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Ribonucleic Acid Isolated by Cesium Chloride Centrifugation†

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ABSTRACT: A quick and simple procedure based upon CsCl ultracentrifugation for the isolation of RNA from disrupted cells or subcellular fractions is described. rRNA is obtained intact and can be recovered in yields close to 100%. It is shown that the translation of rabbit globin mRNA is not impaired by exposure to this treatment. The yield of poly(A) containing globin mRNA extracted by this method and partially enriched on a poly(U)-Sepharose column was assayed in wheat germ S-30 extracts along with that extracted with phenol undergoing identical treatment. RNA from the CsCl extraction yields RNA six times more active in globin synthesis. It is observed that specific aggregation takes place during CsCl centrifugation at high gravitational fields diminishing the amount of the 16S or 18S rRNA component but not 26S or 28S component. The aggregation can be avoided by slow-speed centrifugation or reversed by heating at 60° for 30 sec. The CsCl centrifugation appears to be superior to phenol extraction in terms of simplicity, processing time, yield, intactness, and biological activity of resulting RNA. The suitability of the method for small amounts of starting material and one step separation of macromolecules from each other seem to recommend the CsCl centrifugation as a general method of isolating RNA over other methods currently in use.

The isolation of RNA from tissues, whole cells, or subcellular organelles requires a procedure in which proteins, polysaccharides and DNA are removed and in which endogenous ribonucleases are not released in an active form. Phenol extraction (Kirby, 1968) has been most generally

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used to isolate RNA from a wide variety of biological materials.

However, this method is not ideal in that degradation of the RNA is not always suppressed, phenol is subject to varying impurities, the sequences of shaking and transfer are difficult to make completely reproducible and contribute to variable and often substantial losses, and the method cannot be reliably scaled down for very small samples.

Since the introduction of CsCl equilibrium density gradient centrifugation of DNA (Meselson *et al.*, 1957) it has been apparent that RNA can be isolated as well (Kurland, 1960). (For a comprehensive list of applications of isopycnic centrifugation in cesium salts in characterization of nucleic acids, see recent reviews by Szybalski and Szybalski (1971) and Flamm *et al.* (1972).) However, the RNA can only be obtained as a pellet because there is no attainable CsCl concentration at which it will band. Ordinarily pelleting occludes other components of a mixture but since RNA is likely to be the most dense component the possibility remains that in this case the pellet will represent highly purified RNA. The work described here supports this expectation and shows CsCl centrifugation to be a very simple widely applicable procedure for isolating RNA with negligible degradation or loss of biological activity. Moreover, it has the advantage of very effectively eliminating nucleases and other proteins as these migrate in the opposite direction and it is applicable to samples of all sizes. Furthermore, the CsCl centrifugation technique can be applied to biological materials not readily available in sufficient quantities for phenol extraction. No difficulty is experienced in redispersing the pellet, in which the RNA concentration is about 12% by weight. Another advantage of this method is that DNA is removed without the use of DNase. Since most commercially available DNases are contaminated with RNase, this is particularly beneficial.

In order to assess this procedure adequately it has been applied to the isolation of several quite different RNA species including globin mRNA whose ability to be translated into globin could be monitored.

Materials and Methods

General CsCl Centrifugation Method of RNA Extraction. Washed cells were gently homogenized at 0° with a Duall homogenizer in 3–10 volumes of 0.1 M Tris-HCl, pH 8, 4% (w/v) sarkosyl (sodium lauryl sarconsinate, obtained from Schwarz/Mann Co.). The homogenate was allowed to remain in ice during intermittent homogenization for 10–15 min; then 1 g/ml of solid CsCl was added and mixed until all of the CsCl had dissolved. The homogenate (4 ml) was then layered onto 1.2 ml of a 5.7 M CsCl–0.1 M EDTA cushion in a cellulose nitrate centrifuge tube. The cushion of CsCl had a density of 1.7070 and had been previously filtered through a Millipore filter to remove impurities. The sarkosyl buffer (0.25 ml) was then layered on the top and the tube was centrifuged in a Beckman SW 50L rotor at 35,000 rpm for 12 hr at 25°. After centrifugation, the DNA banding at the interface of the two CsCl solutions was removed with a Pasteur pipet. The tube was inverted, and all but the bottom 1 cm was sheared off. The RNA in the clear pellet was then dissolved in buffer and either used immediately or precipitated from 2.5 volumes of 80% ethanol–0.2 M NaCl and stored at −20°.

