Centrifugation of Nuclear Ribonucleoprotein Particles of Sea Urchin Embryos in Cesium Sulfate†

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ABSTRACT: The hatching blastulae of sea urchin embryos synthesize predominantly heterogeneous nuclear RNA (HnRNA). Methods were developed for the isolation of nuclear ribonucleoprotein particles containing HnRNA that prevent adventitious binding of protein and nucleic acid. The entire nucleus is dispersed in 0.5 M NaCl containing 0.5% deoxycholate, and may then be banded in a Cs_2SO_4 density gradient without fixation or sedimented through sucrose gradients containing 0.5 M NaCl. More than 90% of the HnRNA, regardless of the time of labeling with ribonucleosides, exists in the form of aggregates composed of protein–RNA. (4–5:1). The density of the ribonucleoprotein is not due to adventitious binding of protein, to association with chromatin, nor presence of phospholipid. The particles sediment rapidly in sucrose gradients at these ionic strengths, over one-half of them exceeding 500 S. The chromatin is extensively deproteinized under these conditions, although a small portion (10–15%) retains protein and bands in Cs_2SO_4 in the same region as ribonucleoprotein. The chromatin which is resistant to deproteinization is apparently not enriched for the actively transcribed portion of the genome.

An understanding of the types of structures that contain non-rRNA in the nucleus will affect our views of how HnRNA[1] is processed in the nucleus, and how portions of it are transported to the cytoplasm, as well as bear on models for the function of HnRNA. Experiments by several groups of investigators (Samarina *et al.*, 1968; Faiferman *et al.*, 1970) using nuclei from a variety of cell types support the idea that HnRNA is associated with protein rich aggregates. The ribonucleoprotein aggregates found in the nucleus, sometimes called "informosomes," are very sensitive to degradation by nucleases. They are usually extracted from the nuclei of cells previously treated with actinomycin, which reduces rRNA synthesis although it is not known if the drug affects RNP structure. The dilute buffers used for extraction allow adventitious association of RNA and protein (Baltimore and Huang, 1970). Furthermore, it is not known whether chromatin and/or the inner nuclear envelope is associated with the ribonucleoprotein aggregates, nor have the aggregates been quantitatively recovered from nuclei.

The sea urchin embryo blastula is favorable material for reinvestigation of this problem. Nuclei and cytoplasm of considerable purity may be easily obtained (Hinegardner, 1962; Aronson and Wilt, 1969), the differential rate of accumulation of rRNA is so low it is very difficult to detect (Emerson and Humphreys, 1970), and HnRNA present in large nucleoprotein aggregates has already been demonstrated in this material (Aronson *et al.*, 1972). In the present experiments ribonucleoprotein complexes have been quantitatively extracted from nuclei and characterized in 0.5 M NaCl, which prevents adventitious binding of protein and RNA, and the buoyant density of the aggregates determined in Cs_2SO_4 without fixation. We conclude that *all* the HnRNA exists in nucleoprotein aggregates, containing about protein–RNA (4–5:1). Chromatin and membrane phospholipid are not an integral part of the complex as isolated.

Materials and Methods

Obtaining Embryos and Nuclei. Specimens of *Strongylocentrotus purpuratus* were collected locally, while those of *Lytechinus pictus* were purchased from Pacific Biomarine, Venice, Calif. All the experiments reported were conducted with embryos of both species, and no species differences were noticed. Gametes were obtained and embryos raised to the hatching blastula stage (about 18-hr postfertilization at 15°) by conventional methods (Kijima and Wilt, 1969).

Nuclei were prepared by the method of Hinegardner (1962) as modified by Aronson *et al.* (1972). The nuclei obtained from a step sucrose gradient were suspended in 1 M dextrose to which was added 0.15 volume of a mixture of Tween-40–DOC (2:1) (Penman *et al.*, 1968). The nuclei were then sedimented at 15,000*g* for 12 min. The detergent washed nuclei were dispersed by suspending them in 1 M dextrose containing 1% DOC–0.5% Lubrol W (Rohm & Haas). An equal volume of 1 M NaCl–0.025 M magnesium acetate–0.02 M Tris (pH 7.8) (double strength HS buffer) was added and the mixture was homogenized with five strokes in a tight-fitting Dounce homogenizer. The nuclei should be suspended in at least 10 ml of HS buffer (containing dextrose and detergents) for every 7×10^8 nuclei (= 1.15 mg of DNA).

Gradients. Stock 60% (w/v) solutions of reagent grade sucrose (Merck) were purified by autoclaving in the presence of 0.1 mg/ml of bentonite and 0.1 mg/ml of activated Norit A, followed by passage through a 0.45 μ Millipore filter.

Cesium sulfate was obtained from Mann/Schwarz, The Henley Co. or American Potash. Regardless of the stated purity, it was necessary to further purify the cesium sulfate. The technical grade material was recrystallized according to the directions of Szybalski (1968). Optical grade or recrystallized technical grade material was autoclaved in the presence of 0.1 mg/ml each of activated Norit A and bentonite, followed by passage through a 0.45 μ Millipore filter. If this precaution was not followed, recovery of radioactive material from gradients was often less than 80% of the input radioactivity.

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[1] Abbreviations used are: HnRNA, heterogeneous nuclear RNA; DOC, sodium deoxycholate; RNP, ribonucleoprotein; HS buffer, 0.5 M NaCl–0.013 M magnesium acetate–0.01 M Tris (pH 7.8).

Gradients were preformed from solutions of cesium sulfate in 0.01 M NaCl–0.015 M magnesium acetate–0.01 M Tris (pH 7.8)–0.01% Triton-X-100. The density of the heavy solution was 1.65 g/cm^3 and of the light solution was 1.25 gm/cm^3. One milliliter of dispersed nuclei (in HS buffer) was laid over a 4-ml linear cesium sulfate gradient and centrifuged for 16 hr at 39,000 rpm in the Spinco SW 39 rotor; the density gradient was determined by refractometry of aliquots (Szybalski, 1968). Gradients were fractionated by dripping through a needle inserted into the bottom of the tube, and pellets were always recovered from the bottom of the centrifuge tubes and shown on the figures as fraction "0." The top 0.9 ml of the contents of the centrifuge tube was collected as a single fraction and is shown on the figures as fraction "23."

