Polyribosomes from Peas

AN IMPROVED METHOD FOR THEIR ISOLATION IN THE ABSENCE OF RIBONUCLEASE INHIBITORS[1]

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ABSTRACT

Profiles of polyribosomes were obtained from etiolated stem segments of *Pisum sativum* L. var. Alaska isolated in various buffers. Tissue homogenized in a medium containing 0.2 M tris-HCl, pH 8.5, 0.2 M sucrose, 30 mM MgCl₂, and 60 mM KCl yielded polyribosomes exhibiting far less degradation than tissue homogenized in conventional media containing tris-HCl at lower ionic strength and pH. A further decrease in degradation was found when polyribosomes were sedimented through a sucrose pad buffered at pH 8.5 prior to centrifugation. Increased separation was obtained using heavy (125–500 mg/ml), linear sucrose gradients. Using these techniques, messenger RNA species bearing up to 12 ribosomes (dodecamers) were resolved, with messenger RNA chains bearing 9 ribosomes (nonamers) being the most abundant (having the highest absorption peak). The data presented suggest that buffer of high ionic strength and high pH was more effective in preventing degradation of polyribosomes than was diethyl pyrocarbonate and, furthermore, that ratios involving large polyribosomes (hexamers and larger) were more accurate indices of degradation than were ratios involving total polyribosomes.

Weeks and Marcus (9) and Anderson and Key (1) reported that many published polyribosomal profiles were indicative of considerable degradation due to endogenous ribonuclease acting during isolation. Conclusions concerning the functional status of the tissue based on information from degraded polyribosomes may, therefore, be misleading. Because the proportion of polyribosomes increased when tissue was ground in the presence of the ribonuclease inhibitor, diethyl pyrocarbonate, Weeks and Marcus (9) and Anderson and Key (1) suggested that such an inhibitor should be employed routinely for work involving polyribosome distribution in plants. This inhibitor might be used profitably with peas, as they have been shown to contain high levels of endogenous RNase (5), some of which is firmly associated with the microsomes (2, 6).

Situations exist, however, which preclude the use of DEP.[2] These include assay of polyribosome-associated enzymes such as RNase itself (2), assay of other enzymes which may be DEP-

sensitive, *e.g.*, cellulase (unpublished data), and measurement of protein synthetic capacity *in vitro* (1, 9). In such cases, therefore, methods must be made available which permit the isolation of undegraded polyribosomes in the absence of DEP. We report the development of such a method.

MATERIALS AND METHODS

Ten to 20 apical 10-mm segments (200 to 450 mg tissue) from the third internode of dark-grown Alaska pea seedlings (*Pisum sativum* L.) were frozen on Dry Ice and ground in a mortar in at least 10 volumes of grinding buffer (buffer A). The composition of buffer A is given in the text for each experiment. The resulting brei was clarified by centrifugation for 20 min at 29,000g, and the supernatant was gently layered over a 4-ml pad of 1.5 M sucrose in buffer B (40 mM tris-HCl, pH 8.5; 10 mM MgCl₂; 20 mM KCl) and centrifuged for 90 min at 95,000g (average) in the 40 rotor of a Spinco Model L ultracentrifuge. The pellet was rinsed gently and resuspended in 0.5 ml buffer B by means of a Vortex-Genie mixer. Aliquots (usually 0.5 ml) of resuspended polyribosomes were layered on linear (125–500 mg/ml) sucrose gradients in buffer C (20 mM tris-HCl, pH 8.5; 10 mM MgCl₂; 20 mM KCl) and spun for 75 min at 122,000g (avg) in an SW-36 rotor. The gradients were prepared by layering 2 ml of sucrose at 500 mg/ml in cellulose nitrate tubes followed by 4 ml at 375 mg/ml, 4 ml at 250 mg/ml, and 2 ml at 125 mg/ml and equilibrated for 24 to 48 hr at 2 C (3). Lower concentration gradients were used (*e.g.*, 75–300 mg/ml and 100–400 mg/ml) and spun for shorter periods of time, but poor resolution was obtained. All operations were conducted at 0 to 4 C except where stated otherwise.

