RIBOSOME PRECURSOR PARTICLES IN NUCLEOLI

MING C. LIAU and ROBERT P. PERRY

From the Institute for Cancer Research, Philadelphia, Pennsylvania 19111

ABSTRACT

Ribonucleoprotein (RNP) particles containing the precursors of ribosomal RNA were extracted from L cell nucleoli and analyzed under conditions comparable to those used in the characterization of cytoplasmic ribosomes. Using nucleoli from cells suitably labeled with ^{3}H-uridine, we detected three basic RNP components, sedimenting at approximately 62S, 78S, and 110S in sucrose gradients containing magnesium. A fourth particle, sedimenting at about 95S, appears to be a dimer of the 62S and 78S components. When centrifuged in gradients containing EDTA, the 62S, 78S, and 110S particles sediment at about 55S, 65S, and 80S, respectively. RNA was extracted from RNP particles which were prepared by two cycles of zonal centrifugation. The 62S particles yielded 32S RNA and a detectable amount of 28S RNA, the 78S structures, 32S RNA and possibly some 36S RNA, and the 110S particles, a mixture of 45S, 36S, and 32S RNA's. When cells were pulsed briefly and further incubated in the presence of actinomycin D, there was a gradual shift of radioactivity from heavier to lighter particles. This observation is consistent with the scheme of maturation: 110S → 78S → 62S. The principal buoyant densities in cesium chloride of the 110S, 78S, and 62S particles are 1.465, 1.490, and 1.545, respectively. These densities are all significantly lower than 1.570, which is characteristic of the mature large subunit of cytoplasmic ribosomes, suggesting that the precursor particles have a relatively higher ratio of protein to RNA, and that ribosome maturation involves, in addition to decrease in the size of the RNA molecules, a progressive decrease in the proportion of associated protein.

INTRODUCTION

It is now well established that nucleoli contain the cistrons which direct the transcription of ribosomal RNA (rRNA). In mammalian cells the rRNA is initially synthesized in the form of a 45S precursor, and then is subsequently converted in the nucleolus via several intermediates into mature 18S and 28S ribosomal RNA (for references see reviews by Perry, 1967, 1969). Several studies have indicated that the nucleolar "preribosomal RNA's" exist as ribonucleoprotein complexes (Tamaoki, 1966; Yoshikawa-Fukada, 1967; Warner and Soeiro, 1967; Izawa and Kawashima, 1968; Rogers, 1968). Warner and Soeiro (1967) have established that these complexes exist as discrete particles, which sediment at 80S and 55S in the presence of EDTA, and contain 45S and 32S RNA, respectively. However, since a satisfactory description of these particles has not been obtained under the variety of conditions conventionally employed in the study of cytoplasmic ribosomes, there is still some uncertainty as to the exact nature of the *in situ* nucleolar particles, and a detailed mechanism for the process of ribosome maturation cannot yet be formulated.

In the series of experiments reported here ribosome precursor particles were extracted from isolated nucleoli and fractionated in the presence of concentrations of magnesium and monovalent

ions which preserve the functional integrity of the cytoplasmic ribosomes. These nucleolar particles were compared to those obtained under dissociative conditions, and were further characterized with respect to their buoyant densities and the species of RNA which they contain. Of the three species of nucleolar particles identified, the one containing the 45S RNA component appears to possess the highest proportion of protein to RNA, thus suggesting that during ribosome maturation the reduction in RNA size is accompanied by a progressive diminution in the protein to RNA ratio.

A brief report of these experiments was presented previously (Liau and Perry, 1968).

MATERIALS AND METHODS

Buffer Designations

$TM_{1.5}$ and TM_5: 0.01 M Tris-acetate, pH 7.0, with 1.5 mM and 5 mM $MgCl_2$, respectively.

TKMD: 0.01 M Tris-HCl, pH 7.4, 0.01 M KCl, 0.5 mM $MgCl_2$, and 0.02 M dithiothreitol (Calbiochem, Los Angeles, Calif.).

RSB: 0.01 M Tris-HCl, pH 7.4, 0.01 M NaCl, 1.5 mM $MgCl_2$.

Labeling and Preparation of Nucleoli

L cells were grown in spinner cultures (Perry and Kelley, 1966 *a*) to concentrations of about 4×10^5 cell/ml. The cells were concentrated four-fold, incubated for 45 min with 1–5 μc/ml of ^{3}H-uridine (9.5–10 C/mmole), quickly chilled, sedimented and washed once with balanced salts solution. From this point, all operations were carried out at 0°–4°C, unless stated otherwise. Nucleoli were prepared as described previously (Liau et al., 1965), with a slight modification to reduce the contamination by cytoplasmic ribosomes. Approximately 1 ml of packed cells (obtained from an 800 ml culture) was homogenized with 15 strokes of a Potter-Elvehjem device in 20 volumes of TM_5 containing 0.1 M sucrose. This treatment gives almost complete breakage of the cells, although most nuclei are still surrounded by a thin rim of cytoplasm. The nuclei were sedimented at 900 *g* for 3 min, washed once with TM_5–0.1 M sucrose, suspended in 10 ml of TM_5 containing 0.2 mM $CaCl_2$, and passed through a previously chilled Aminco-French Pressure Cell (American Instrument Co., Silver Spring, Md., Cat. No. 4-3399) at 4000–5000 psi. The pressed brei was mixed with an appropriate volume of concentrated sucrose solution to give a final concentration of 0.25 M, and centrifuged at 600 *g* for 5 min. After removal of the supernatant, the sediment was packed more tightly by recentrifugation for 10 min at 900 *g*. The sediment was suspended by homogenization in 30 ml of $TM_{1.5}$ containing 2.2 M sucrose, and centrifuged at 88,700 *g* for 10 min in a Spinco No. 30 rotor, (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.). The pellet was washed once with 10 ml of $TM_{1.5}$–0.25 M sucrose, and sedimented at 900 *g* for 10 min to give the final nucleolar preparation.

