SELECTIVE PRECIPITATION OF MONOMERIC RIBOSOMES BY BENTONITE

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Any study of ribosomes and polyribosomes from the point of view of tissue development demands a quantitative extraction of all classes of these particles from the tissue. The use of bentonite, a commonly used inhibitor of RNase, has been found to influence considerably the total yield of the ribosomal complement of germinating oat tissues, and to influence the yield of monomeric ribosomes relative to polyribosomes.

To prepare ribosomes and polyribosomes from these tissues, oat seeds were germinated in darkness for 72 hours after which time the coleoptile sheath and its enclosed primary leaf were excised from the plantlet at the coleoptilar node. This material, hereafter referred to as sections, was immediately homogenized with a loose fitting, motor driven Duall homogenizer at 1-2°C. Generally 100 to 200 sections were utilized for each preparation and homogenized in 15 to 30 ml of medium respectively. The homogenizing medium was composed of 0.1 M tris-succinate buffer, pH, 7.6 at 5°C, 0.005 M $MgCl_2$, 0.001 M spermidine, 0.25 M sucrose and other additives as mentioned in the text. This homogenate was centrifuged for 20 minutes at 10,000 gravities and sodium deoxycholate (Na Doc) added to the supernatant to give a concentration of 0.5%. The supernatant was next layered over a concentrated sucrose solution (5 ml over 7 ml) in Spinco #40 rotor centrifuge tubes. If the entire ribosome population was to be sedimented in the subsequent centrifugation, the sucrose concentration was 1 molar; if polyribosomes, predominately free of monomeric ribosomes, were to be collected, the sucrose con-

centration was 2 molar. This latter procedure for preparing polyribosomes is a slight modification of the method of Wettstein, et. al. (1963), for preparing "C' ribosomes, and consequently these preparations of polyribosomes are referred to as C ribosomes. The concentrated sucrose solutions also contained 0.1 M tris-succinate buffer, pH 7.6, 0.005 M $MgCl_2$ and 0.001 M spermidine.

The tubes were centrifuged for 2 hours at 40,000 RPM in a Spinco #40 rotor. The pellets were resuspended in a small volume of the tris, $MgCl_2$, spermidine solution and layered immediately over a 23 ml linear sucrose density gradient (10 - 34%) which in turn had been layered over 2 ml of 34% sucrose. After centrifuging for 2 hours at 25,000 RPM, the profiles of absorbancy at 254 mμ of the sucrose gradients were obtained with a flow-through gradient fractionator (ISCO Instrument Co). The effluent from the gradient containing only the monomeric ribosomes was collected and the UV absorption spectrum of an aliquot determined. Maximum and minimum absorptions were 259 mμ and 236 mμ respectively and the ratio of maximum to minimum was 1.6 ± 0.05. The extinction coefficient at 260 mμ of a 1% ribosome solution is 120 ± 5/cm. These ribosomes contain 55% RNA and 45% protein (± 2.5%) as determined by the orcinol assay and the biuret assay respectively.

In order to establish the total amount of ribosomal RNA (rRNA) present in the tissue, most all of which should be extractable in ribosomes, rRNA was extracted from the tissue by the phenol method, purified on MAK columns (Sueoka and Cheng, 1962) and expressed as mg rRNA per 100 sections in Table 1.

Initially the homogenizing medium utilized contained 1 mg/ml of bentonite, sized by the method of Fraenkel - Conrat (1961). Although these preparations gave striking sucrose gradient profiles of absorbancy which depicted the bulk of the ribosomal complement as existing as polyribosomes in these tissues, (Fig. 1a, solid line) the yield of rRNA from these preparations represented only 1/4 of the total rRNA in these tissues as revealed by phenol extraction (Table 1).

This missing rRNA was not in the 10,000 gravities supernatant. Considering that the failure to solubilize all the ribosomes of the sections might be caused

Table 1. Yield of r RNA

Preparation	mg rRNA/100 sections
From phenol extraction	2.9
From sucrose gradients of ribosomes	
-Bentonite	2.76
+Bentonite 0.5 mg/ml	1.80
+Bentonite 1.0 mg/ml	0.75
From sucrose gradients of C ribosomes	
-Bentonite	0.64
+Bentonite 1.5 mg/ml	0.53
+Bentonite 2 mg/ml	0.26
-Bentonite, -Na Doc	0.40

by a failure to disrupt membraneous structures that contained ribosomes, Na Doc was added to the initial homogenization medium. This procedure failed to increase the yield of rRNA in the ribosome preparations. If the amount of homogenizing medium used was increased relative to the amount of tissue homogenized, the yield of rRNA decreased further, suggesting that some component of the homogenizing medium was responsible for the failure to recover the entire ribosome complement. Decreasing the amount of bentonite utilized in the homogenizing medium to 0.5 mg/ml brought about a substantial increase in ribosome yield (Figure 1b, dotted line; Table 1), and complete omission of bentonite resulted in an almost quantitative extraction of the ribosome complement. (Fig. 1b, solid line; Table 1). (Note scale change in Figure 1).