After density gradient centrifugation, the contents of the tubes were analyzed in an ISCO Model 640 fractionator and the $A_{254 \text{ nm}}$ monitored continuously. The areas in different regions of the polyribosomal profiles were determined from the average of three measurements with a planimeter. The different regions were: total absorbing material (T), *i.e.*, polyribosomes plus monosomes plus sub-units; polyribosomes (P), *i.e.*, material sedimenting faster than monosomes; and large polyribosomes (LP), *i.e.*, material sedimenting faster than pentamers. The ratios P/T, LP/T and LP/P were calculated. Equilibrated blank gradients were always monitored because it was found that the baseline varied from time to time. The baseline is reported for each figure and the area below excluded from calculations.

Sucrose solutions were clarified by filtering through activated charcoal to remove UV-absorbing materials and endogenous RNase (3). Diethyl pyrocarbonate (diethyl oxydiformate) was obtained from Fisher Scientific Co., and bovine pancreatic ribonuclease (type II-A) was obtained from Sigma Chemical Co.

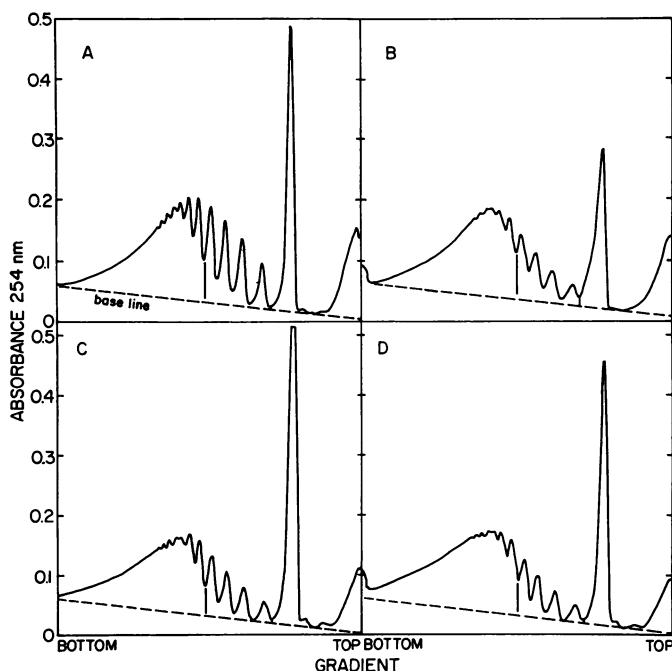[2] Abbreviation: DEP: diethyl pyrocarbonate.

FIG. 1. Polyribosome profiles obtained using grinding buffers at varying pH and concentration and a sucrose pad buffered at pH 8.5. Profiles represent the polyribosomes isolated from 20 segments (450 mg tissue). Apical 10-mm segments of etiolated pea stems were frozen on Dry Ice and ground in 5 ml buffer A (0.4 M sucrose, 20 mM KCl, 5 mM $MgCl_2$, and 5 mM β-mercapatoethanol—Birmingham and Maclachlan [2]) containing tris-HCl at varying concentration and pH. After clarification at low speed, the supernatant (5 ml) was layered on a sucrose pad in buffer B and centrifuged at 95,000g (average) for 90 min. The pellets were resuspended in 0.5 ml buffer B and layered on sucrose gradients (125 to 500 mg/ml) in buffer C and spun for 75 min at 122,000g (avg). Concentration and pH of tris-HCl in the grinding buffer; A: 50 mM, pH 7.5; B: 100 mM, pH 7.5 (as used by Birmingham and Maclachlan [2]); C: 50 mM, pH 8.5; D: 100 mM, pH 8.5.