Extraction and Zonal Centrifugation of Ribonucleoproteins (RNP) from Isolated Nucleoli

The nucleolar pellet was suspended by repeated pipetting in 4 ml of 0.25 M sucrose-2 mM $MgCl_2$-0.05 M KCl-0.01 M sodium acetate, pH 6.0, and its 260 mμ absorbance was determined by solubilizing an aliquot in 20 volumes of 0.3 N KOH. The nucleolar suspension was adjusted to a concentration of 3.3 A_{260} units/ml, made 40 μg/ml in polyvinylsulfate (K & K Laboratories, Inc., Plainview, N.Y.), allowed to stand in the ice bath for 10 min with occasional pipetting, and then sedimented at 20,000 *g* for 10 min in a Servall SS-34 rotor (Ivan Sorvall, Inc., Norwalk, Conn.). The gelatinous pellet was dispersed in a Dounce homogenizer (Kontes Glass Co., Vineland, N. J.) in about 3 ml of TKMD, and ribonucleoproteins were extracted by incubation at room temperature (~24°C) for 15 min. After centrifugation at 20,000 *g* for 15 min, the supernatant (nucleolar S20) was layered onto a 28-ml 15–30% (w/v) sucrose gradient prepared in 0.5 mM $MgCl_2$–0.01 M KCl-0.001 M dithiothreitol-0.01 M triethanolamine-HCl, pH 7.4. After centrifugation at 22,000 rpm for 10 hr in a SW 25 rotor, 60% sucrose was introduced into the bottom of the tubes and the gradients were displaced through a 5 mm flow cell of a Gilford Spectrophotometer (Gilford Instrument Corporation, Oberlin, Ohio). The absorbance at 260 mμ was recorded, and 1 ml fractions were collected. Aliquots were assayed for radioactivity and the remainder was used for buoyant density analysis or RNA extraction.

Preparation of Cytoplasmic Particles

The cytoplasmic particles used as markers were obtained from cells labeled with $Na_2P^{32}O_4$ for 24 hr. The cells were washed once with balanced salts solution, and ruptured by Dounce homogenization in RSB. Nuclei were removed by centrifugation at 900 *g* for 3 min. The supernatant was adjusted to 5 mM $MgCl_2$, 0.04 M KCl and 0.25 M sucrose, and centrifuged at 20,000 *g* for 15 min. The supernatant (postmitochondrial fraction) was mixed in an appropriate proportion with the nucleolar S20 and layered onto sucrose gradients as described above.

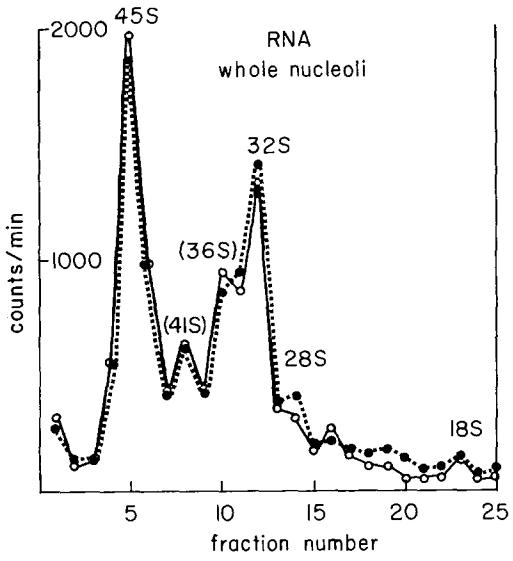

FIGURE 1 Preservation of nucleolar RNA components under the conditions of RNP extraction. Nucleoli were isolated from L cells that were labeled with ^{3}H-uridine for 45 min and divided into several portions: One sample (●-----●-----●) was washed with PVS and incubated at room temperature for 15 min in TKMD; another (○——○——○) was kept on ice. RNA was extracted from both and analyzed by electrophoresis on acrylamide gels (5 hr at 5 ma/gel). Positions of 28S and 18S RNA were obtained with marker ^{32}P–rRNA's which were included in the same gels.

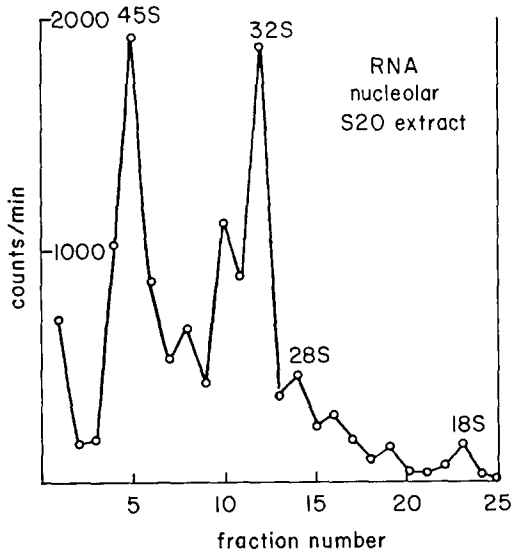

FIGURE 2 RNA constituents of the nucleolar S20. A nucleolar S20 was prepared from the same batch of nucleoli used for Fig. 1. RNA was extracted and analyzed by acrylamide gel electrophoresis as in Fig. 1.

Buoyant Density Analysis in Cesium Chloride

RNP particles in selected fractions of the sucrose gradients were fixed in 6% formaldehyde, and banded in CsCl density gradients as described previously (Perry and Kelley, 1966 *a*). Densities were determined from measurements of refractive index.

Extraction of RNA and Analysis by Gel Electrophoresis

For the extraction of RNA from isolated nucleoli or nucleolar S20, the preparations were suspended in 1–2 ml of 0.14 M NaCl-0.05 M sodium acetate, pH 5.0, made 0.5% with sodium dodecyl sulfate (SDS), and shaken at room temperature for 30 min with an equal volume of phenol. The phenol was saturated with extraction buffer, and contained 0.1% of 8-hydroxyquinoline. RNA was precipitated from the aqueous phase with two volumes of ethanol after adding 30–50 μg ribosomal RNA as carrier. Extraction of RNA from sucrose gradient fractions was performed in the same way except that the fractions were diluted with an equal volume of extraction buffer, and SDS was added to a final concentration of 1%. Gel electrophoresis of RNA was carried out according to Weinberg et al. (1967), with 0.04 M Tris-0.02 M sodium acetate-1 mM EDTA-0.2% SDS, pH 7.4, as the electrophoresis buffer.