Linear chloral hydrate gradients were constructed from a heavy solution (48 g of chloral hydrate; 8.9 g of sucrose; 10 ml of 0.5 M NaCl and 0.025 M magnesium acetate; 4 ml of 3.5 M Tris base; density about 1.42 g/cm^3, pH 7.8) and a light solution (23 g of chloral hydrate; 4.14 g of sucrose; 13 ml of 0.5 M NaCl and 0.025 M magnesium acetate; 2 ml of 3.5 M Tris base; density about 1.32 g/cm^3, pH 7.8). Since chloral hydrate reacts with the Lucite gradient maker, the density gradient was preformed by pipetting mixtures of light and heavy solutions in ten "steps," and the gradients were aged overnight at 4°. In all other respects the gradients were processed like Cs_2SO_4 gradients. We appreciate the advice of Drs. Hossainy, Zweidler, and Bloch with regard to the details of chloral hydrate centrifugation.

Radioactivity Determination. [5-^{3}H]Uridine, which is converted to deoxycytidylate by these cells, is an efficient precursor of DNA. Hence, aliquots of samples were hydrolyzed in 0.5 N NaOH at 67° for 1 hr prior to acid precipitation to correct for the incorporation of ^{3}H into alkaline stable material. This correction has been applied to all data presented in the figures. Samples were precipitated with cold 10% trichloroacetic acid, collected on glass-fiber filters (Reeve Angel type AH3), washed with 7% trichloroacetic acid, followed by methanol, and air-dried. In experiments involving incorporation of choline, one set of filters was washed only with trichloroacetic acid, and a duplicate set with acid followed by $CHCl_3$–methanol (3:1). Dry filters were covered with 5 ml of a toluene-based fluor containing 2% of the commercial solubilizer NCS (Amersham-Searle). All radioactivity was determined in a Nuclear-Chicago scintillation counter with appropriate corrections made for isotope contamination in different channels and correction for quenching by the channels ratio method.

Analytical Methods. RNA was determined by the modification of the Schmidt–Thannhauser technique (Munro and Fleck, 1962), protein by the Folin reaction (Lowry *et al.*, 1951) using bovine serum albumen as a standard, and DNA by the indole reaction (Ceriotti, 1952) using calf thymus DNA (Worthington) as a standard. RNA was prepared by a conventional phenol–sodium dodecyl sulfate method, as reported previously (Aronson *et al.*, 1972). Chromatin was prepared by the method of Shaw and Huang (1970). Sedimentation constants were estimated with an accuracy of ±10% by the method of McEwen (1967).

Radioactive Labels. [5-^{3}H]Uridine (20 Ci/mol) and [methyl-^{3}H]choline (1.8 Ci/mmol) were obtained from New England Nuclear. [2-^{14}C]Thymidine (7.3 Ci/mol) and [*methyl*-^{3}H]thymidine (6 Ci/mmol) were obtained from Schwarz. DNA of embryos was labeled by culturing them in the presence of 0.04 μCi/ml of [^{14}C]thymidine; the label is fully withdrawn from the sea water and incorporated into DNA by the time the seventh division, some 6–10 hr before the hatching blastula stage. Pulse labeling of RNA with uridine was accomplished by adding 1–5 μCi/ml of isotope to the sea water.

TABLE I: Chemical Composition of Nuclei.

	Partially Purified	Detergent Washed
DNA	1	1
RNA	0.60	0.14
Protein	2.15	1.12

[a] DNA, RNA, and protein of nuclei of blastulae of *Lytechinus pictus* were determined and their weight ratios are expressed relative to DNA. Partially purified nuclei are prepared by the method of Hinegardner (1962) and detergent washing involves treatment with DOC and Tween 40.

Results

Sedimentation of Nuclear RNP in Cs_2SO_4. Many of the experiments involve isolation of nuclei from embryos in which the DNA has been prelabeled with thymidine, which serves both as a marker for DNA and for calculation of recoveries; shortly before nuclear isolation the RNA is pulse labeled with ribonucleosides. The partially purified nuclei obtained from a step sucrose gradient possess both nuclear membranes with some cytoplasmic tags, and the membranes and cytoplasmic adnexa are removed by using the detergent mixture recommended by Penman (*cf.* Aronson *et al.*, 1972). Table I presents typical data on the composition of the partially purified and detergent washed nuclei. The composition of the detergent washed nuclei does not differ very much from that of chromatin prepared by conventional low ionic strength procedures (Marushige and Ozaki, 1967), except for a slightly higher RNA content. About 90% of the RNA pulse labeled with uridine for 1 hr is still retained in the preparation of detergent-washed nuclei (Aronson *et al.*, 1972).

It is useful to disperse the entire nucleus to quantitatively examine its physical properties. A systematic examination of different concentration of NaCl and detergent showed that only a narrow range of concentrations effectively dispersed the nuclei without extensively deproteinizing the RNA. We have employed 0.5 M NaCl–0.013 M magnesium acetate–0.01 M Tris (pH 7.8) containing 0.5% DOC and 0.25% Lubrol W to disperse the nuclei.

The buoyant density distribution of nuclear RNP was determined. Nuclei were obtained from embryos pulse labeled for 1.5 min, washed in detergent, dispersed in HS buffer with detergent, and banded without fixation in Cs_2SO_4 (Figure 1a). When preformed gradients were used centrifugation times from 5 to 48 hr gave the same results (*cf.* Baltimore and Huang, 1968); 80% of the labeled DNA is found in a sharp peak at about 1.37 g/cm^3. If only DNA and protein contribute to the density of the material, then the peak is composed to 80% DNA and 20% protein (Table II) and probably represents partially deproteinized chromatin. Another 20% of the DNA is found in a broad peak with an average density of about 1.27 g/cm^3, and is presumably chromatin composed of protein–DNA (*ca.* 1:1).

