Influence of Ionic Strength, pH, and Chelation of Divalent Metals on Isolation of Polyribosomes from Tobacco Leaves

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ABSTRACT

A procedure was developed for extracting polyribosomes from tobacco (Nicotiana sp) leaves. Unexpanded leaves ground in a medium consisting of 200 mM tris-HCl, pH 9, 400 mM KCl, 200 mM sucrose, and 35 mM MgCl₂ yielded larger amounts of polyribosomes with less degradation than polyribosomes from leaves extracted with buffers of lower ionic strength or pH. Extraction of polyribosomes from expanded leaves required the inclusion of ethyleneglycol-bis(2-aminoethyl ether)tetracetic acid (EGTA), a divalent cation chelator with a high affinity for Ca²⁺, Cu²⁺, and Zn²⁺. EGTA also improved isolation of polyribosomes from unexpanded leaves. Addition of 25 mM Ca²⁺, Cu²⁺, or Zn²⁺ to extracts from young leaves precipitated polyribosomes, and density gradient profiles of polyribosome preparations from the cation treatments mimicked profiles from expanded leaves which were extracted without EGTA. Polyosome precipitation by Ca²⁺ was prevented by EGTA. Endogenous Ca²⁺ was present in unexpanded leaves in sufficient concentrations (25 mM) to cause some precipitation of polyribosomes during extraction, and this cation increased by 60% in expanded leaves. Cu²⁺ and Zn²⁺ were not present in amounts sufficient to cause polyribosome precipitation. The results show that recovery of polyribosomes may be reduced by divalent cations in leaf tissue, and this can be overcome by chelation of these ions with EGTA.

Polyribosome metabolism and synthesis of mRNA can be expected to be altered in plants undergoing changes in metabolism during stress, development, or viral infection. An understanding of these processes requires polyribosome isolation procedures which provide high yields of polyribosomes and minimum nuclease activity. Many extraction media inhibit ribonuclease activity during the isolation of polyribosomes from plant, fungal, and animal tissues, and these might be applied to tobacco leaf tissues. The extraction media have contained: protein inactivators (1, 21), RNAse absorbents in the presence (6, 18) and absence (13, 17, 20, 21) of detergents, polyamions (6, 16), high ionic strength (4, 9, 13, 19), and high pH (4, 9, 13). The combination of high ionic strength and high pH reduces polyosome degradation (4, 9, 13), but these seem to be more effective on germinating seeds (18), etiolated tissue (8), and immature leaf tissue (4), than on mature leaf tissue (2).

The difficulties in recovering polyribosomes from mature leaves may be caused by factors other than RNAse activity, such as polyosome precipitation due to accumulation of divalent cations during leaf expansion. Aggregation of polyribosomes is caused by low levels of Cu²⁺ and Zn²⁺ in sucrose gradients (15), and by high concentrations of Ca²⁺ in extraction media (10, 12). Ca²⁺ also stimulates the degradation of pea polyribosomes, possibly by activating a heat-labile nuclease (12).

We report methods for improving polyosome isolation by grinding young and expanded tobacco leaves in high pH and high ionic strength buffers containing EGTA, a divalent cation chelator with a high affinity for metallic cations (14).

MATERIALS AND METHODS

Plant Material. Polyribosomes were isolated from a Nicotiana hybrid (N. clevelandii x N. glatinoidea) developed by Christie (5). Plants were grown in a greenhouse in 15-cm pots containing autoclaved soil (50% sandy clay soil and 50%peat moss). Leaves for polyosome extraction were: (a) unexpanded leaves, 1 to 3 cm long, surrounding the growing point, or (b) expanded leaves, 7 to 12 cm long, one-third to one-half way down from the top of the plant. Plants were sometimes transferred to a growth chamber (27 C, 40 to 45 klux) for 2 to 4 hr before use. Leaves were usually harvested in the morning immediately before extraction.