Assay of Radioactivity

Aliquots of ribonucleoprotein particles from sucrose and CsCl gradients were trapped on millipore filters (Infante and Nemer, 1968) and washed with cold 5 mM $MgCl_2$ solution. The filters were dried and counted in a toluene phosphor solution with a liquid scintillation spectrometer. For the measurement of radioactivity from acrylamide gels, 2 mm slices were placed in counting vials, digested with 0.5 ml of conc NH_4OH for 1 hr, and counted in a toluene phosphor-methoxyethanol mixture (6:4).

RESULTS

Effect of Polyvinylsulfate (PVS) Treatment, Extraction Conditions and Labeling Time on Release of Nucleolar RNP

Nucleoli isolated by the method used here contain degradative enzymes which can quickly destroy RNA, particularly the 45S component, when the nucleoli are incubated at room temperature in TKMD (Liau et al., 1968). Fortunately this degradation is avoided when the nucleoli are washed before incubation with a medium containing PVS (Liau et al., 1968). The effectiveness of PVS in preserving the integrity of nucleolar RNA is illustrated in Fig. 1. From a comparison of acrylamide gel profiles, we see that the RNA from nucleoli washed in PVS and incubated for 15 min at room temperature (the conditions used for extraction of RNP) is not detectably different from the RNA of unincubated nucleoli.

Nucleoli appear to retain their gross structure when suspended in PVS medium, although after centrifugation at 20,000 *g* they form an aggregated gelatinous pellet. Nevertheless, the PVS washing actually facilitates the subsequent extraction of nucleolar RNP. As shown in Table I, the release of RNP from untreated nucleoli is considerably less than from nucleoli treated with PVS. There is a correlation between the amount of PVS used and the amount of RNP extracted. However, with amounts of PVS in excess of 20 μg per A_{260} unit of nucleoli, the released RNP was considerably deproteinized, as evidenced by the pelleting of a considerable proportion of the material when analyzed on CsCl gradients. In all experiments to be subsequently described, the nucleoli were exposed to 12 μg per A_{260} unit. Under these conditions there is no detectable deproteinization of the RNP particles, nor is there any significant release of particles from the nucleoli prior to incubation in TKMD.

TABLE I

Effect of PVS Treatment and Temperature of Extraction on Amount of RNP Released from Nucleoli

Treatment	Temperature of TKMD extraction medium	Per cent radioactivity extracted
None	0°	5
PVS 40 μg/ml	0°	25
None	24°	44
PVS 25 μg/ml	24°	43
40 μg/ml	24°	65–75
100 μg/ml	24°	75–85

Nucleoli were isolated from cells which had been labeled for 45 min with ^{3}H-uridine, and divided into six portions, each containing 3.3 A_{260} units/ml of nucleoli. Each portion was given the treatment indicated, and sedimented at 20,000 *g* for 10 min. The pellets were then extracted in TKMD for 15 min at 0° or room temperature (24° C), and centrifuged at 20,000 *g* for 15 min. The acid-precipitable radioactivity in the supernatants was determined and compared with that contained in the nucleoli before extraction. PVS treatment alone, without subsequent extraction in TKMD, caused less than 5% of the radioactivity to be released from the nucleoli.

The facilitation of RNP release by PVS washing can be mimicked to some extent by exposure of nucleoli to relatively high concentrations of monovalent salt, e.g. 0.5 M NaCl. In preliminary experiments we found that the amount of ribonucleoprotein released after incubation of nucleoli in an EDTA-containing buffer (Warner and Soeiro, 1967) was increased more than three-fold when the nucleoli were previously washed in HSB (0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris-HCl, pH 7.4).

After incubation in TKMD the nucleolar structure, as viewed microscopically, is considerably dispersed. If dithiothreitol is omitted from the extraction medium, the nucleoli remain intact, and little RNA-containing material appears to be released. It is clear from the data of Table I that the extraction is much more effective at room temperature than at 0°C. At room temperature some RNP is extracted, even from nucleoli not washed with PVS, although, as mentioned previously, the bulk of this material contains partly degraded RNA.

The types of labeled RNA in a nucleolar S20 extract from cells incubated with uridine for 45

TABLE II

Extractability of Nucleolar RNP at Different Stages of Maturation

Duration of labeling of nucleolar RNP with ^{3}H-uridine	Per cent radioactivity extracted
15 min	44
45 min	70
75 min	85

Nucleoli were isolated from cells which were labeled as indicated, washed with 40 μg/ml PVS (12 μg/A_{260} unit), and extracted in TKMD for 15 min at room temperature. The per cent radioactivity extracted was measured as in Table I.

min are shown in Fig. 2. The proportion of labeled 45S component in the nucleolar S20 is reduced as compared to the overall nucleolar RNA (Fig. 1), suggesting that some of the 45S component is not extractable as RNP. This was verified by the finding that the RNA in the residue that is pelleted at 20,000 *g* after RNP extraction consists predominantly of the 45S component and only a small amount of 32S RNA. These observations may be correlated with other data (Table II) which show the proportion of total radioactive RNA that is extracted into the S20 from nucleoli labeled for different durations of time. For example, from nucleoli of cells pulsed for 15 min 44% of the radioactivity is released, whereas 85% is released from nucleoli of cells labeled for 75 min. After a 15-min pulse the 45S component is the major labeled species; after 75 min most of the nucleolar label is in the 32S component. It thus seems likely that the most newly formed 45S RNA is not in a state which renders it extractable as RNP, but that extractability increases as maturation proceeds.