Preparation and Assay of Rabbit Hemoglobin mRNA. Rabbit reticulocyte lysates were obtained by the method of McDowell *et al.* (1972) and then layered on a 7-ml cushion of 36% (w/v) sucrose in buffer A (0.1 M KCl–0.03 M Tris-HCl (pH 7.5)–0.003 M $MgCl_2$) and centrifuged for 90 min in the Beckman 60 Ti rotor at 56,000 rpm at 3°. The polysomal pellets were briefly rinsed with buffer A and stored under liquid nitrogen. Pellets from the same batch were dissolved in buffer B (0.12 M NaCl–0.02 M Tris-HCl (pH 7.5)–0.5% SDS[1]) and either layered on top of 1.2 ml of 5.7 M CsCl − 0.1 M EDTA cushion and spun in a SW 50 rotor at 39,000 rpm for 12 hr or extracted with an equal volume of phenol saturated with H_2O and 0.1% in 8-hydroxyquinoline at room temperature. The resuspended pellets were shaken briefly for 3–5 min, and the phases were separated by centrifugation in a Sorvall RC2B centrifuge at 10,000 rpm for 10 min. The phenol phase was then reextracted with buffer B and the combined aqueous phases were reextracted once with phenol and once with phenol–chloroform–isoamyl alcohol (49:49:1). The RNA was then precipitated with two volumes of ethanol from the final aqueous phase. The RNA pellet from CsCl centrifugation was dissolved in buffer B and heated at 60° for 30 sec and then precipitated with two volumes of ethanol.

Thereafter the two RNA preparations were treated identically and applied to a poly(U)-Sepharose column. The preparation of the column and elution steps have been described (Firtel *et al.*, 1972; Firtel and Lodish, 1973). The fraction of total reticulocyte polysomal RNA that binds to the column in 0.3 M NaCl was operationally termed "Hb mRNA."

The activity of globin mRNA was tested in a heterologous wheat germ cell-free system. The extracts were prepared and RNAs tested as outlined by Roberts and Paterson (1973). The polypeptide product made in wheat germ extract with rabbit globin mRNA was characterized by CMC chromatography (Schrier *et al.*, 1973) and SDS polyacrylamide gel electrophoresis (Weber and Osborn, 1969).

Polyacrylamide gel electrophoresis of RNA was performed in 2.85% (w/v) gels according to the procedure outlined before (Boedtker *et al.*, 1973).

Sucrose Gradients. Sucrose gradients (17 ml; 5–30% (w/v)) in buffer B were run for 12 hr at 27,000 rpm at 22° in the Beckman SW 27.1 rotor. The absorbance at 254 nm was continuously monitored with an Instrumentation Specialties Co. Model UA ultraviolet optical unit and the UA-2 ultraviolet analyzer was coupled to a recorder.

Sea Urchin Eggs. Strongylocentrotus purpuratus was obtained from Pacific Bio-Marine Supply Co., Venice, Calif. Ovulation was induced by injecting 0.5 M KCl into the peritoneal cavity. Eggs were washed by repeated settling in several hundred volumes of synthetic sea water.

Results

Biological Activity of RNA. Since the most stringent requirements in RNA isolation are met in preparing mRNA, we undertook a comparison of the translational activity of mRNA from rabbit reticulocytes as prepared by the CsCl and phenol methods. The wheat germ S-30 *in vitro* system was used for the assay as described above.

Polyribosomal pellets obtained from the same preparation of rabbit reticulocytes were divided and processed by the CsCl and phenol extraction procedures. The two resulting RNA preparations were then passed through columns containing poly(U) covalently linked to Sepharose beads. Only the RNA which binds to the column at high salt concentration (0.3 M) and is eluted with H_2O or 60% formamide was assayed

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; mRNA, messenger RNA; CMC, carboxymethylcellulose.

TABLE I: Translational Capacity in Wheat Germ S-30 Extract.

Method of Deproteinization	Poly(U)-Sepharose Column			Amount of "Bound RNA" Added/ 50 μl of Reaction Mix.	
	A_{260} Units			1×10^{-6} g Nonsaturating Condn	3×10^{-6} g Saturating Condn
	Input	Bound	% Bound	Incorporation into Globin (pmol of Leu)[a]	
CsCl centrifugation A	165	5.0	3.03	46.9	146.2
Phenol extraction B	150	1.26	0.84	28.7	87.2
A/B			3.6	1.63	1.67

[a] 1 pmol of leucine incorporated = 2750 cpm. Background of 5500 cpm subtracted; figures rounded to nearest thousand.

for its capacity to stimulate globin synthesis. The results are summarized in Table I where it is seen that 3.6 times as much RNA was eluted from the poly(U)-Sepharose column used for the CsCl preparation. Moreover, the incorporation of radioactive leucine was 1.65 times higher per unit weight of RNA eluted from such columns. Thus in terms of equal amounts of polysomal pellets, the translational capacity of poly(U)-Sepharose processed product is intrinsically six times greater.