The labeled RNA is primarily located in the region of the density gradient from 1.25 to 1.31 g per cm^3, but about 10% of the labeled RNA is spread throughout denser portion of the gradient, more dense than pure DNA at 1.41 g/cm^3, but

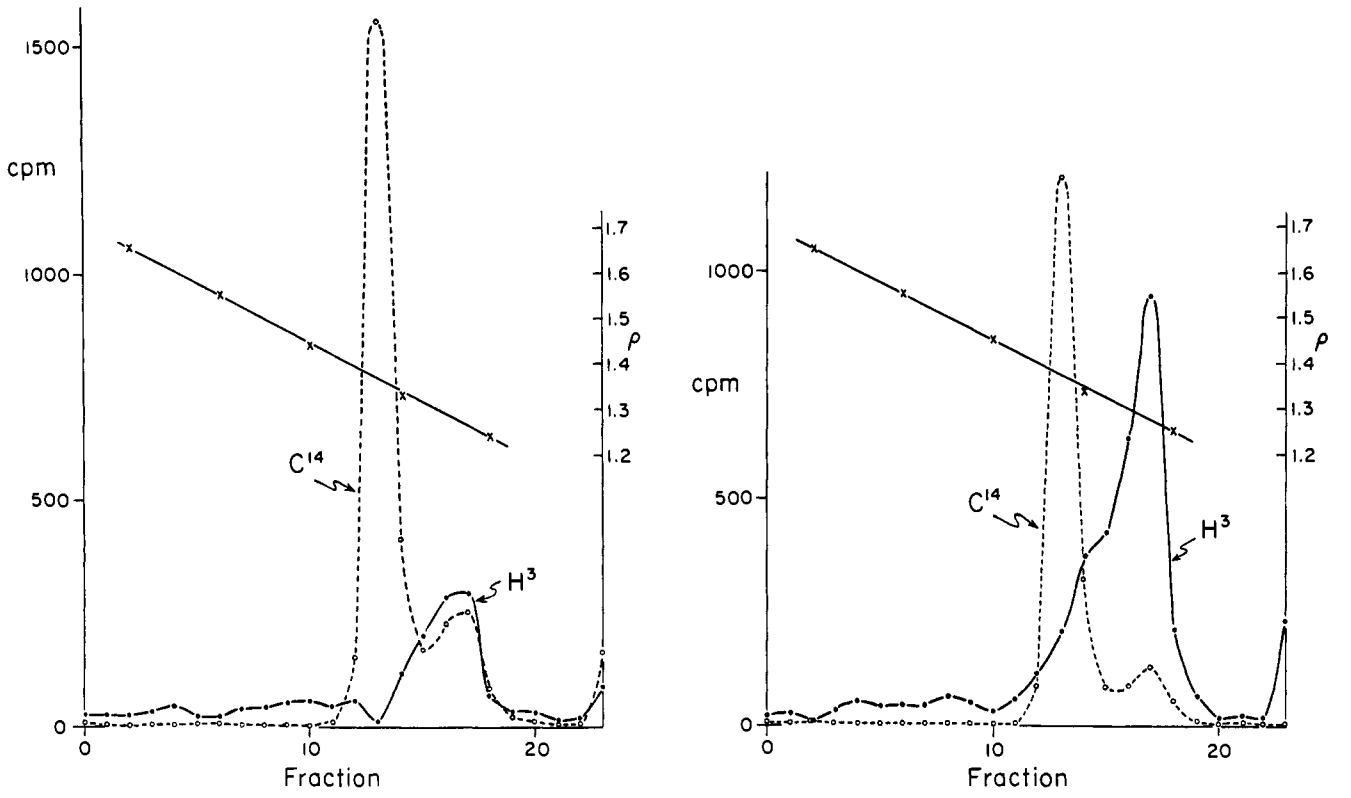

FIGURE 1: *Lytechinus pictus* embryos were labeled by development in the presence of 0.04 μCi/ml of [^{14}C]thymidine and were pulsed after hatching with 2 μCi/ml of [5-^{3}H]uridine for 1.5 (a, left) and 10 (b, right) min. Nuclear extracts were prepared and banded in Cs_2SO_4.

less dense than pure RNA at 1.61 g/cm^3. We presume this latter heterodense RNA represents ribonucleoprotein with widely varying protein contents. A comparison of Figure 1a,b shows the same general distribution of labeled RNA is evident at both 1.5- and 10-min labeling. In fact, the same distribution is found with labeling times varying from 40 sec to 2 hr; an indication of two distinct classes of ribonucleoprotein in the density region from 1.25 to 1.31 g/cm^3 is often seen with longer labeling times (*cf.* shoulder in Figure 1b). Figure 2 shows a comparison of the kinetics of labeling of the RNA found in the 1.25–1.31-g/cm^3 region of the Cs_2SO_4 gradient with whole cell RNA. It is evident that the kinetics of labeling in the two instances is similar; this is consistent with the hypothesis that the labeled RNP in the density region of the gradient may be the principle vehicle for metabolism of HnRNA in these cells.

The approximate composition of nuclear RNP (and chromatin) may be determined if the buoyant densities of protein and nucleic acids in the Cs_2SO_4 gradients are known. In order to supplement the data already available (*cf.* Szybalski, 1968), sea

TABLE II: Buoyant Density of Some Materials in Cs_2SO_4 (g/cm^3).[a]

Material	No Fixation		Glutaraldehyde Fixation	
	Max.	Range	Max.	Range
Protein	1.15	1.21–1.08	1.22, 1.12	1.23–1.10
Polyribosomes	1.47	1.51–1.41	1.41	1.43–1.36
DNA	1.41	1.43–1.39	1.41	1.44–1.38
RNA	1.61	1.64–1.52	1.62	1.64–1.51

[a] Radioactive DNA ([^{14}C]thymidine), RNA ([^{3}H]uridine), polyribosomes ([^{3}H]leucine), or whole cell protein ([^{3}H]-leucine) were dissolved in HS buffer and banded in Cs_2SO_4 as described in Methods. Aliquots were fixed at 0° for 10 min with 6% neutralized glutaraldehyde. The range refers to the apparent densities which included 90% of the banded material. The maximum refers to the position of the peak of radioactivity. Polyribosomes were unstable without fixation.