Sedimentation of Nucleolar RNP

When the nucleolar S20 is sedimented through a sucrose gradient containing magnesium ion, profiles such as those shown in Fig. 3 are obtained. The most abundant particle, seen by its A_{260} absorbance, sediments at about 62S. After 45 min of labeling, this particle is well labeled, as are three other particles whose approximate sedimentation coefficients are 78S, 95S, and 110S. The sedimentation coefficients were estimated, by assuming proportionality between sedimentation velocity and distance sedimented, and by using as markers ^{32}P-labeled cytoplasmic monoribosomes (74S) and subunits (60S and 40S) which were sedimented in the same gradient.

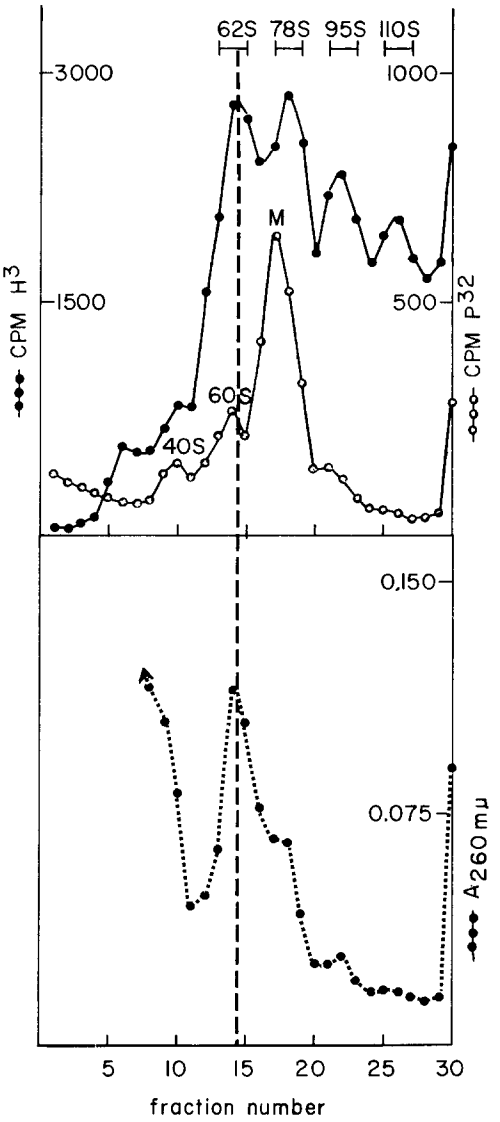

FIGURE 3 Sedimentation profile of nucleolar RNP in a sucrose density gradient containing magnesium ion. A nucleolar S20, prepared from cells labeled with ^{3}H-uridine for 45 min, was mixed with ^{32}P cytoplasmic ribosome marker (postmitochondrial fraction) and centrifuged in a sucrose gradient containing 0.5 mM Mg^{++} as described in Materials and Methods. For the recording of absorbance at 260 mμ a duplicate sample of nucleolar S20 was run in a parallel gradient without cytoplasmic marker. The horizontal bars in the upper panel indicate the fractions selected for Figs. 4 and 5.

About 15–20% of the radioactive material was pelleted under these conditions. Analysis of the RNA of this pellet indicated that it contained the same species as those found in the 110S particles,

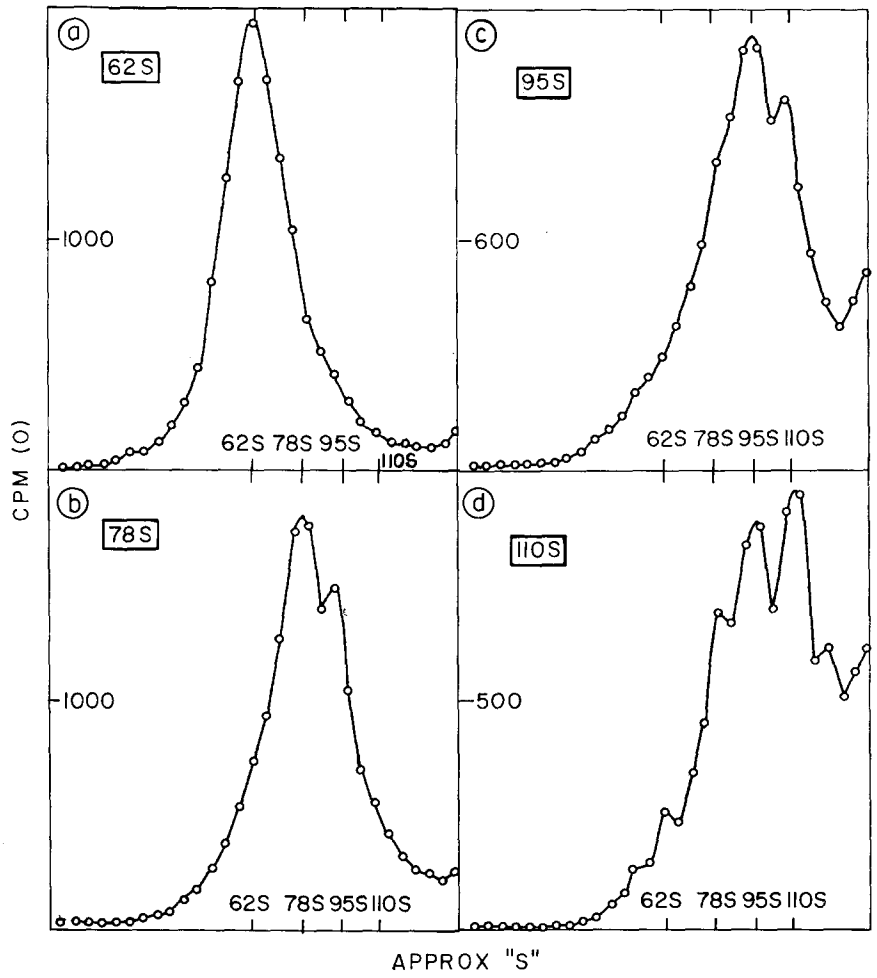

FIGURE 4 Resedimentation of nucleolar particles in sucrose gradients containing magnesium ion. Three 1 ml fractions from the peaks shown in Fig. 3 were pooled and diluted with an equal volume of gradient buffer. Half of each sample was centrifuged through a sucrose gradient under conditions identical to those of Fig. 3. The equivalent positions of the various peaks in Fig. 3 are shown on the abcissa. Panels *a*, *b*, *c*, and *d* show re-runs of the 62S particles, 78S particles, 95S particles, and 110S particles, respectively.

thus suggesting that some of the 110S particles are in the form of larger aggregates. The tendency of preribosomal particles to aggregate in larger clusters has also been noted by others (Vesco and Penman, 1968).