We took the precaution of showing in separate experiments using CMC column chromatography and SDS gel electrophoretic analysis that the mRNAs prepared by both methods stimulate the synthesis of completed globin chains almost exclusively. Hence, the six-times higher translational activity refers specifically to the globin mRNA and indicates the superiority of the CsCl method for this purpose.

Some of the Physical-Chemical Properties of RNA. The DNA contamination was measured by diphenylamine colorimetric test (Burton, 1956) in RNAs isolated from whole tissues only. A good example is provided in analyzing the RNA isolated from unfertilized sea urchin eggs and the corresponding gastrula stage embryos. In the latter the amount of DNA per embryo increases by a factor of 1000 while the RNA remains constant. No DNA could be detected in the RNA isolated from the unfertilized egg while the DNA contamination in gastrula RNA did not exceed 0.25%.

All RNA preparations isolated by CsCl centrifugation method had a ratio of absorbances at 260/280 nm higher than 2.05. The same was true for the ratios at 260/235 nm, thus indicating (Warburg and Christian, 1941) that the protein contamination is negligible. The direct (Lowry *et al.*, 1951) colorimetric test showed that protein contamination was in the range of 0.3–0.5%.

Polysaccharides and glycogen have a buoyant density in CsCl solutions of about 1.67 g/cm^3 (Counts and Flamm, 1966; Anderson *et al.*, 1966; as cited in Flamm *et al.*, 1972) therefore they are the major contaminant of DNA (densities about 1.7 g/cm^3) rather than of RNA (buoyant density of about 1.9 g/cm^3) (Piko *et al.*, 1967).

The sedimentation profiles and polyacrylamide gel electrophoretograms of RNA isolated from different organisms by phenol extraction and CsCl centrifugation are compared in Figure 1. Phenol-extracted RNA from *Escherichia coli* (1A), sea urchin eggs (1B), and chick polyribosomal RNA (1C) are seen to have a ratio of about 2:1 in absorbance between the large and small ribosomal subunit RNA. In contrast to this, the ratio of the large to small rRNA species extracted by CsCl centrifugation (*E. coli*, 1D; sea urchin egg RNA, 1E; chick polyribosomal RNA, 1F) appears to be higher. The absorbance ratios were 3:1 and, in some cases, even 4:1.

One interpretation of this apparent difference could be that the small rRNA component is not quantitatively recovered in the CsCl centrifugation while the large 26S or 28S RNA is. To test this possibility we extracted RNAs from different organisms by the phenol method, the deproteinized RNA was layered on the CsCl cushion and the RNA was collected after centrifugation from the bottom of the tube. The recovery of the RNA in all these cases was 100%. Again, as in the case when the RNA was isolated from whole cells, we found the same disproportionate ratio (not shown) between the large and the small rRNA species. Also, some larger aggregates with sedimentation constants greater than 28 S were ob-

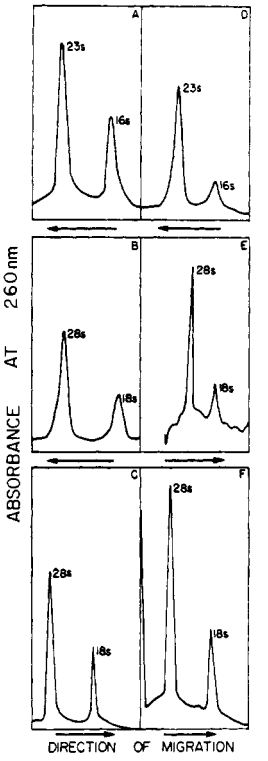

FIGURE 1: Absorbance profiles of RNAs extracted by phenol (panels A, B, C) or CsCl centrifugation (D, E, F). A and D sucrose gradient analysis of *E. coli* RNA; B and E sucrose gradient and gel electrophoresis analysis of sea urchin *S. purpuratus* RNA; C and F gel electrophoresis of chick embryo polysomal RNA. CsCl centrifugation was performed at 150,000*g*. For details, see the text.