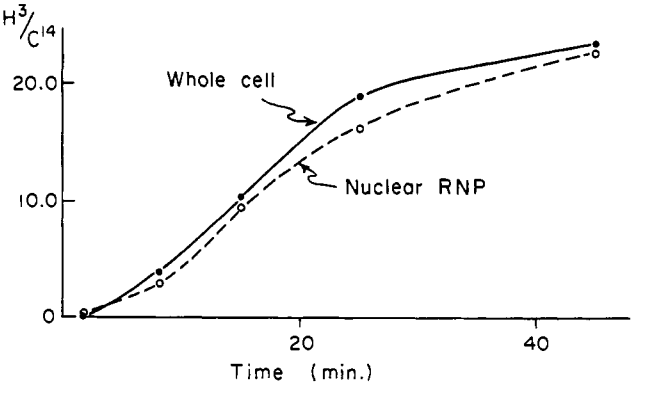

FIGURE 2: Embryos were prelabeled with [^{14}C]thymidine and then exposed to 2 μCi/ml of [5-^{3}H]uridine. The ratio of ^{3}H incorporated into RNA:^{14}C in DNA is plotted as a function of time of exposure to uridine. The ratio for nuclear RNP was obtained from Cs_2SO_4 gradients like those shown in Figure 1. The ^{3}H in RNP with a density of 1.25–1.31 g/cm^3 was divided by the total recovered ^{14}C in the gradient.

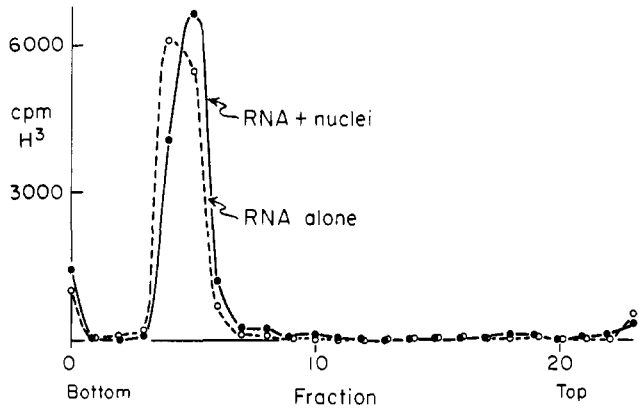

FIGURE 3: Purified [^{3}H]RNA was banded in Cs_2SO_4, either alone, or after mixing with dispersed nuclei from the equivalent of 0.1 ml of packed embryos. Both RNA and dispersed nuclei were suspended in HS buffer. The recovery of the RNA was virtually identical in both gradients, and both gradients are plotted together since the shapes of the Cs_2SO_4 gradients in the two instances were very similar. A visible precipitate (with an apparent density of 1.61 g/cm^3) was visible in both gradients, which was collected in fractions 4 and 5.

urchin embryos were labeled with various radioactive precursors and the purified, radioactive RNA and DNA, or protein soluble in HS buffer, or polyribosomes prepared by sucrose density gradients, were banded in Cs_2SO_4. Table II shows that these materials exhibited characteristic buoyant density distributions. If a combination of protein and RNA were responsible for the observed density of the [^{3}H]RNA banding at 1.25–1.31 g/cm^3, there would be about a protein to RNA ratio of 4–5 to 1 in the RNP.

The RNP observed in Cs_2SO_4 gradients is prepared at high ionic strengths without fixation, making it unlikely the RNP is formed by adventitious association of protein and nucleic acid. Several other lines of evidence support this contention. First, purified RNA may be added during various stages of the preparation to determine whether artefactual binding occurs. Figure 3 shows the results of an experiment in which purified [^{3}H]RNA was added to [^{14}C]thymidine-labeled nuclei prior to their dispersal. The banding patterns of the RNA in Cs_2SO_4 are nearly identical, whether nuclear contents are present or not. In both instances RNA forms a hypersharp band (some precipitate is visible) at about 1.61 g/cm^3, indicating that there is no association of RNA with protein or other nuclear constituents.

Second, the RNA banding at 1.25–1.31 g/cm^3 can be isolated and rebanded. Figure 4 shows that the RNP and deproteinized chromatin occupy the same position in a Cs_2SO_4 gradient after a recentrifugation, even if the previously isolated RNP is mixed with an excess of unlabeled, dispersed nuclei prior to the second centrifugation.

Finally, the DNA can be hydrolyzed by extensive treatment with DNase. Figure 5 shows that if the dispersed nuclei are treated with the enzyme prior to banding in Cs_2SO_4, the observed buoyant density of the radioactive RNP is identical with the control. Thus, RNA banding at 1.25–1.31 g/cm^3 represents a ribonucleoprotein structure whose density does not depend on interaction with DNA or chromatin, and is not due to some adventitious interaction of protein and RNA.

The presence of membrane lipid in the RNP was examined by labeling the developing embryos with [^{3}H]choline throughout cleavage. Table III shows the distribution of radioactivity in various cell fractions of the blastula. Of the incorporated acid-precipitable choline, only 1.5% is present in the detergent-washed nuclei. When these washed nuclei are dispersed in HS buffer and 0.5% DOC, and banded in Cs_2SO_4, none of the acid-precipitable, chloroform-soluble ^{3}H was found associated with the RNP, but rather was located at the top of the density gradient. This does not necessarily mean that lipid is