For better resolution of the labeled nucleolar particles we resedimented selected fractions on a second series of sucrose gradients (Fig. 4). Each particle sedimented to its proper position in the gradient, although, except for the 62S particle, all were contaminated with their neighboring components. In particular, the fractions containing 110S particles were grossly contaminated by other particles.

To investigate whether any of the peaks observed in these gradients might be due to simpler structures which are complexed in the presence of magnesium ion we resedimented a portion of the same samples used for Fig. 4 into sucrose gradients containing EDTA. Under these conditions, in which cytoplasmic monoribosomes dissociate into 30S and 50S subunits, the nucleolar 62S particles sediment at 55S, the 78S particles at 65S, 95S particles at 55S and 65S, and the 110S particles at 80S (Fig. 5). In each sample the presence of particles other than those mentioned above can be attributed to the contaminating components normally found in a particular cut of the primary sucrose gradient. For example, the minor peak of 55S particles found in the 78S cut could be con-

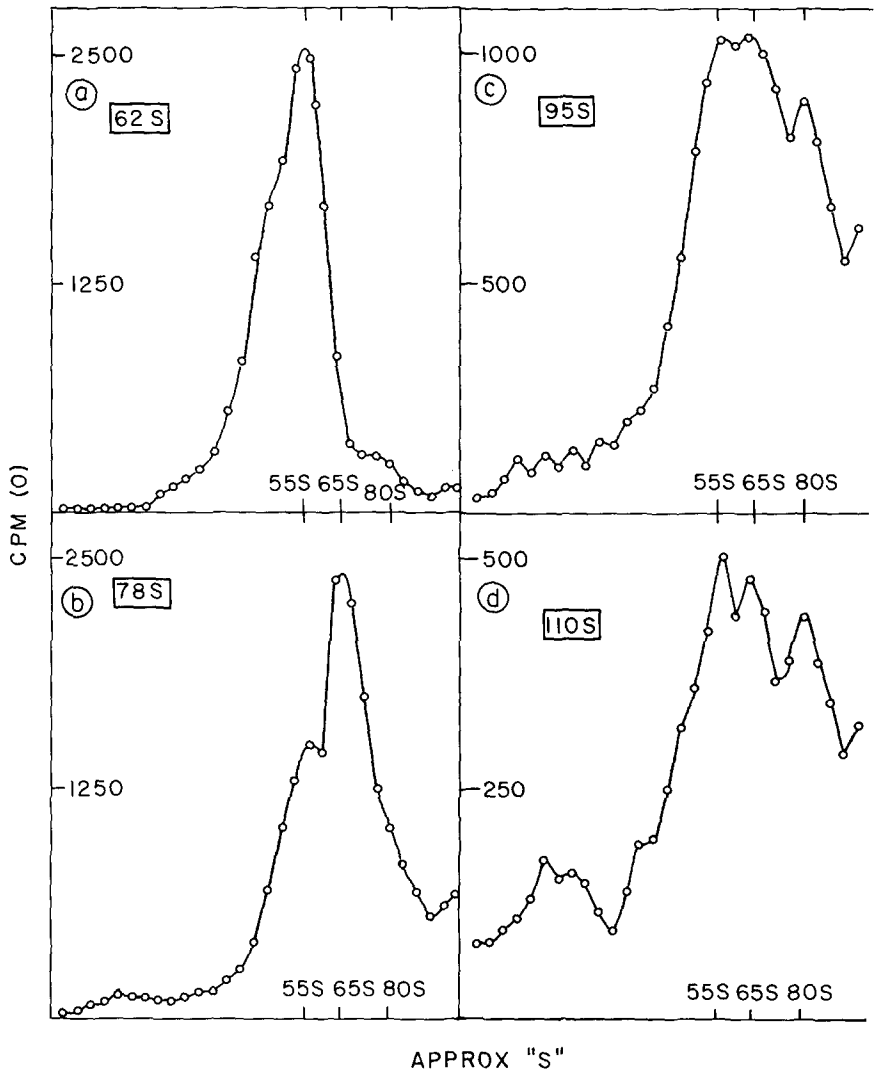

FIGURE 5 Resedimentation of nucleolar particles in sucrose gradients containing EDTA. The other halves of the samples used for Fig. 4 were centrifuged for 17 hr at 22,000 rpm through 15–30% sucrose gradients made up in a buffer consisting of 0.01 M EDTA, 0.01 M KCl, 0.001 M dithiothreitol, and 0.01 M Tris, pH 7.4. The approximate sedimentation coefficients shown on the abcissa were determined with a marker of ^{32}P-labeled monoribosomes which dissociated into 50S and 30S subunits in this gradient (not shown). Panels *a*, *b*, *c*, and *d* show reruns of 62S, 78S, 95S, and 110S particles, respectively.

tributed either by the neighboring 62S or 95S particles. Similarly, the presence of 80S particles in the 95S cut and the presence of both 55S and 65S particles in the 110S cut can be accounted for by cross contamination.

From these data and those of Fig. 4 it can be concluded that there are three basic ribonucleoprotein structures in nucleoli. These sediment at 62S, 78S, and 110S in sucrose gradients containing magnesium ion, or at 55S, 65S, and 80S, respectively, in sucrose gradients containing EDTA. The 95S particles do not yield unique derivatives in EDTA, and thus probably represent dimers composed of 62S and 78S particles.