Table I. *Distribution of Polyribosomes Obtained from Peas Ground in a Medium Buffered with Tris-HCl at Varying pH and Concentration and Sedimented through a Sucrose Pad at pH 8.5 and 7.5*

	Buffering Conditions in the Grinding Medium (tris-HCl at)			
	pH 7.5		pH 8.5	
	50 mM	100 mM	50 mM	100 mM
	% polyribosome distribution			
Extract sedimented through a sucrose pad at pH 8.5[1]				
P/T	78	79	75	80
LP/T	56	63	60	66
LP/P	72	80	80	82
Polyribosome class size with greatest absorbance				
	6–7	8–9	7	8–9
Extract sedimented through a sucrose pad at pH 7.5[2]				
P/T	67	74	72	72
LP/T	47	51	58	58
LP/P	69	72	81	81
Polyribosome class size with greatest absorbance				
	6	6	6	7

[1] Tissue identical to that illustrated in Figure 1.

[2] Tissue isolated in the same manner as that in Figure 1, except that the pH of the sucrose pad was 7.5. For definition of symbols, see "Materials and Methods."
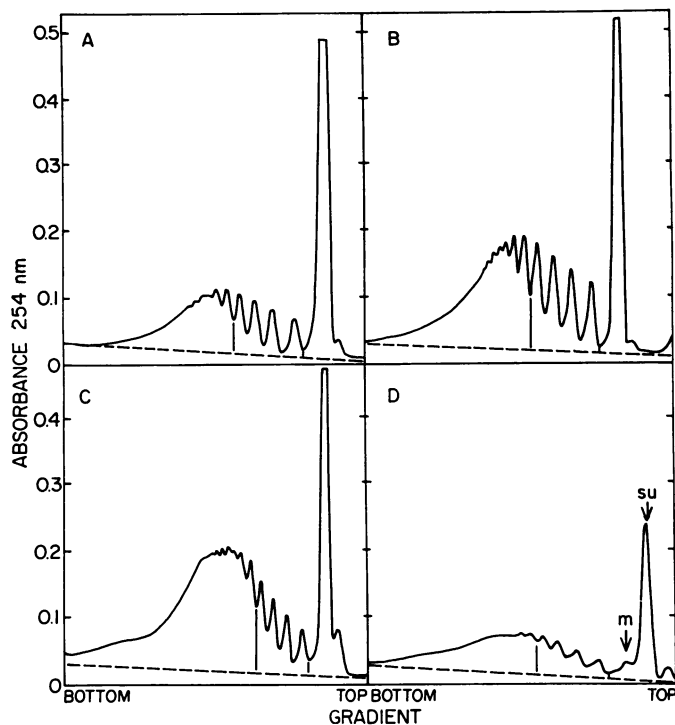

FIG. 2. A comparison of polyribosomal profiles obtained with our buffer in the presence or absence of DEP or with conventional buffers. Profiles represent the polyribosomes isolated from 20 segments (except for 2A, where only 15 were used). Buffers B and C were the same as outlined in "Materials and Methods," except the latter was at a pH of 7.5. The grinding buffers used were: A: that of Anderson and Key (1), (0.25 M sucrose; 50 mM tris-HCl, pH 7.5; 5 mM $MgCl_2$; 15 mM KCl and 0.67 mM β-mercaptoethanol); B: that of Birmingham and Maclachlan (2) (0.4 M sucrose; 100 mM tris-HCl, pH 7.5; 5 mM $MgCl_2$; 20 mM KCl; 5 mM β-mercaptoethanol); C: our buffer (0.2 M sucrose; 200 mM tris-HCl, pH 8.5; 30 mM $MgCl_2$; 60 mM KCl); D: same as C, except 20 μl DEP added to 5 ml buffer. Note the small monosome peak (m) and the large subunit peak (s.u.) obtained after DEP treatment (Fig. 1D).

Table II. *A Comparison of Polyribosomal Profiles Obtained Using Buffers of High Ionic Strength and pH in the Presence or Absence of DEP or Using Conventional Buffers*

See Figure 2 for experimental procedures. Tissue was ground in either buffer A (0.2 M sucrose; 200 mM tris-HCl, pH 8.5; 30 mM $MgCl_2$; 60 mM KCl ± 0.4% (v/v) DEP) or the buffer of Birmingham and Maclachlan (2) (100 mM tris-HCl, pH 7.5) or Anderson and Key (1) (50 mM tris-HCl, pH 7.5).