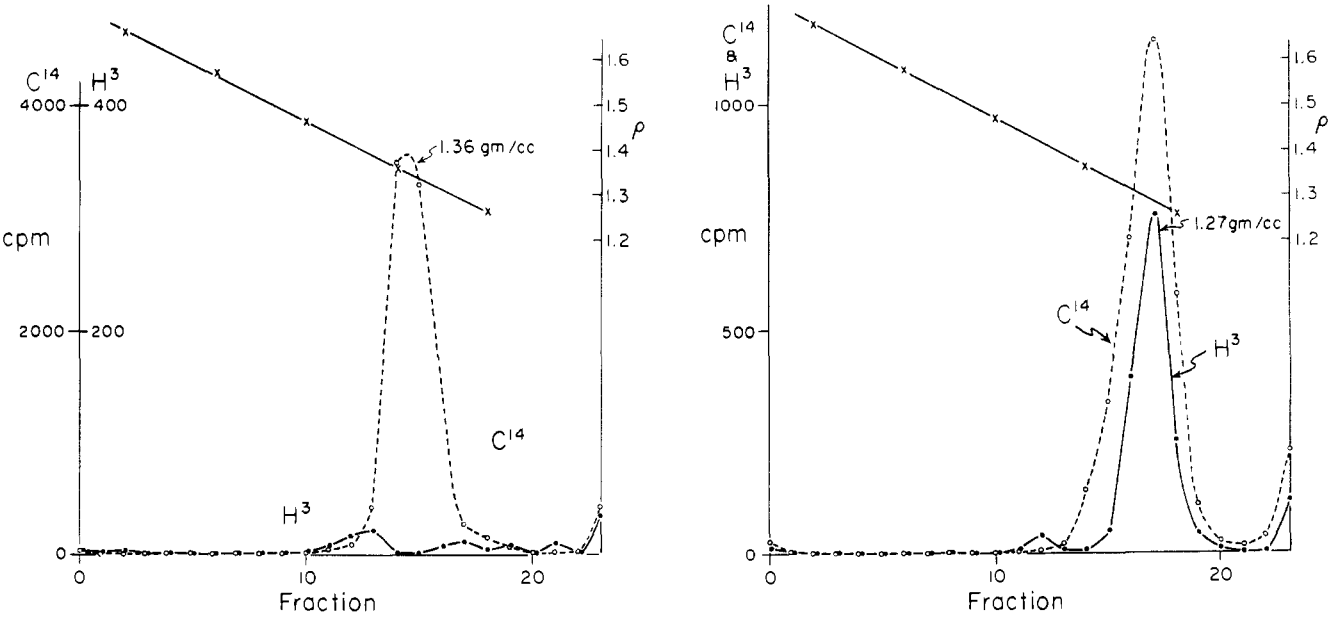

FIGURE 4: *Lytechinus* embryos were labeled for 16 hr with 0.1 μCi/ml of [^{14}C]thymidine and pulsed for 10 min at the blastula stage with 5 μCi/ml of [^{3}H]uridine. Dispersed nuclei were prepared and banded in Cs_2SO_4. Deproteinized RNP (corresponding to tubes 1–10 of Figure 1b), dense chromatin (tubes 12–14 of Figure 1b) and light chromatin and RNP (tubes 15–20 of Figure 1b) were grouped, and dialyzed for 4 hr against HS buffer; 0.5 ml of each group was mixed with 0.5 ml of nonradioactive dispersed nuclei and a second Cs_2SO_4 centrifugation was carried out. (a, left) Recentrifugation of dense chromatin; (b, right) recentrifugation of light chromatin and RNP. Deproteinized RNP is not figured, but rebanded in its original density position.

TABLE III: Distribution of [^{3}H]Choline in Homogenates of Embryos.[a]

Acid-Precipitable Radioactivity	%	Proportion of Acid-Precipitable Material Soluble in $CHCl_3$–MeOH (%)
Homogenate	100	88
Nuclei from step sucrose gradient	15.2	90
First detergent wash of nuclei	8.0	91
Second detergent wash of nuclei	5.5	99
Nuclei after detergent washing	1.5	90
RNP from Cs_2SO_4	0.22	0

[a] Embryos were cultured in the presence of 0.04 μCi/ml of [^{14}C]thymidine and 2 μCi/ml of [^{3}H]choline for 16 hr, after which the blastulae were washed in dextrose and various cell fractions were prepared. Aliquots of fractions were normalized for yields by the content of DNA, and the recovery of acid-insoluble ^{3}H is shown above. The original homogenate contained 1.06×10^6 cpm. The amount of acid-precipitable ^{3}H rendered soluble by $CHCl_3$–methanol (3:1), presumably authentic phospholipid, is shown in the second column.

not associated with the RNP or chromatin, *in vivo*, but does suggest that the buoyant densities observed here are probably not due to associated choline containing lipids.

It is possible that the conditions employed have removed components from the RNP. The effects of Cs_2SO_4 were examined by fixation of the dispersed nuclei with glutaraldehyde prior to centrifugation, and the RNP had the same

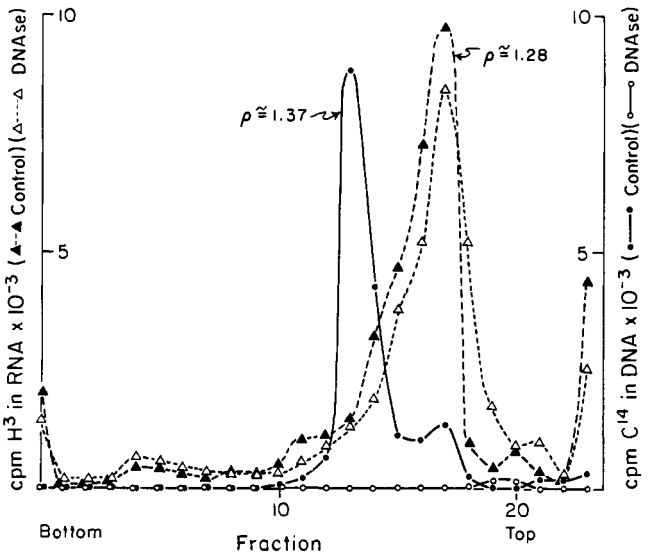

FIGURE 5: Embryos were prelabeled with 0.04 μCi/ml of [^{14}C]-thymidine and pulsed with 5 μCi/ml of [^{3}H]uridine for 25 min. Nuclei were prepared and dispersed, and one aliquot was treated with 250 μg/ml of DNase for 30 min at 15°, and both the control and DNase-treated sample were centrifuged in Cs_2SO_4. Both control and DNase-treated patterns are shown on the same graph: (●——●) control [^{14}C]DNA; (○——○) DNase-treated [^{14}C]DNA; (▲- - -▲) control [^{3}H]RNA; (△- - -△) DNase-treated [^{3}H]RNA.