Polysome Extraction Procedure. The best polyribosomes were obtained by exciting leaf midribs with a razor blade and grinding 0.6 g of leaf blades in a mortar containing 6 ml of extraction buffer (200 mM tris-HCl, pH 9; 400 mM KCl; 200 mM sucrose; 35 mM MgCl₂; and 25 mM EGTA). All pH adjustments were made at room temperature; buffers and gradients were cooled to 0 to 4 C before use. Sucrose (RNase-free) was from Schwarz-Mann. The brei was clarified by centrifuging at 15,500 rpm for 10 min in the SS-34 rotor of a Sorvall RC2B centrifuge. The polyribosomes were pelleted from the supernatant through a 4-ml layer of 1.75 M sucrose (in 40 mM tris, pH 9; 200 mM KCl; 30 mM MgCl₂; and 5 mM EGTA) at 60,000 rpm for 60 min at 4 C in the type 65 rotor of a Beckman L2-65 centrifuge. The pale green opalescent pellets were recovered by aspirating the supernatant from the centrifuge tubes. Either the pellets were stored at –20 C or 0.2 ml of resuspension buffer (40 mM tris-HCl, pH 8.5; 200 mM KCl; 30 mM MgCl₂; and 5 mM EGTA) was immediately added to them. Polyribosomes were resuspended by flushing resuspension buffer over the pellet with a Pasteur pipette and stirring the mixture with a vortex mixer. The suspensions were then layered on gradients equilibrated in nitrocellulose centrifuge tubes. The gradients were formed by layering 0.8, 1.6, 1.6, and 0.8 ml of 150, 300, 450, and 600 mg sucrose/ml, respectively, of gradient buffer (40 mM tris-HCl, pH 8.5; 20 mM KCl; and 10 mM MgCl₂). The preparations were centrifuged at 45,000 rpm in an SW 50.1 rotor at 4 C for 40 or 50 min. In some instances resuspended polyribosome pellets were centrifuged in the SW 41 rotor for 65 min at 39,000 rpm through gradients formed by layering 2, 4, 4, and 2 ml of 125. 250, 375, and 500 mg su-

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2 Abbreviation: EGTA: ethylene glycol bis(2-aminoethyl ether)tetraacetic acid (from Sigma Chemical Co., dissolved in H₂O and adjusted with NaOH to pH 9 before use).

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crose/ml, respectively, of gradient buffer. Gradients for SW 50.1 and SW 41 rotors were allowed to equilibrate for at least 18 hr at 0 to 4 C before use. All operations were conducted as described above except where otherwise specified in the text. Most experiments were repeated at least three times. The gradients were analyzed with an ISCO model D density gradient fractionator attached to an ISCO model UA5 absorbance monitor. The area of different polysome constituents was measured with a planimeter for quantitative comparison of their absorbance in density gradients.

**Determination of Divalent Cations in Leaf Tissue.** The concentration of divalent cations in unexpanded and expanded leaves was compared by atomic absorption spectrophotometry of leaf extracts. Blades of unexpanded or expanded leaves were ground in extraction buffer and centrifuged in the SS-34 rotor as described in the polysome extraction procedure. Ca\(^{2+}\), Cu\(^{2+}\), and Zn\(^{2+}\) contents of the supernatants were determined with a Perkin-Elmer atomic absorption spectrophotometer, model 214.

**RESULTS**

**Effects of pH and Ionic Strength on Polysome Recovery and Stability.** Because salt concentration (4, 13, 19), buffer concentration (9), and pH (4, 9, 13, 18) affect polysome recovery from various sources, the KCl concentration, tris concentration and pH were varied to determine the most appropriate combination for use with tobacco leaves. Polysomes were obtained by grinding unexpanded leaves in different extraction buffers (Fig. 1). Several components with different sedimentation rates have been identified by their sedimentation behavior and RNase sensitivity (Figs. 1 and 3). These are ribosomal subunits (s), monomeric ribosomes (m), and various classes of polymerized ribosomes which have been called "mers" (8). The mers are presumably various numbers of ribosomes attached to mRNA, and sediment as dimers (2-mers), trimers (3-mers), through 7-mers or 8-mers, to aggregates which sediment more rapidly and are not resolved on the gradients (Figs. 1 and 3).

![Graph](https://example.com/graph.png)

**Fig. 1.** Effects of ionic strength and pH on sucrose density gradient profiles of polysomes extracted from unexpanded tobacco leaves. Polysomes were isolated as described under "Materials and Methods"; pH and ionic strength of the extraction buffer varied and EGTA was omitted from the buffer. Polysomes were centrifuged through density gradients in the SW41 rotor for 65 min. Extraction buffer contained: A: 40 mm tris, 60 mm KCl, pH 8; B: 40 mm tris, 400 mm KCl, pH 8; C: 40 mm tris, 400 mm KCl, pH 9; D: 200 mm tris, 60 mm KCl, pH 8; E: 200 mm tris, 400 mm KCl, pH 8; F: 200 mm tris, 400 mm KCl, pH 9. Subunits are designated s, monosomes and mer peaks as 2, 3, 4, 5, 6, and 7.