	Concn and pH of tris-HCl in Grinding Buffer			
	200 mM, pH 8.5		100 mM, pH 7.5	50 mM, pH 7.5
	+DEP	−DEP	−DEP	−DEP
	% polyribosome distribution			
P/T	71	79	76	66
LP/T	51	66	48	37
LP/P	71	83	64	56
Polyribosome size class of maximum peak height				
	6–7	9	6	6

RESULTS AND DISCUSSION

Effects of Concentration and pH of Buffer on Polyribosome Distribution. The profiles depicted in Figure 1 demonstrate the distribution of polyribosomes obtained from apical segments of etiolated peas ground in a medium containing tris-HCl at varying pH and concentration and sedimented through sucrose pad at pH 8.5 prior to density gradient centrifugation. The profiles show decreased degradation of large polyribosomes when buffers of higher concentration and pH were used. The relative distribution between total polyribosomes, large polyribosomes (hexamers and greater), and monosomes is reported in Table I. Polyribosomes, as a percentage of total $A_{254\ nm}$ material (P/T), varied from 75 to 80% with no consistent differences resulting from the isolation technique. The pro-
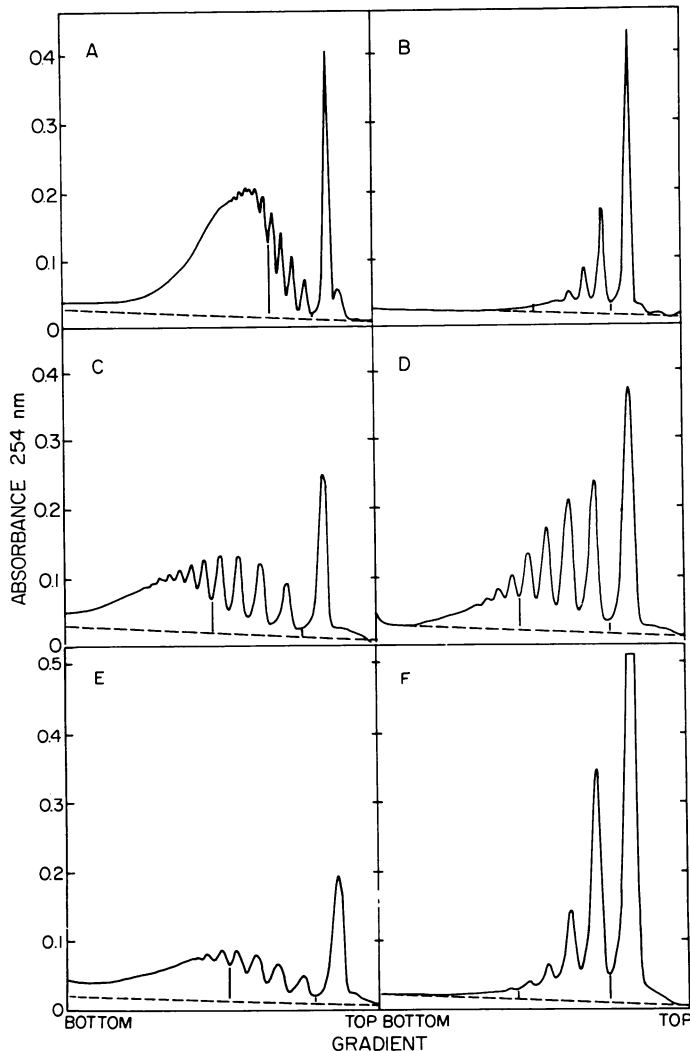

FIG. 3. Comparative resistance of polyribosomes to degradation by endogenous or exogenous ribonuclease when isolated in different buffers. Profiles represent the extract from 20 segments (except for 3B, where only 10 were used, because of the increase in the monosome fraction after RNase treatment). Buffers B and C were as outlined in "Materials and Methods;" A, B, C, and E: ground in buffer containing 200 mM tris-HCl, pH 8.5 (Fig. 2); D and F: ground in buffer containing 100 mM tris-HCl, pH 7.5 (Birmingham and Maclachlan [2] [Fig. 1]). Modifications from the regular procedure were: A: control, no RNase; B: 0.2 μg RNase added to resuspended pellet; C and D: resuspended polyribosomes incubated for 2 hr at 25C; E and F: 0.2 μg RNase added to original grinding medium (5 ml).