The sucrose gradient absorbancy profile of the total ribosome population shows a considerably different distribution of monomeric ribosomes relative to polyribosomes than was encountered when bentonite was used. Over 70% of the population exists at this stage in germination as monomeric ribosomes. Plotting the absorbancy profile of the bentonite treated ribosome preparation on the 5 fold reduced scale of Fig. 1b and c (Figure 1a, dotted line) reveals that the bentonite does not substantially decrease the yield of polyribosomes. These data suggest that the presence of bentonite caused a preferential failure to recover monomeric ribosomes in these preparations.

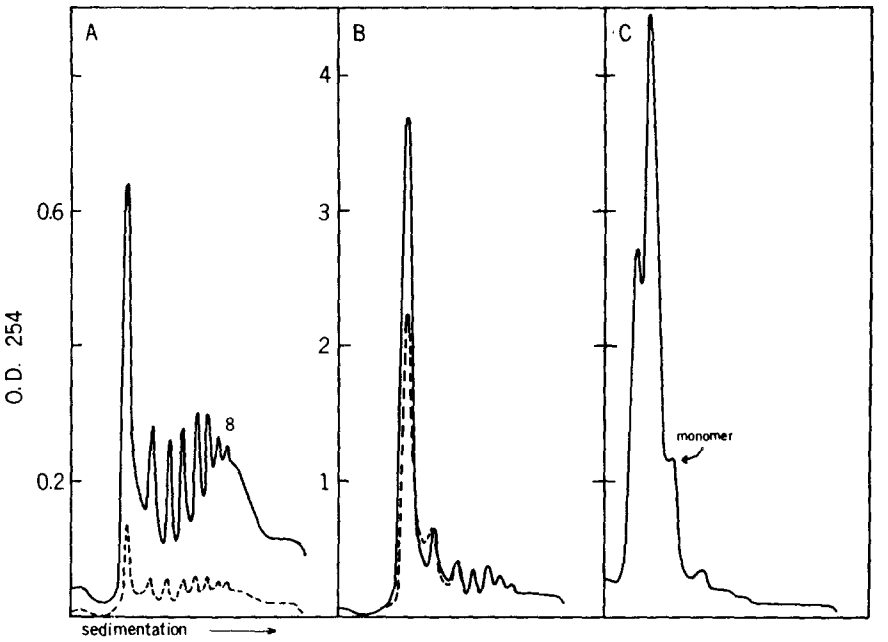

Figure 1. Sucrose gradient profiles of absorbancy of total ribosome complement of 100 sections. A. Solid line, prepared with bentonite 1 mg/ml. Dotted line, same preparation plotted on scale of B and C. B. Solid line, prepared without bentonite. Dotted line, prepared with bentonite 0.5 mg/ml. C. Prepared without bentonite and with 1mM $MgCl_2$. Note scale change.

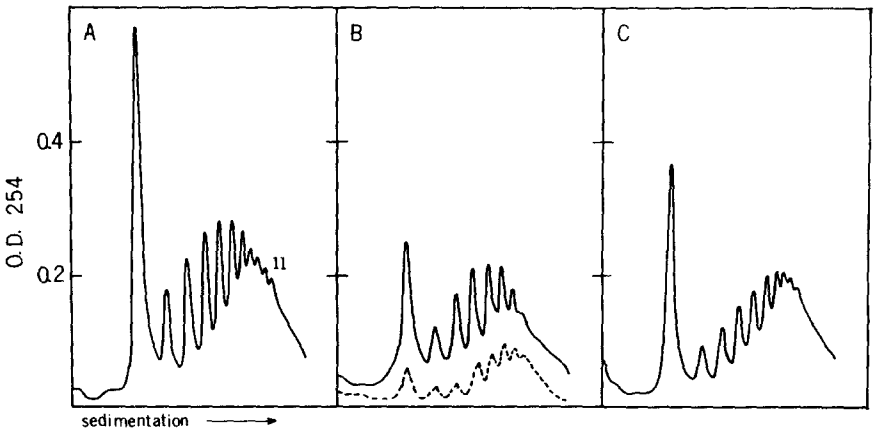

Figure 2. Sucrose gradient profiles of absorbancy of C ribosomes from 100 sections. A. Without bentonite. B. Solid line, prepared with bentonite 1.5 mg/ml. Dotted line, prepared with bentonite 2 mg/ml. C. Without bentonite, without Na Doc.