Polysomes extracted in 40 mm tris had higher absorbance (Table I) but showed evidence of more RNase degradation than polysomes prepared with 200 mm tris (Fig. 1). The increased stability of polysomes extracted in 200 mm tris is numerically illustrated by the higher ratios of >5-mers to ≤5-mers (Table I). Polysome degradation was reduced when KCl was increased from 60 mm to 400 mm (Table I). The total recovery of polysomes increased when the levels of KCl were increased (Table I). At higher ionic strengths (Fig. 1, C, E, and F) the increased ratios of subunits to monosomes illustrate the variation of these components in buffers of different ionic strength.

Recovery of polysomes, as measured by increased absorbance in the gradients, improved when the pH of the extraction medium increased from 8 to 9 in the presence of 400 mm KCl (Table I). The polysomes sedimented deeper in the gradients at pH 9 (Fig. 1, B versus C and E versus F), and the ratios of the large to small polysomes increased (Table I). Polysome profiles were not appreciably altered when the pH of the extraction buffer increased from 9 to 9.2, but polysomes were more stable at pH 9 than at pH 8.5 in both 60 and 400 mm KCl. Thus, the extraction medium for subsequent experiments consisted of 200 mm tris, 400 mm KCl, and pH 9.

Triton X-100 (0.5%), sodium deoxycholate (1%), 0.15 μM spermidine, yeast RNA (0.1–1 mg/ml), or 0.01 μM dithiothreitol in the extraction medium did not improve polysome recovery. The NaCl and bentonite extraction buffer of Leaver and Lovett (13) decreased the yield of polysomes, as did bentonite (10 mg/ml) when added to our high pH, high KCl buffer. We did not use bentonite in subsequent experiments because it absorbs some nucleoproteins and RNA (3).

**Effect of EGTA on Polysome Isolation from Expanded and Unexpanded Leaves.** Attempts to extract polysomes from expanded leaves with pH 9 extraction medium containing 400 mm KCl and other additives were unsuccessful. Density gradient profiles of these preparations were similar to those of Figure 2D. When the extraction medium was supplemented with 25 mm EGTA, a divalent cation chelator with a high affinity for metals (14), large polysomes were recovered with little evi-
gence of degradation (Fig. 2F). Addition of 5 mM EGTA to the extraction medium increased recovery of monosomes but did not always improve polysome recovery from expanded leaves (Fig. 2E). In three of five trials, polysome recovery from expanded leaves was improved with 5 mM EGTA, but not to the same extent as with 20 or 25 mM EGTA (Fig. 2F). Density gradient profiles of EGTA extracts from unexpanded leaves had more polysomes and fewer monosomes (Fig. 2B, C). When the concentration of EGTA was increased to 50 mM without increasing the concentration of Mg<sup>2+</sup> in the extraction medium, the polysomes were destroyed and a single component was produced which sedimented slightly above the subunit peak. The concentration of Mg<sup>2+</sup> must exceed the concentration of EGTA in the extraction procedure. Polydisperse profiles were obtained from leaves extracted in 50 or 100 mM EGTA containing 60 or 120 mM MgCl<sub>2</sub>, respectively. Polysome recovery or stability was not improved by using high concentrations of EGTA, so the lower concentration (25 mM) was subsequently used for routine isolation.

Increased sedimentation of polysomes extracted with buffers of high ionic strength and high pH, probably is due to decreased RNase scission of mRNA which links the ribosomes in a chain. Similar sedimentation patterns might occur if the extraction buffer induced protein-protein interactions between ribosomes so that different ribosomal aggregation states formed. In the former case, the polysomes should be susceptible to RNase and, in the latter case, the aggregated polysomes should be resistant to RNase. The relative RNase susceptibility of polysomes isolated from unexpanded leaves in the high pH buffer containing high KCl and EGTA was determined (Fig. 3). In the absence of RNase (Fig. 3A), subunits, monosomes, and polysomes with absorbance increasing to about 10-mers were detected in the gradients. When the preparations were treated with 3 (Fig. 3B), 30 (Fig. 3C), or 300 ng (Fig. 3D) of pancreatic RNase per ml of resuspension buffer, the absorbance of large polysomes decreased so that the polysome size-class of maximum absorbance became the 7-mer, 3-mer, and 2-mer, respectively. The progressive shift to smaller polysome size with increasing levels of RNase supports the hypothesis that the polyribosomes are linked by mRNA rather than protein-protein association. A minor component of unknown composition (u) which sedimented between the monomer and dimer appeared after RNase treatments of the polysomes (Fig. 3, B, C, and D). We did not identify this component, but it appeared to be a product of RNase treatment, as indicated by its progressive increase in concentration with high RNase concentrations.