RNA Composition and Buoyant Density of Nucleolar RNP

For characterization of RNA constituents and determination of buoyant densities, nucleolar particles were purified by two cycles of gradient centrifugation. Half of each sample of purified particles was used for the extraction of RNA, and

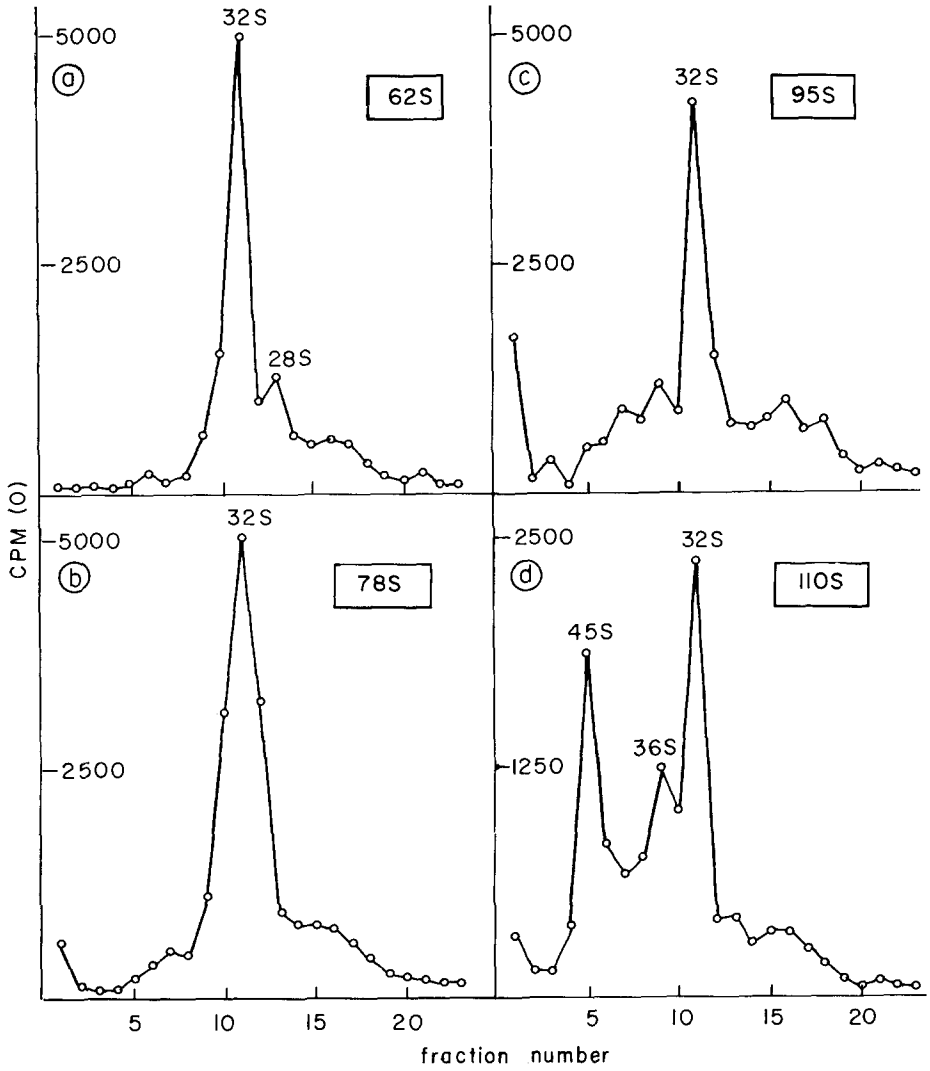

FIGURE 6 The RNA constituents of the various nucleolar particles. Nucleolar RNP's were purified by two cycles of zonal centrifugation as in Figs. 3 and 4. RNA was extracted from selected fractions and analyzed by electrophoresis on acrylamide gels (5 ma for 5 hr). Panels *a*, *b*, *c*, and *d* illustrate gel patterns of RNA from 62S, 78S, 95S, and 110S particles, respectively. The peak designations were determined from the mobilities of ^{32}P rRNA markers as in Fig. 1.

the remainder was fixed in formaldehyde and banded in CsCl. The RNA, analyzed by acrylamide gel electrophoresis, is shown in Fig. 6. The 62S fraction contains 32S RNA and a small, but reproducible amount of 28S RNA. The RNA of the 78S fraction forms a somewhat broader band and may consist of a mixture of 36S and 32S RNA. The 95S particles contain predominantly 32S RNA. The 110S particles yield a mixture of 45S, 36S, and 32S RNA's, and possibly some 41S RNA which could be concealed under the trough between the 45S and 36S peaks. The overall recovery of 45S RNA from the RNP particles was rather poor when compared to the amount present in the S20 extract, suggesting that some of this highly labile RNA component was probably degraded during isolation of the particles.

The buoyant density profiles of the four nucleolar ribonucleoproteins are illustrated in Fig. 7. The 62S particles exhibit a principal sharp band at $\rho = 1.545$. This is 0.025 g/cm^3 less than the band ($\rho = 1.570$) of mature cytoplasmic 60S

