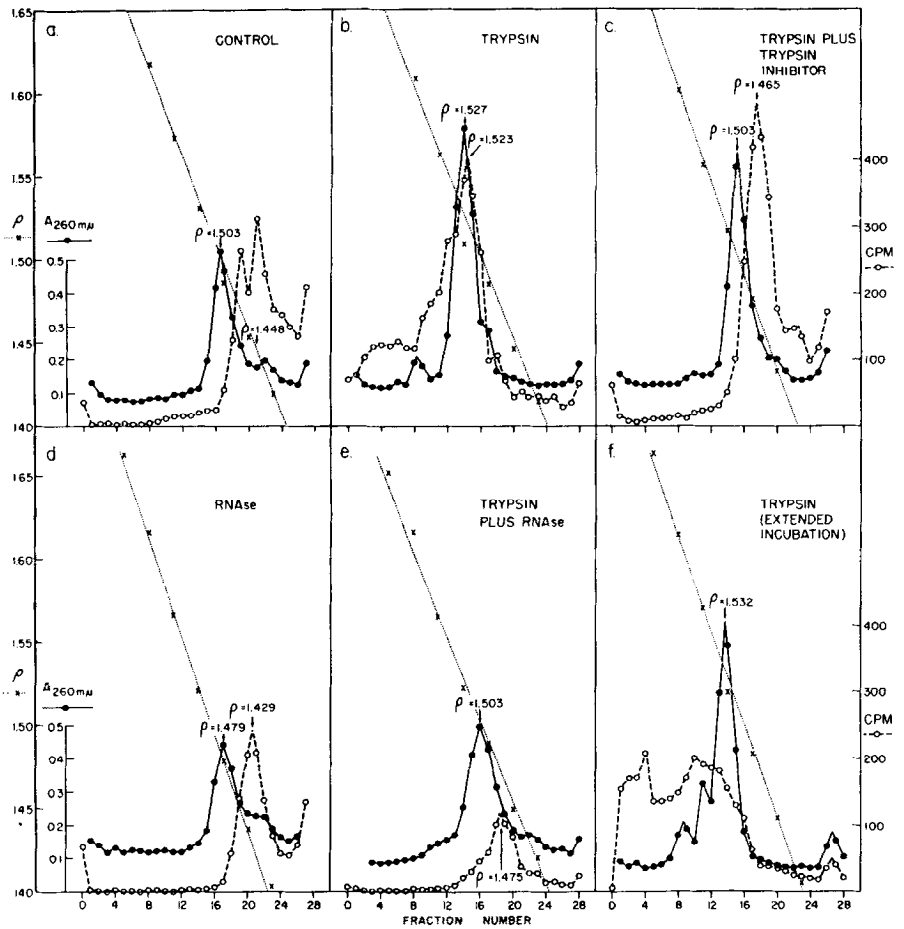


Fig. 1: A series of CsCl density gradient profiles from one preparation of 40S particles obtained from L cells pulsed for 30 min with $^{3}$H-uridine.
●: Absorbance at 260 mμ representing mature particles; 0: radioactivity representing newly formed particles (measured as material not filtrable through millipore filters, hence ribonucleoprotein is counted but not free RNA); X: density calculated from refractive index measurements. Particles incubated for 10 min at 28°C in TEA buffer with a) nothing added, b) 6 μg/ml trypsin, c) 6 μg/ml trypsin plus 12 μg/ml soybean trypsin inhibitor. d) Incubation for 2 min at 28°C with 6.6 μg/ml ribonuclease. e) Incubation as in b) followed by incubation as in d). f) Incubation with 6 μg/ml trypsin for 30 min at 28°C.

by about $0.03_5$ density units (cf., for example Fig. 1c). This suggests that the radioactive component of lower density ($\rho \simeq 1.44_5$) may be converted to the higher one ($\rho \simeq 1.46_5$) by loss of some rather easily dissociable constituent whose buoyant density is less than that of particles themselves. The $\rho = 1.46_5$ component, on the other hand, was apparently not altered by these procedures nor by incubation in β-mercaptoethanol (which should favor dissociation of S-S linkages) or in phospholipase (which could be expected to eliminate attached phospholipid).

When the preparations were incubated with trypsin a striking effect was observed (Fig. 1b, Table I). After treatment with 6 μg/ml of trypsin the radioactive and absorbance peaks were practically coincident. One tenth the concentration of trypsin produced a smaller effect, whereas a 10-fold higher concentration or prolongation of the incubation to 30 min at 28°C caused extensive deproteinization and a consequent shift to higher density, particularly of the newly synthesized particles (Fig. 1f). Trypsin also induced a certain amount of deproteinization of the mature particles, resulting in a relatively small displacement of the absorbance peaks toward higher density. These effects were absent when the incubation with trypsin was performed in the presence of a twofold excess of trypsin inhibitor (Fig. 1c).