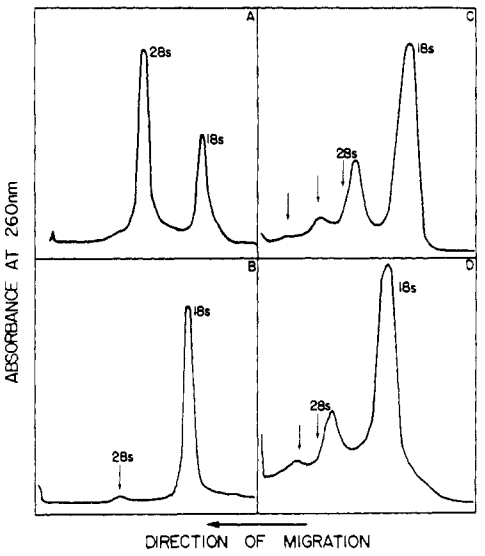

FIGURE 2: Sucrose gradient analysis of chick embryo 18S RNA after high-speed centrifugation in a CsCl step gradient: (A) phenol-extracted total chick embryo RNA; (B) fractionated 18S RNA; (C) 18S RNA centrifuged through a CsCl step gradient; (D) the nonaggregated 18S RNA recentrifuged through a CsCl step gradient.

served as well. This suggests that part of the small rRNA may undergo aggregation and thereby remove itself from the appropriate peak.

To test this hypothesis the small (18S) fraction of the chick polyribosomal RNA was analyzed directly. In the first control experiment (Figure 2A) we found that the phenol extracted RNA contained the usual 2:1 ratio of two rRNAs. Fractionation of the 18S RNA and the subsequent analysis on a sucrose gradient showed that the 18S RNA was only very slightly contaminated with the 28S RNA (2B). The fractionated 18S RNA was then precipitated with alcohol and centrifuged through a CsCl cusion. The sedimentation profile of this RNA was found to have been changed significantly (2C). In addition to 18S RNA, higher molecular weight aggregates were formed as well. The three additional peaks observed could correspond to aggregates containing two, three, and four 18S monomers. The dimers represent about 30% of the remaining 18S RNA. The same is true if one compares the ratio of trimers to dimers or tetramers to trimers, respectively.

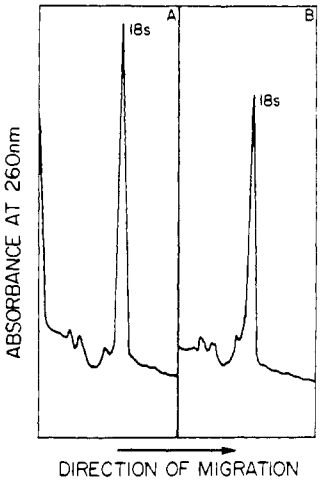

FIGURE 3: Polyacrylamide gel electrophoresis of 18S chick rRNA centrifuged through a CsCl step gradient at 35,000*g*: (A) unheated; (B) heated at 60° for 30 sec.

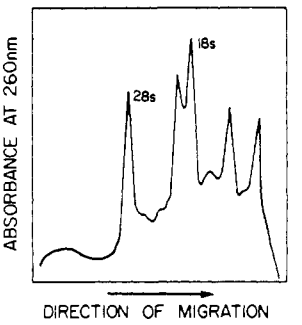

FIGURE 4: Gel electrophoresis of *S. purpuratus* RNA. RNA extracted by CsCl centrifugation and heated at 60° for 30 sec.

To see if the aggregation of the 18S RNA was selective or not, the remaining nonaggregated 18S RNA was subjected to CsCl centrifugation. The result of this experiment is shown in Figure 2D. Again, a similar pattern of aggregates could be recognized. Thus the involvement of a unique subclass of 18S RNA has been excluded. After heating at 60° for 30 sec these high molecular weight aggregates dissociated into the original 18S rRNA component. The only exception to this was *Strongylocentrotus purpuratus* rRNA, which will be discussed later.

Two additional questions need to be answered. (a) Are the aggregates formed due to precipitation in high CsCl salt or are they formed because of the high gravitational force to which the RNA pellet has been exposed to? (b) Does the 28S RNA show the same tendency to aggregate as the 18S RNA does?