Table III. *Increased Resistance of Polyribosomes to Degradation by Endogenous and Exogenous Ribonuclease when Isolated in Buffers of High Molarity and High pH*

See Figure 3 for experimental procedure. Resistance of polyribosomes to endogenous RNase was tested by incubating resuspended pellets for 2 hr at 25 C. Resistance to exogenous RNase was tested by adding varying amounts of RNase to grinding buffer (5 ml) prior to homogenization.

	Concentration and pH of tris-HCl in grinding buffer							
	200 mM, pH 8.5				100 mM, pH 7.5			
	2 hr, 25 C	RNase added (μg)			2 hr, 25 C	RNase added (μg)		
		0	0.05	0.2		0	0.05	0.2
	% polyribosome distribution							
P/T	81	85	78	76	73	75	60	47
LP/T	51	73	57	47	18	53	6	5
LP/P	63	85	73	63	24	70	10	10
Polyribosome size class of maximum peak height								
	4	9	8	5	2	6	2	2

portion of large polyribosomes (LP/T or LP/P), however, varied more conspicuously and increased with both the molarity and pH of the grinding buffer (Table I). There was a similar increase in the polyribosome class size of maximum peak height, which varied with the buffer from 6 to 7 (50 mM, pH 7.5, Fig. 1A) to 8 to 9 (100 mM, pH 7.5, Fig. 1B) to 7 (50 mM, pH 8.5, Fig. 1C) to 8 to 9 (100 mM, pH 8.5, Fig. 1D). Certain features were apparent: grinding buffers of higher pH and molarity increased the proportion of fast sedimenting material; the ratios involving large polyribosomes (LP/T and LP/P) were a more accurate index of degradation than the commonly used ratios involving total polyribosomes (P/T) (4, 8); and the polyribosome class size showing the highest absorbance peak was also a good index of degree of degradation. Identical trends were obtained when material was sedimented through a sucrose pad buffered at pH 7.5, but both the proportion of large polyribosomes and the polyribosome size class of greatest absorbance were decreased when pads of low pH were used (Table I).

Other components of the homogenizing medium were found to be less critical than the ionic strength and pH of the buffer for the isolation of undegraded polyribosomes. β-Mercaptoethanol was found to be without effect, K^+ had little effect over the range 0 to 100 mM, and Mg^{2+} had little effect over the range 10 to 50 mM, although when less than 1 mM or more than 80 mM Mg^{2+} was used, virtually no material appeared on the gradient (data not shown here). The levels used henceforth (30 mM $MgCl_2$, 60 mM KCl) appeared to yield the greatest total amount of material on the gradient.

Effects of DEP and of Previously Published Buffers on Polyribosome Isolation. Besides Trewavas (8), who used NH_4Cl rather than sucrose as an osmoticum, the only investigators who have published profiles of polyribosomes isolated from etiolated pea stem segments are Birmingham and Maclachlan (2). In addition, the most thorough examination of the effects of DEP on plant polyribosomes was published by Anderson and Key (1). Accordingly, an experiment was designed in which the profiles obtained using Anderson and Key's, Birmingham and Maclachlan's, and our grinding buffers were compared. Because DEP increases the acidity of aqueous solutions (9), and because higher strength buffers alone prevent some degradation of polyribosomes (Fig. 1, Table I), we increased the ionic strength of the grinding buffer to 200 mM,