Precipitation of Polysomes by Divalent Metals. In unexpanded leaves, EGTA reduces polysome degradation (Fig. 2, B and C). Because EGTA is known to chelate metallic cations, we postulated that divalent cations in expanded leaf tissue might precipitate or degrade polysomes during extraction. To test this hypothesis, unexpanded leaves were ground in extraction buffer without EGTA, the extracts were separated into six aliquots and CaCl<sub>2</sub>, CuCl<sub>2</sub>, or ZnCl<sub>2</sub> were added. The preparations were clarified, pelleted by centrifugation, and the resuspended pellets were centrifuged through sucrose gradients. The recovery of polysomes was reduced by about 25% in media containing 5 mM Ca<sup>2+</sup> and 5 mM Cu<sup>2+</sup> (Fig. 4, B and D). But, polysome degradation was not stimulated because the ratios of the >5-mer s to ≤5-mers were similar. Ca<sup>2+</sup> and Zn<sup>2+</sup> at 25 mM reduced polysome recovery by about 90% (Fig. 4, C and F) and the

### Table 1. Planimeter Measurements of Sucrose Density Gradient Profiles of Polyribosomes Extracted in Media of Varied Ionic Strength and pH

Extraction procedure was as described in "Materials and Methods" except that EGTA was omitted.

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Relative Area of Polysome Profiles</th>
<th>5-mers ≤5-mers</th>
<th>5-mers &gt;5-mers ≤5-mers</th>
<th>5-mers &gt;5-mers/ ≤5-mers</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 mM tris, 60 mM KCl, pH 8</td>
<td>1.54 0.90 0.64 0.71</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 mM tris, 400 mM KCl, pH 8</td>
<td>2.22 1.09 1.13 1.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 mM tris, 400 mM KCl, pH 9</td>
<td>2.52 0.69 1.83 2.65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 mM tris, 60 mM KCl, pH 8</td>
<td>0.81 0.21 0.60 2.86</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 mM tris, 400 mM KCl, pH 8</td>
<td>2.15 0.51 1.64 3.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 mM tris, 400 mM KCl, pH 9</td>
<td>2.35 0.39 1.96 5.02</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 The relative areas were measured by planimeter scanning of polysome profiles. Monosomes and subunits were not included in these measurements.

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**Fig. 2.** Effect of EGTA on extraction of polysomes from unexpanded and expanded leaves. Polysomes were extracted in 200 mM tris, 400 mM KCl, pH 9 buffer as described in "Methods" but the amount of EGTA in the extraction buffer was varied. The polysomes were centrifuged in an SW50.1 rotor for 40 min as described. A: unexpanded leaves (UL), no EGTA; B: UL, 5 mM EGTA; C: UL, 25 mM EGTA; D: expanded leaves (EL), no EGTA; E: EL, 5 mM EGTA; F: EL, 25 mM EGTA.
polysome patterns on density gradients were similar to those of preparations from expanded leaves (Fig. 2D). Polysome recovery was reduced 70% by 25 mM Cu\textsuperscript{2+}, but the ratios of large to small polysomes did not indicate polysome degradation. In one control experiment up to 60 mM Mg\textsuperscript{2+} added to the extraction medium did not reduce the recovery of polysomes. It is concluded that the metallic cations act specifically to reduce recovery of polysomes, and polysome loss is not due to increases in the total concentration of divalent cations.

Different cations in leaves might have a synergistic effect on the reduction of polysome recovery. This possibility was tested by comparing polysome recovery in preparations containing 5 mM each of Ca\textsuperscript{2+}, Cu\textsuperscript{2+}, and Zn\textsuperscript{2+} with the recovery in preparations which contained 15 mM Ca\textsuperscript{2+}, 15 mM Cu\textsuperscript{2+}, or 15 mM Zn\textsuperscript{2+}. The polysomes recovered by the cations acting together was about 80% as high as controls which did not contain divalent metals. Polysome recoveries after 15 mM Cu\textsuperscript{2+}, Zn\textsuperscript{2+}, or Ca\textsuperscript{2+} treatments were about 80%, 70%, and 40%, respectively, as high as the control preparation. These results show that precipitation by different divalent cations is an additive effect of the specific cations' concentration, and suggests that low levels of divalent metals in tobacco leaves do not interact synergistically to cause polysome precipitation.

The ability of EGTA to prevent precipitation of polysomes by Ca\textsuperscript{2+} in the extraction buffer was tested (Fig. 5). When 15 mM Ca\textsuperscript{2+} was added to a preparation, the absorbance of the recovered polysomes was about 30% of the control (Fig. 5B), but polysome recovery was greater than 95% when 25 mM EGTA was added before (Fig. 5C) or after (Fig. 5D) Ca\textsuperscript{2+} addition. Thus, EGTA is able to reverse as well as prevent polysome precipitation by Ca\textsuperscript{2+}.