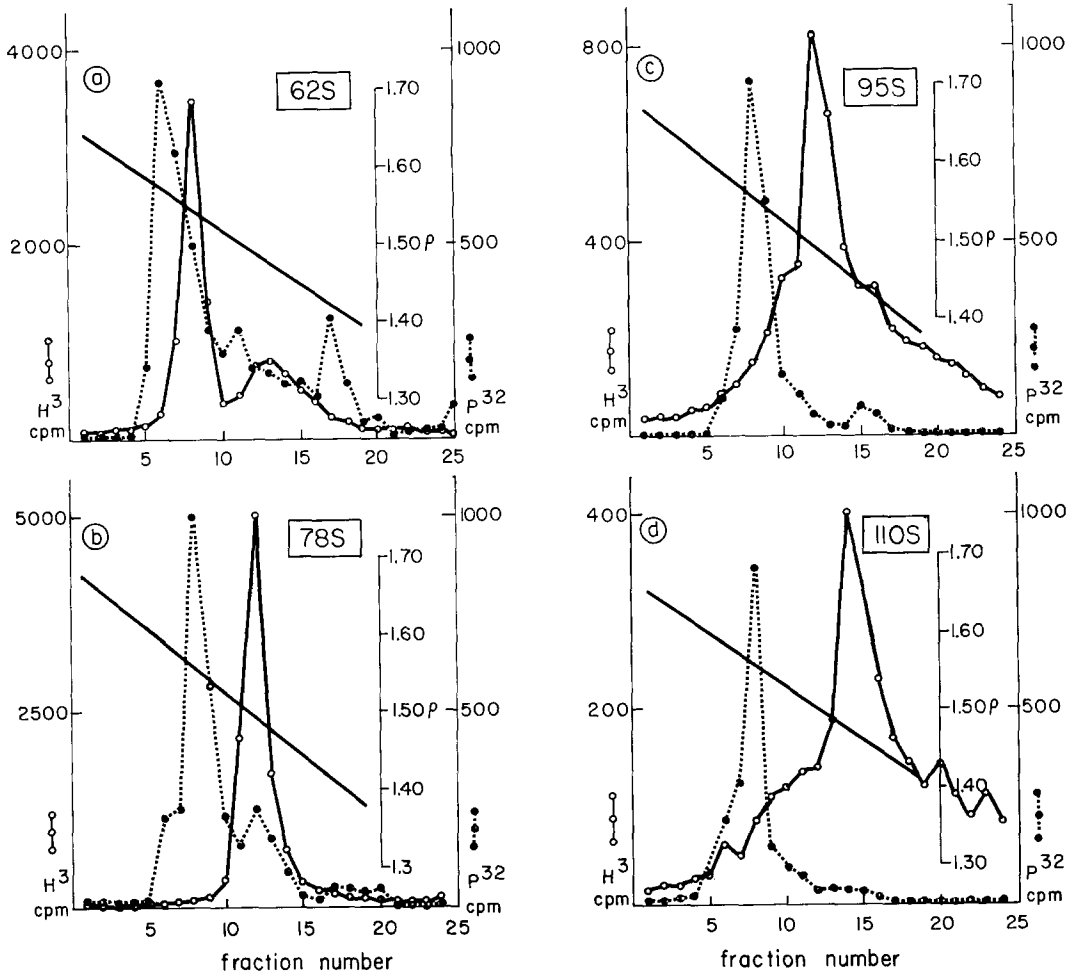

FIGURE 7 Buoyant densities of nucleolar particles. Nucleolar RNP's were purified by two cycles of zonal centrifugation as in Figs. 3 and 4. Pooled fractions of the designated particle were fixed in formaldehyde for 24 hr, and mixed with a sample of fixed, ^{32}P-labeled 60S cytoplasmic subunits *a* or 74S monosomes *b-d*. After dialysis against buffer to remove sucrose and formaldehyde, the particles were centrifuged in preformed CsCl density gradients at 35,000 rpm (SW 50 rotor) for 15 hr.

particles which were sedimented in the same gradient. The 78S particles form a single sharp band at $\rho = 1.490$. The 95S and 110S particles form principal bands at $\rho = 1.500$ and $\rho = 1.465$, respectively, although both profiles exhibit rather high and broad backgrounds, indicating a considerable amount of heterogeneity in these preparations.

These data clearly show that $\rho_{110S} < \rho_{78S} < \rho_{62S}$. The same relationship was found to hold for the EDTA derivatives of these particles, i.e. $\rho_{80S} < \rho_{65S} < \rho_{55S}$.

Kinetic Studies

The labeling sequence of nucleolar particles was studied in cells given a 15-min pulse of uridine and chased for various times in the presence of actinomycin D and cold uridine. The proportion of total nucleolar label in each particle was measured, and the results are summarized in Table III, together with comparable data for the RNA constituents. After a 15-min pulse the highest proportion of label is in the 110S particles. As the chase proceeds there is an increased proportion of label in the slower

TABLE III

Distribution of Nucleolar Radioactivity in a Pulse-chase Experiment

Duration of chase	RNP particles			RNA		
	110S	78S	62S	45S	41-36S	32S
min						
0	45	39	16	57	21	21
15	29	45	26	35	31	34
30	17	31	52	19	18	63
60	10	31	59			

Cells labeled for 15 min with ^{3}H-uridine and chased for designated periods with medium containing 0.5 mg/ml unlabeled uridine and 2 μg/ml actinomycin D. Nucleolar RNP particles were analyzed as in Fig. 3, RNA as in Fig. 6. Values represent the percentage of radioactivity in the various components.

sedimenting species, first in the 78S particles and later in the 62S particles. The 62S component clearly represents the most mature stage of ribosome precursor still associated with the nucleolus.

DISCUSSION

Extractability of Nucleolar RNP

The treatment of isolated nucleoli with appropriate amounts of PVS is a crucial part of our procedure for RNP extraction. The PVS serves two purposes: it prevents degradation of nucleolar RNA, presumably by eliminating nuclease activity, and it facilitates the release of RNP. The ability of PVS to inhibit ribonuclease is generally assumed to be related to its ability to bind basic proteins; thus it is maximally effective at a slightly acidic pH where the basic groups carry a positive charge. The choice of a pH 6 buffer for PVS treatment was prompted by this fact (cf. Philipson and Kaufman, 1964), plus a further observation that 0.1 mg/ml PVS in a pH 7.8 buffer causes dissociation of cytoplasmic monoribosomes, whereas the same concentration of PVS in a pH 6 buffer does not induce dissociation. It should also be remarked that in previous studies by others (Vanyushin and Dunn, 1967; Clark et al., 1964), dissociation of ribosomes by PVS was obtained with concentrations in the range of 0.4–10 mg/ml and with relatively pure preparations of ribosomes. The conditions of PVS treatment used in the present study (0.04 mg/ml, pH 6.0) were thus judged to be appropriate for preserving cytoplasmic ribosomes in the intact state.