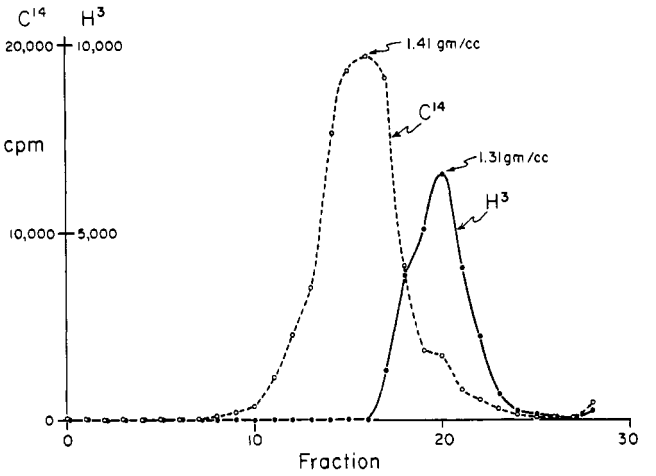

FIGURE 6: Embryos were prelabeled with [^{14}C]thymidine and pulsed for 15 min with [^{3}H]uridine, and nuclei were dispersed in HS buffer and detergent by the usual procedure. The extract was layered over a preformed linear chloral hydrate gradient (made in HS buffer) and centrifuged for 16 hr before collecting fractions.

apparent densities as without fixation (Wilt and Ekenberg, 1971). If a nonionic material, chloral hydrate, is used to produce the density gradient, the RNP shows the buoyant density expected of particles composed of protein–RNA (approximately 4–5:1), which is shown in Figure 6. A thorough study

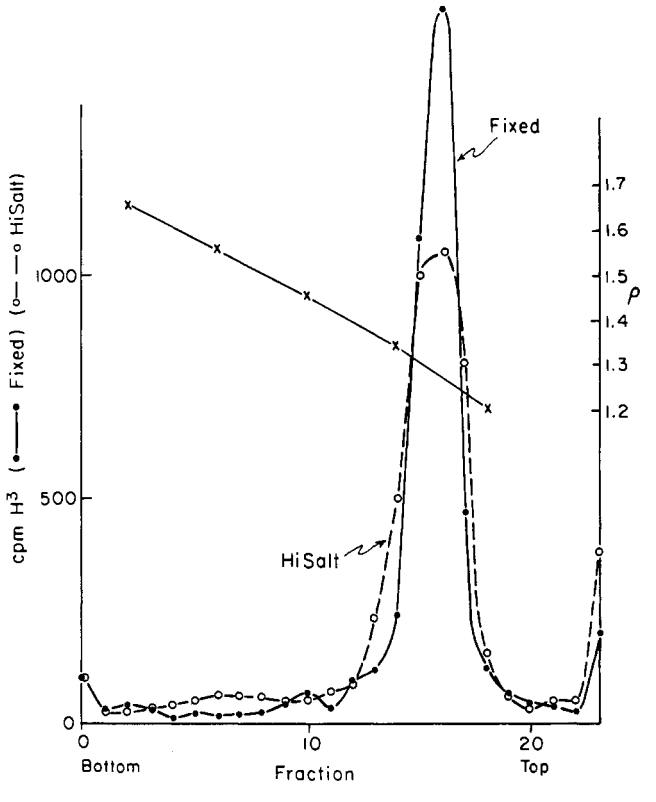

FIGURE 7: Embryos were prelabeled with [^{14}C]thymidine and pulsed for 15 min with [^{3}H]uridine, and the chromatin was isolated exactly as described by Shaw and Huang (1970). The chromatin was layered over linear Cs_2SO_4 gradients and analyzed as described in Methods. The chromatin was either fixed with glutaraldehyde prior to centrifugation, or adjusted to contain the salt composition of HS buffer and 0.5% DOC–0.025% Lubrol W. Only the radioactive RNA from the two gradients are shown, which are superimposed since the Cs_2SO_4 gradients were identical.

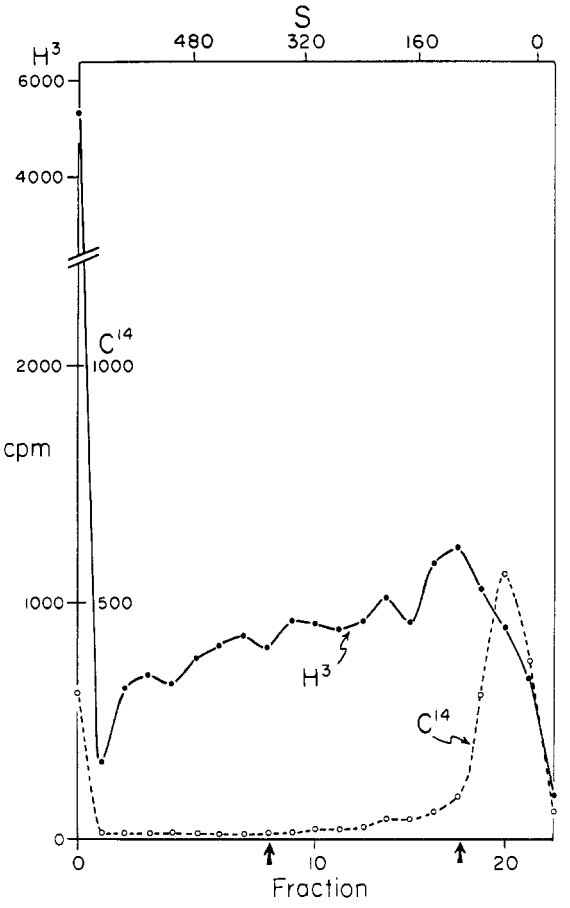

FIGURE 8: *Lytechinus* embryos were cultured 16 hr with 0.08 μCi/ml of [^{14}C]thymidine and pulsed for 10 min with 5 μCi/ml of [^{3}H]-uridine. Nuclei were prepared and dispersed, and 0.1 ml of extract was layered over a sucrose gradient (in HS buffer) and centrifuged for 40 min at 39,000 rpm through a 15–30% gradient. Notice the large accumulation of radioactivity in the pellet. Sedimentation constants are approximate and calculated by the method of McEwen (1967) assuming a particle density of 1.4 g/cm^3.