In agreement with earlier results (2) both new and mature 40S particles were relatively insensitive to a brief incubation with ribonuclease, i.e. there was little drop in total absorbance or radioactivity (Fig. 1d). The limited amount of nuclease action which did occur (detected by a shift to lower density) appeared to be equivalent for both types of particles. On the other hand, when the ribonuclease treatment was preceded by an incubation with trypsin, then, as is seen in Fig. 1e, there was no measurable change in the density of the absorbance band - slight amounts of both RNA and protein being removed - but an appreciable reduction in the quantity of radioactive ribonucleoprotein. This suggests that the trypsin is capable of modifying the newly formed particles so as to render them more sensitive to nuclease attack than the

Table I

EFFECT OF VARIOUS TREATMENTS ON THE BUOYANT DENSITY OF NEW AND MATURE 40S PARTICLES

| Preparation of 40S particles | Treatment | $\rho_{mature}$ | $\rho_{new}$ | $\Delta\rho \times 10^3$ |
|---|---|---|---|---|
| I | none | 1.492 | 1.466 | 26 |
| | | | 1.444 | 48 |
| | none | 1.500 | 1.468 | 32 |
| | | | 1.445 | 55 |
| | Rerun on sucrose (before fixation) | 1.494 | 1.467 | 27 |
| II | Rerun on sucrose (before fixation) | 1.507 | 1.475 | 32 |
| III | 10 min 30°C | 1.500 | 1.468 | 32 |
| | " " with 0.05 M β-mercapto-ethanol | 1.506 | 1.479 | 27 |
| IV | $10^{-2}$ M $Mg^{++}$ | 1.510 | 1.473 | 37 |
| | 0.5 M NaCl, $10^{-2}$ M $Mg^{++}$ | 1.538 | 1.493 | 45 |
| | 0.5 M NaCl | No discrete bands. Material shifted to high ρ. | | |
| | 200 μg/ml phospholipase C | 1.513 | 1.475 | 38 |
| V | 10 min 28°C | 1.490 | 1.464 | 26 |
| | | | 1.437 | 53 |
| | Trypsin 0.6 μg/ml | 1.510 | 1.498 | 12 |
| | " 6 μg/ml | 1.518 | 1.515 | 3 |
| | " 60 μg/ml | 1.532 | No sharp band. $\rho_{new} > \rho_{mature}$ | |
| VI | 10 min 28°C | 1.503 | 1.468 | 35 |
| | | | 1.448 | 55 |
| | Trypsin 6 μg/ml | 1.527 | 1.523 | 4 |
| | " " + soybean trypsin inhibitor 12 μg/ml | 1.503 | 1.465 | 38 |
| | RNAse (boiled) 6.6 μg/ml | 1.479 | 1.429 | 50 |
| | Trypsin 6 μg/ml + RNAse 6.6 μg/ml | 1.503 | 1.475 | 28 |
| | Trypsin 6 μg/ml (30 min 28°C) | 1.532 | No sharp band. $\rho_{new} > \rho_{mature}$ | |

mature particles which are also treated with trypsin. This could help explain why extended treatment with trypsin also leads to a preferential degradation of the newly formed particles.

## DISCUSSION

The foregoing results suggest that the comparatively lower buoyant density of newly synthesized 40S particles is due to their association with additional protein-containing material. From the better resolved density gradient profiles it may be inferred that there are at least two separately attached components of this material, giving rise to two states of decreased buoyant density. One component is more labile than the other and is usually lost under conditions favoring dissociation of adsorbed material. The more stable component has so far been removed only by proteolytic cleavage.

Assuming that the newly synthesized particles consist of a structure with a density equivalent to the mature particles plus an additional protein component, the weight per cent of protein, f, necessary to produce a particular shift in buoyant density can be calculated from $f = 100\ \rho_p(\rho_m-\rho_n)/\rho_n(\rho_m-\rho_p)$ where $\rho_m$, $\rho_n$ and $\rho_p$ are the buoyant densities of mature particles, new particles, and pure protein, respectively. For $\rho_m = 1.49$, $\rho_n = 1.46$ and $\rho_p = 1.25$, f is 10.7%, which would be equivalent to about 140,000 daltons of protein if the molecular weight of the mature 40S particle is taken to be $1.3 \times 10^6$. If this additional protein does not significantly alter the conformation of the particle one would expect the newly formed particles to have a sedimentation coefficient about 8% greater than that of the mature ones. That this has not been detected may simply be due to insufficient resolution of the sucrose gradients. On the other hand it could mean that the new particles possess a more open configuration than the mature ones so that the increase in mass is compensated for by an increased frictional coefficient. Alternatively, the additional component might be composed of an especially low buoyant density material, for example lipoprotein, in which case it could be less than 56,000 daltons and still

cause the observed shift in buoyant density.

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