The first question was answered by comparing the sedimentation profiles of the 18S chick RNA centrifuged through the CsCl cushion at low gravitational fields (35,000*g*) with that exposed to the usual higher fields (150,000*g*). The result is shown in Figures 3A (unheated) and 3B (heated at 60°, 30 sec), respectively. The RNA pelleted at 35,000*g* remained completely in its original 18S monomeric form. Thus, the high CsCl concentration is not sufficient to induce aggregation. The exposure to higher gravitational fields and the denser packing that results is evidently the cause of the aggregation.

Finally, in a direct test it has been shown that the large 28S RNA component does not aggregate at the high gravitational force at which the small RNAs component does.

The behavior of the sea urchin egg RNA requires an additional comment. Nemer and Infante (1968) showed some time ago that in the eggs of *S. purpuratus* the 18S rRNA can be converted to two approximately equal 13S fragments by heating briefly at 60°. This has only been observed so far for *S. purpuratus*. Using the CsCl centrifugation method for isolating the total egg RNA we have confirmed their basic result. Upon heating, the RNA disperses into a complex pattern of different molecular sizes as shown in Figure 4. In addition to the 18S and 28S molecules, 14S, 9S, and 7S molecules appear as well. The 28S peak has decreased to an amount smaller than the remaining 18S. Obviously, the complex pattern obtained upon heating in contrast to the typical pattern of other sea urchin RNAs can be explained if one assumes that both the 28S and the 18S components contain thermolabile subunits. Careful examination of the data obtained by Nemer and Infante is also consistent with the idea that in this case 28S RNA is thermolabile. In their experiments, however, it appears that the 28S RNA was broken into smaller pieces only when heated at 75°. The observed differences between the two results can most probably be attributed to differences in heating conditions used (Nemer and Infante, 1968). To see if this thermolability might exist in other sea

urchin species we analyzed the RNA extracted from egg and embryos of the Adriatic Sea species *Paracentrotus lividus* and *Arbacia lixula*. No fragile rRNA was observed (data not shown). Thus, the significance of the lability of *S. purpuratus* is unclear.

Discussion

Before CsCl centrifugation could be widely used to isolate RNAs it was necessary to show that the procedure yields biologically active molecules. Our experiments with rabbit globin mRNA isolated by the CsCl centrifugation have shown that this procedure does not adversely affect the mRNA in its ability to perform its coding function. Furthermore, the RNA obtained subsequently by poly(U)-Sepharose column elution was 6 times more active in *in vitro* synthesis of globin.

Part of the lower translating activity of the phenol prepared RNA may be due to the loss of poly(A) segments during this procedure as reported by Perry *et al.* (1972). This would account for the lower yield from the poly(U)-Sepharose column but not the lower intrinsic incorporating activity. This is presumably due to the generation of more fragments of RNA that bind to poly(U) in the phenol procedure.

The finding that the CsCl centrifugation at high fields induced aggregation among 16S or 18S rRNA molecules is of some interest but does not affect the usefulness of the method as the aggregation can be eliminated by mild heating or avoided by using lower gravitational fields.

There is no obvious explanation for this aggregation phenomenon, but several points should be considered. First, the concentration of RNA in the pelleted gel, about 10–12% (w/w), is unusually high and as a result the RNA molecules in the gel are very close to one another. At lower gravitational fields the pellets do no become so compacted and aggregation does not occur. Second, the aggregates might be formed as a consequence of the interactions of the adenine-uracil (A + U)-rich regions of the molecules involved. Recently it has been shown by Wellauer and Dawid (1973) that when 28S RNA is exposed to strong denaturation conditions (80% formamide and 4 M urea) it retains a significant amount of its secondary structures. In marked contrast, however, the 18S RNA retains none. This reflects the difference in G + C content or sequences of the two molecules. Therefore, a possible interpretation of our results is that, in very high concentration the 18S RNA molecules in the gel can interact with one another and the intramolecular hydrogen bonds may be replaced with the corresponding *intermolecular* ones. However, the stronger intramolecular hydrogen bonds of the 28S RNA are not disrupted and the molecules cannot be forced to aggregate with each other.

The higher aggregates observed (trimers, tetramers) presumably arise as a consequence of "annealing" of 18S RNA at different places along the molecule. The opportunity for such annealing should decrease as the number of monomers in the aggregates increases. The decreasing amount of trimers and tetramers is then self-explanatory.

In conclusion, CsCl centrifugation seems to meet all experimental requirements as a method of choice for isolating RNA free of proteins, DNA, polysaccharides, and other cellular components. The method is extremely simple. It offers distinct advantages over the phenol as generally used in respect to degradation, yield, and the preservation of translational activity.

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