Divalent Cation Levels in Unexpanded and Expanded Leaves.
Ca\(^{2+}\), Cu\(^{2+}\), and Zn\(^{2+}\) content was determined by atomic absorption spectrophotometry by extracting leaf tissue with polysome buffer containing EGTA to determine if the metal concentration in expanded leaves was sufficient to affect polysome recovery. Only Ca\(^{2+}\) is concentrated enough to interfere with polysome extraction (Table II). Ca\(^{2+}\) concentrations in expanded leaves (Table II) are probably sufficient to cause some polysome aggregation when the cells are disrupted. The concentration of Ca\(^{2+}\) increased nearly 60% during leaf expansion, and this level, even when reduced 10-fold by dilution with extraction buffer, caused decreases in polysome recovery (Fig. 4B). Similar cation levels were measured when EGTA was omitted from the extraction media. The effects of pool sizes and intracellular location of Ca\(^{2+}\) pools were not investigated, but these may also influence polysome recovery, as may enzymes such as RNases or polyphenol oxidase which may require metals for optimal activity.

**DISCUSSION**

We have described conditions for reproducible isolation of highly polymerized polysomes from tobacco leaf tissue. The pH and ionic strength of the extraction medium influenced recovery of undegraded polysomes from unexpanded leaf tissue as reported in studies with different tissue (4, 9, 18). Polysome degradation was inhibited at high pH and at high concentration of tris in the extraction medium. The polysome recovery was improved by increasing the concentration of KCl. Increased polysome recovery in high KCl may be caused by elution of polysomes from membranes (11) at the high salt concentration, but this was not investigated.

Addition of EGTA to the extraction medium was essential for recovery of polysomes from expanded leaf tissue, and it also reduced polysome degradation from unexpanded leaves. This additive presumably reverses polysome aggregation caused by Ca\(^{2+}\) in leaf tissue. EGTA may substantially improve polysome recovery in plant and animal tissues which contain high Ca\(^{2+}\) concentrations, and it should be tested as an additive to extraction media when poor recovery of polysomes occurs.

Addition of Ca\(^{2+}\) to polysome extracts of pea was reported to degrade polysomes by activating RNase (12). We did not detect polysome degradation in extracts which were supplemented with metal ions. This may be due to the high ionic strength of tris used in the grinding buffer, since a similar inhibition was also found for the Ca\(^{2+}\)-activated polysome degradation reported in peas (12).

The ratio of monosomes to polysomes is frequently used to estimate the integrity of polysomes. We have observed that this ratio and that of subunits to monosomes varies with ionic strength of buffers to such an extent that the monosome to polysome ratio was not useful for measuring polysome integrity. The monosome level decreased with increasing tris concentration. KCl concentration, and pH while the subunit concentration was altered only slightly. Comparison of ratios of the 2-mers, 3-mers, 4-mers, and 5-mers with more highly polymerized polysomes (7) reflect RNase degradation more reliably than the ratio of monosomes to polysomes. When this value is combined with the absorbance of the polysomes in density gradients it provides a more consistent and reliable measure for distinguishing between polysome degradation and polysome precipitation such as that induced by Ca\(^{2+}\).

**Acknowledgments**—We thank M. A. Young for technical assistance and H. E. Parker for assistance with atomic absorption spectrophotometry.

**LITERATURE CITED**

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**Table II. Calcium, Copper, and Zinc Concentrations of Polysome Extracts from Unexpanded and Expanded Tobacco Leaves**

<table>
<thead>
<tr>
<th>Metal</th>
<th>Cation Conc</th>
<th>Unexpanded leaves</th>
<th>Expanded leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+})</td>
<td>26.55 (±1.03) mm</td>
<td>41.87 (±1.45) mm</td>
<td></td>
</tr>
<tr>
<td>Zn(^{2+})</td>
<td>61.65 (±0.05) µM</td>
<td>72.65 (±6.53) µM</td>
<td></td>
</tr>
<tr>
<td>Cu(^{2+})</td>
<td>&lt;20 µM</td>
<td>&lt;20 µM</td>
<td></td>
</tr>
</tbody>
</table>

Samples for mineral analysis were obtained by grinding leaf tissue with extraction buffer containing 25 mm EGTA. Extracts were centrifuged at 15,000 rpm in the SS-34 rotor for 10 min and the supernatants were stored at 4 °C until analyzed by atomic absorption spectrophotometry. The data represent the concentration of divalent cations based on fresh weight of leaf tissue ± standard error of the mean.