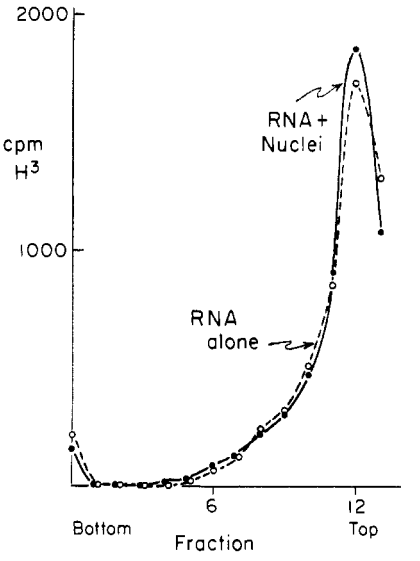

FIGURE 9: Purified [^{3}H]HnRNA was sedimented for 40 min in a 15–30% sucrose gradient (made in HS buffer), either alone or after mixing with dispersed nuclei from the equivalent of 0.05 ml of packed embryos. The distribution of radioactivity in both gradients is superimposed. The approximate sedimentation constant of material in tube 12 is 35 S.

of the relationship of the chemical composition of nucleoproteins to their buoyant density in chloral hydrate has not yet been carried out, but preliminary results indicate the relationships are similar to those of aldehyde fixed nucleoprotein banded in CsCl (E. Hossainy, A. Zweidler, and O. Bloch, personal communication). It is significant that no RNP is seen in these chloral hydrate gradients of Figure 6 with a density greater than DNA, suggesting that the 10% of the RNP banding heavier than DNA in Cs_2SO_4 may be produced by the action of the cesium salt. Another less likely possibility is that chloral hydrate somehow alters the more dense RNP to change its apparent density.

Another possibility is that the combined treatment with HS buffer and detergent during nuclear dispersal may strip protein from the RNP, just as it does to some of the chromatin. Earlier experiments in which nuclei were dispersed by sonication did not support the idea that such stripping is extensive (Wilt and Ekenberg, 1971). We have also "solubilized" nuclei by extensive treatment with low ionic strength buffers, a conventional method of preparing chromatin (Shaw and Huang, 1970). The washing of nuclei with dilute buffers only leaves one-sixth to one-eighth of the [^{3}H]RNA in the final preparation, the remainder being extracted and degraded. The remaining RNA was examined by centrifugation in Cs_2SO_4, and the results are shown in Figure 7. If the nuclear contents are fixed with glutaraldehyde prior to centrifugation in Cs_2SO_4, the RNP bands sharply at 1.27 g/cm^3. On the other hand, if the preparation is adjusted to contain HS buffer and 0.5% DOC, and then centrifuged without fixation, the peak density is 1.28 g/cm^3, an insignificant difference. But treatment of RNP prepared at low salt with HS buffer and detergent does increase the bandwidth of the RNP, from 1.25–1.31 to 1.24–1.34 g/cm^3, and a somewhat increased amount of RNP more dense than DNA is observed after the high salt and detergent treatment. The RNP surviving the preparation at low ionic strength does, then, show evidence of limited deproteinization by the treatment with HS buffer.

The ribonucleoprotein observed in Cs_2SO_4 has the following additional properties. First, treatment of the dispersed nuclei with 25 μg/ml of RNase at 15° for 15 min converts all the labeled RNA to an acid-soluble form. Second, addition of EDTA (0.05 M) to the nuclear extracts and inclusion of 0.05 M EDTA in the Cs_2SO_4 density gradients do not noticeably change the amount or density of RNP in the gradient. Third, treatment of the nuclear extract with SDS and pronase completely destroys the RNP banding at 1.25–1.31 g/cm^3; the [^{3}H]RNA subsequently bands near the position expected for pure RNA.

Sedimentation of Nuclear RNP in Sucrose Gradients. The RNP of the nuclei dispersed in HS buffer and detergents was also examined in sucrose density gradients. Figure 8 shows that the RNA is in structures that are very large (over one-half exceeding a nominal 600 S) and heterodisperse. The very rapid sedimentation may be due to the effects of salt on the structure of the RNP aggregates (*cf.* Penman *et al.*, 1968). The sedimentation pattern of the RNP has been examined after different periods of labeling of the RNA, and no marked change occurs in the relative distribution of radioactive RNA throughout the gradient. Fractions of a sucrose density gradient may be isolated and recentrifuged in a second sucrose density gradient, whereupon the resultant sedimentation

pattern is the same as that expected from the first gradient. This constancy of sedimentation is observed even if the particles isolated from a primary gradient are mixed with an excess of unlabeled nuclear extract prior to the second centrifugation. Furthermore, purified [^{3}H]RNA may be added to the extracts of [^{14}C]thymidine-labeled nuclei to examine adventitious interaction of RNA and nuclear contents. As shown in Figure 9, the sedimentation of the purified RNA is the same, whether the nuclear extract is present or not; evidently the use of HS buffer largely prevents adventitious interaction of nucleic acids and proteins that are observed at lower ionic strengths.

Most of the labeled DNA sediments near the top of the gradient, and under these conditions the apparent sedimentation constant of the DNA peak is 25 S, as measured by comparison to a 28S rRNA marker. About 10% of the DNA does sediment much more rapidly, and is spread throughout the gradient, just as is the [^{3}H]RNP. The apparent buoyant densities of the fractions taken from a sucrose density gradient were examined. As shown in Figure 10, the [^{3}H]RNA in rapidly sedimenting RNP shows a density of 1.25–1.31 g/cm^3 in Cs_2SO_4; identical results were obtained when the particles from a sucrose density gradient were fixed with glutaraldehyde before isopycnic banding. RNA sedimenting at about 25 S was composed mainly of some particles of a variable density greater than DNA and corresponds to the RNP we believe has been partially deproteinized (Figure 10c). The DNA sedimenting at 25 S has a density corresponding to the extensively deproteinized chromatin banding at 1.37 g/cm^3 (see Figure 1), and the rapidly sedimenting DNA has a density similar to the RNP, corresponding to chromatin composed of equal parts of protein and DNA (see Figure 1). The sedimentation of the labeled RNP is unaffected by complete degradation of the DNA with DNase, and furthermore is not affected by treatment of the extracts with 0.05 M EDTA.