The effect of PVS in facilitating the release of nucleolar RNP may be explained if one assumes that the release of discrete particles requires the removal of certain nucleolar components, perhaps some basic proteins as suggested previously by Tamaoki and Mueller (1965). The removal of these components can apparently be accomplished either by treatment with polyanions (Tamaoki, 1966; Yoshikawa-Fukada, 1967; Izawa and Kawashima, 1968; Rogers, 1968) or by treatment with high concentrations of salt (Warner and Soeiro, 1967).

The extraction in dithiothreitol medium may effect a disruption of disulfide linkages which maintain the nucleolar RNP in a continuous structure. The fact that the release of RNP was considerably greater at room temperature than at O°C leads one to suspect that disulfide reduction may be a necessary step of the extraction process. These proposals are consistent with ultrastructural studies of intact nucleoli (Hay, 1968; Miller and Beatty, in press) which indicate that the nucleolar RNP granules exist as part of a continuous structure.

Characteristics of Nucleolar RNP Particles

Using nucleoli labeled for 45 min with ^{3}H-uridine we have detected four types of particle, sedimenting at approximately 62S, 78S, 95S, and 110S in sucrose gradients containing magnesium ion. The 95S particles are apparently dimers of 62S and 78S particles, judging from the fact that they yield the same derivatives, i.e. 55S and 65S particles, when exposed to EDTA, and that they do not contain a distinctive RNA consituent. Ignoring the 95S particle, which may be an artifact created during the experimental procedure, we may consider that there are three basic RNP's in the nucleolus: 62S, 78S, and 110S particles. The 62S particle is the most abundant species, and is easily detectable by its absorbance at 260 mμ. Since the particles seem to bear a precursor-product relationship to each other *vide infra*, their relative abundance can be considered to be related to their relative metabolic lifetimes.

The sedimentation coefficients of the nucleolar particles are diminished when they are sedimented through gradients containing EDTA. The 55S and 80S derivatives of the 62S and 110S structures are comparable to those described by Warner and Soeiro (1967) for HeLa cell nucleoli. These

TABLE IV

Properties of Nucleolar Particles—Comparison with Ribosomal Subunits

Source	Particle: Native	Particle: EDTA derivative	Weight % protein (calculated from ρ values)*		RNA constituents
Nucleolus	110S	80S	57.0 (Principle peak)		45S; 41S(?); 36S; 32S
	78S	65S	53.0		36S(?); 32S
	62S	55S	44.2		32S; 28S
			New	Mature	
Cytoplasm‡	60S	50S	43.4	40.4	28S
	40S	33S	58.3	53.0	18S

* Calculated from wt % protein = $100\ \frac{\rho_p(\rho_n - \rho)}{\rho(\rho_n - \rho_p)}$ where ρ is the measured buoyant density and ρ_n and ρ_p are the buoyant densities of RNA and protein, taken to be 1.900 and 1.250 respectively.

‡ Data from Perry and Kelley, 1968.

authors did not report on the 65S species which we find to be a derivative of the 78S particle. A possible reason for this discrepancy is the lability of the 78S structures in the presence of the relatively high salt concentrations used in their nucleolar isolation procedure. Indeed, we noted that if nucleolar S20 extracts were made 0.5 M in KCl before layering on gradients containing EDTA, then the 55S and 65S peaks were merged into a single band of somewhat broader width.

In Table IV the properties of these particles are summarized and compared with those of the cytoplasmic ribosomal subunits. It is rather surprising that more than one species of RNA can be found in what appears to be one class of RNP particle. In some instances this might be attributable to residual cross contamination which persists even after two cycles of zonal centrifugation. For example, the 32S RNA derived from the 110S preparations may actually originate from contaminating 95S particles. However, it is difficult to account for the pluralities of other RNA constituents on this basis, and one is led to consider the following. (1) Unique RNP's may exist for each species of RNA, but they may not all be resolvable with our methods of analysis. (2) Some of the RNA within a particle may be processed before the particle is converted to a recognizably different form. (3) During the isolation of particles some of the RNA precursors may be converted to species resembling their natural products (Liau et al., 1968). From currently available data we cannot make an unequivocal choice among these possibilities.

From the results of isopycnic banding in CsCl it seems clear that each of the basic nucleolar particles has a distinctive buoyant density, which on the basis of earlier studies with cytoplasmic particles (Perry and Kelley, 1966 *a*; 1966 *b*), may be assumed to reflect primarily variations in protein to RNA ratios. From the buoyant density values one can calculate that the 110S, 78S, and 62S particles have protein contents of 57, 53, and 44 per cent, respectively. The buoyant density of the 62S particle is similar to that reported earlier (Perry, 1967) for newly synthesized 60S ribosomal subunits which have just entered the cytoplasm. However, the 60S particles contain 28S rRNA, whereas the majority of 62S particles in the nucleolus contain 32S rRNA.

Ribosome Maturation in Nucleoli

The kinetic data presented in Table III are consistent with the hypothesis that early maturation of the large ribosomal subunit proceeds in the nucleolus according to the sequence: 110S → 78S → 62S. We have not detected any particles other than the 110S structure which would appear to be precursors of the small subunit. Thus, as was inferred from studies of the nucleolar RNA components (Perry, 1965; Penman et al., 1966), we must conclude that the particle destined to become the small subunit is released from the nucleolus at an early stage in the processing sequence, and that the high relative abundance of the 62S particle reflects a relatively rapid formation and slow release.

The earlier the stage of the precursor particle, the higher is its protein to RNA ratio. This relationship holds as well for the EDTA derivatives as

for the native particles, and would appear to suggest that during ribosome maturation the reductions in size of the RNA components are accompanied by proportionately greater losses in their associated protein. Such changes in protein binding capacity would be expected if, as maturation continues, the rRNA precursors assumed progressively more compact conformations.

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