To more clearly visualize the apparent selective interaction between monomers and bentonite, C ribosomes were prepared with and without bentonite. The amount of polyribosomes in the C ribosome preparation (Fig. 2a) approximate the amount of polyribosomes that are obtained in preparations of total ribosomes prepared with 1mg/ml bentonite and sedimented through 1 M sucrose, again demonstrating a selective interaction of bentonite with monomeric ribosomes. When C ribosomes were prepared with 1.5mg/ml bentonite the total yield of rRNA was slightly reduced (Fig. 2b, solid line; Table 1). However, when the bentonite concentration in the homogenization medium was increased to 2 mg/ml, a substantial loss of rRNA was found in C ribosome preparations (Fig. 2b, dotted line; Table 1) showing that at higher bentonite concentrations the yield of all classes of ribosomes is reduced.

A possible explanation for this loss of ribosomes would be the formation of a ribosome - bentonite complex formed by salt bonding of negatively charged ribosomes to the negatively charged surface of bentonite particles through magnesium and or spermidine ions. Since the bentonite is selectively sized so as to sediment in the 10,000 gravity centrifugation, complexed ribosomes would be sedimented in this step also. The fact that omission of spermidine from the homogenizing medium that contained bentonite did not increase the yield of monomeric ribosomes suggests that the loss of ribosomes was due to the formation of a ribosome - magnesium - bentonite complex. Should this be true, lowering the magnesium concentration in the homogenizing medium ought to increase the yield of ribosomes. The effect of lower magnesium concentrations on the structural integrity of oat ribosomes was first determined by homogenizing the sections in 0.001 M $MgCl_2$ in the absence of bentonite. The resulting sucrose gradient absorbancy profile of the ribosome preparation showed both ribosomes and polyribosomes to have been almost totally degraded to the two ribosome sub unit species (Fig. 1c).

This observation ruled out lowering magnesium as a means of increasing ribosome yield, and in addition, further substantiates the relatively high con-

centration of magnesium necessary for plant ribosome stability. This high magnesium requirement has been found also for cotton ribosomes (Waters and Dure, in preparation) suggesting that plant ribosomes are similiar to bacterial ribosomes in this respect (Tissieres et al., 1959; Silman et al., 1965)rather than to mammalian ribosomes whose magnesium requirement is much lower (Silman et. al., 1965).

Bentonite is not commonly used in the preparation of ribosomes, and in those instances where it has been used, a recovery of the total ribosome complement was not imperative. (Peterman and Pavlovec, 1963; Keller et. al, 1964). However, bentonite is commonly used as an RNase inhibitor in RNA purification procedures. We have further noted that higher yields of RNA from plant tissues are obtained when bentonite is used in conjunction with EDTA throughout purification.

The loss of ribosomes caused by bentonite appears to be related to unknown constituents in the tissues used. When ribosomes are extracted from oat coleoptile sections alone (leaf tissue removed), bentonite at 1 mg/ml causes a total precipitation of ribosomes in the 10,000 gravities centrifugation. In other more dense tissues the loss of ribosomes is much less. It would appear that other cellular components absorb onto the bentonite surface, thereby protecting ribosomes from precipitation, and that the loss of ribosomes is related to their concentration as well as to magnesium concentration.

An alternative interaction between bentonite and ribosomes may be visualized as a binding of ribosomes to the surface of the bentonite particle through the positively charged basic amino acids of the ribosomal proteins.

Na Doc was routinely utilized in the preparation of these ribosomes and the point of addition did not appear to effect the yield. In order to determine if any of these ribosomes were enclosed in membraneous structures C ribosomes were prepared without bentonite or Na Doc. A sucrose gradient absorbancy profile of one of these preparations (Fig. 2c) shows a somewhat reduced yield of

all classes of ribosomes. Since both monomeric ribosomes and polyribosomes are extracted in the same relative concentration with and without Na Doc, it is not felt that this reduced yield is necessarily indicative of membrane-bound ribosomes existing in these tissues at this stage of germination.

In many studies which relate ribosome - polyribosomes changes to tissue differentiation, no effort has been made to ascertain whether the total ribosome complement has been extracted, or whether some non-random sample of this complement has been isolated. In our opinion rigorous proof of quantitative extractions or of representative sampling is necessary in studies correlating nucleic acid metabolism with differentiation.

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