Discussion

The rate of accumulation of rRNA is very low in the cells of the sea urchin blastula, and the entire nuclear contents, containing primarily HnRNA, can be fractionated on the basis of sedimentation or buoyant density; the use of relatively high ionic strengths prevents the adventitious association of nuclear RNA with other constituents of the nucleus, which is consistent with the studies of Baltimore and Huang (1970) on the effects of ionic strength on association of proteins and nucleic acids. On the other hand, both deoxycholate and NaCl, if present in sufficient concentration, may deproteinize RNP and chromatin (Smart and Bonner, 1971; Faiferman *et al.*, 1971). It is possible that the results obtained here are due to the increased resistance to salt of the constituents of cells from organisms that live in sea water. Equally likely, however, is that a precise adjustment of ionic strength and detergent concentration is required for maintaining the RNP particles; we have found that only a narrow range of NaCl and DOC concentrations is effective in disrupting the nuclei while leaving the RNP apparently intact. Penman *et al.*

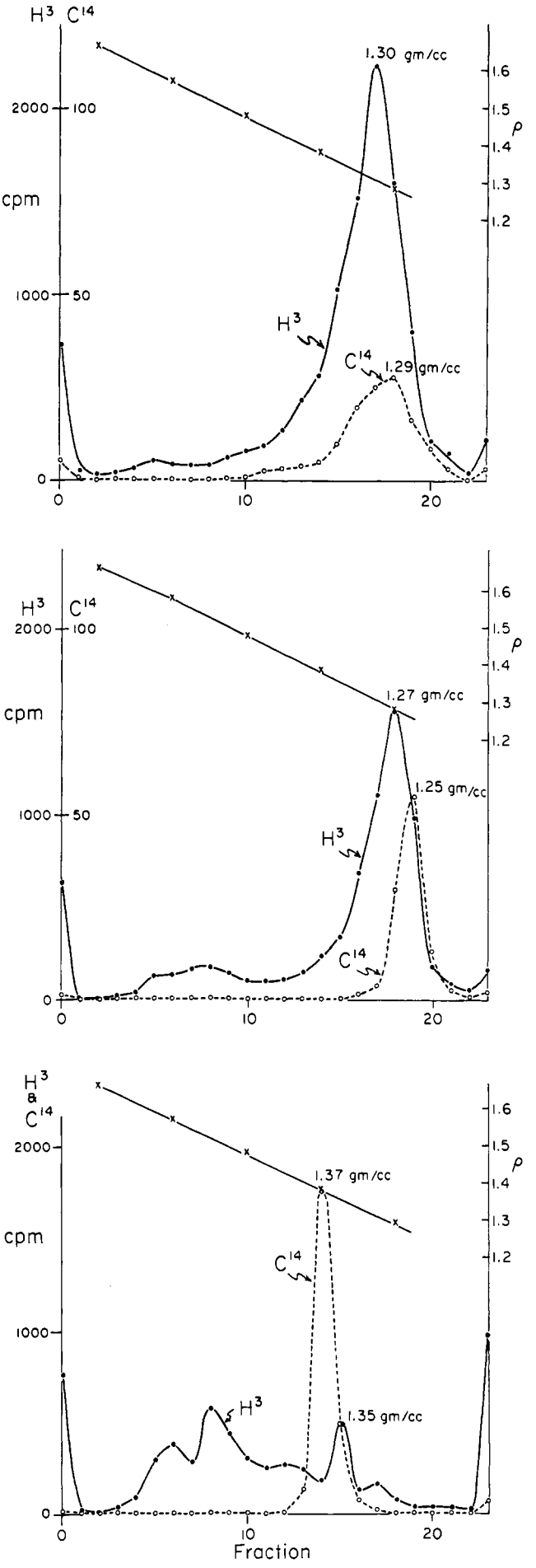

FIGURE 10: Aliquots from tubes 1–8 and the pellet (a, top), tubes 9–16 (b, middle), and tubes 17–20 (c, bottom) of the gradient shown in Figure 8 were layered directly over Cs_2SO_4 gradients and centrifuged to determine the buoyant density of materials at various positions in the sucrose density gradient.

(1968) also found that very large RNP aggregates could be extracted from HeLa cell nuclei in the presence of 0.5 M NaCl. The results obtained here indicate a lower limit for the amount of protein associated with nuclear RNA. With this reservation in mind, the principal conclusions are: (1) at least 90% (Figure 1) and perhaps all (Figure 6) of the nuclear RNA is present in ribonucleoprotein particles; (2) the RNP particles are composed of protein–RNA (approximately 4–5:1), as based on their apparent buoyant density; (3) the RNP particles exist as very large aggregates of heterogeneous size at the relatively high ionic strengths employed; (4) the control experiments presented have made it very likely that nuclear RNP particles are not artefacts.

The chromatin of the nucleus is extensively deproteinized under the conditions employed here, and the DNA may be entirely removed without altering the physical properties of the RNP. This does not prove that DNA may not be associated with some of the RNP, but does show that the physical properties of the RNP do not depend on association with chromatin, nor probably with membranes. The suggestion previously made on the basis of work done in this laboratory (Wilt and Ekenberg, 1971) that the chromatin was being fractionated in Cs_2SO_4 into the nontranscribed, inactive portion and the actively transcribed portion bearing nascent RNA is probably incorrect. Nucleic acid hybridization studies in which "unique" DNA from the chromatin fractions obtained after Cs_2SO_4 fractionation is reacted with an excess of nuclear RNA have ruled out this hypothesis (F. H. Wilt, unpublished). However, the separation of DNA into two fractions is not a random one; the minor DNA fraction composed of equal amounts of protein and DNA that bands in Cs_2SO_4 gradients in the same region as nuclear RNP (1.27 g/cm^3) contains the growing points for DNA synthesis, as revealed in kinetic studies of the incorporation of radioactive thymidine (F. H. Wilt, unpublished).

We believe that the methods presented here which minimize adventitious binding and eschew fixatives will be useful in unraveling the relationships between nuclear RNP and the polyribosome associated particles containing mRNA.

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