keeping the pH at 8.5. The profiles depicted in Figure 2 and the numerical analysis in Table II summarize the results of such an experiment. With both Anderson and Key's buffer (Fig. 2A) and Birmingham and Maclachlan's buffer (Fig. 2B), the hexamer had the highest absorbance, and the proportion of large polyribosomes (LP/T) was less than 50%. With our buffer, the nonamer was the highest and 66% were in the form of large polyribosomes. In the presence of 200 mM tris-HCl at pH 8.5, DEP had no beneficial effects. In fact, even though DEP permitted the same proportion of total polyribosomes (P/T) to be obtained (Table II), it had a number of deleterious effects. It decreased by about 50% the total amount of material recovered on the gradient (Fig. 2D), decreased the percentage of large polyribosomes obtained (Table II), and decreased the polyribosome size class of maximum absorbance from a nonamer in its absence (Fig. 2C) to a hexamer in its presence (Fig. 2D). In addition, monosomes were almost completely converted into subunits (Fig. 2D). Therefore, it would appear that DEP is not as effective as the high concentration and pH of buffer for isolating undegraded polyribosomes from peas. Further work (not included here) indicated that pH 8.5 was superior to pH 7.5 or 8.0 and at least as effective as pH 9.0 for the isolation of undegraded polyribosomes. In addition, increasing the buffer concentration to 0.4 M caused increased conversion of monosomes into subunits while having no beneficial effect on polyribosome distribution. It is not known whether these methods will be widely applicable, although preliminary experiments indicated some decrease in degradation of polyribosomes isolated from corn root tips, which are known to be high in RNase (9).

Susceptibility to RNase. In order to demonstrate that our method was effective in isolating polyribosomes rather than ribosomal aggregates, and also to show that the effectiveness of the buffer was due to its partial inhibition of endogenous RNase, two responses to RNase had to be demonstrated. First, isolated polyribosomes must be susceptible to exogenous RNase and second, low levels of RNase in the grinding medium should cause little degradation. Both these contentions were supported by the profiles depicted in Figure 3 and the numerical analysis in Table III. Exogenous RNase, added during resuspension of polyribosomes, caused severe degradation (Fig. 3B compared with Fig. 3A). The faster sedimenting material was not, therefore, an artifact of ribosomal aggregates caused by high levels of components in the buffer (tris-HCl, MgCl$_2$ or KCl), nor by possible contaminants such as Cu^{2+} or Zn^{2+} contained therein (7). Exogenous RNase (0.2 μg) added to the grinding medium had little effect when 200 mM tris-HCl at pH 8.5 was used (Fig. 3E), but did cause severe degradation when 100 mM tris-HCl at pH 7.5 was used (Fig. 3F, Table III). The comparative resistance of polyribosomes isolated in high strength buffer was even more striking when lower levels (0.05 μg) of RNase were added prior to homogenization. Polyribosomes isolated in low ionic strength buffer were severely degraded; the maximum peak being a dimer and large polyribosomes (LP/T) comprised less than 10% of the total material on

the gradient (Table III). In contrast, polyribosomes isolated in high strength buffer in the presence of the same amount of RNase were almost undegraded; the maximum peak was an octamer and the large polyribosomes comprised more than 50% of the total material (Table III). In addition, resuspended polyribosomes obtained from tissue homogenized in high strength buffer and incubated at 25 C for 2 hr (Fig. 3C) were far more resistant to degradation by endogenous RNase than those obtained from tissue ground in low ionic strength buffer (Fig. 3D). In this experiment (Table III), as in others shown previously (Tables I, II), the percentage of total polyribosomes (P/T) did not give as accurate an indication of polyribosome degradation as did ratios involving large polyribosomes (LP/T or LP/P). For instance, when polyribosomes isolated in either low or high ionic strength buffer were incubated for 2 hr at 25 C, more than 70% of the material remained as polyribosomes (P/T) in each case. However, in the low ionic strength buffer, less than 25% of the polyribosomes were hexamers or larger (LP/P), whereas more than 60% remained as large polyribosomes when the higher ionic strength buffer was used (Table III). Similarly, the polyribosome size class of maximum absorbance was a dimer in low ionic strength buffer (Fig. 3D), but a tetramer in high ionic strength buffer (Fig. 3C).

Isolation of polyribosomes in tris-HCl at high pH (8.5) and concentration (200 mM) followed by sedimentation through a sucrose pad at pH 8.5 yielded remarkably undegraded polyribosomes in the absence of any RNase inhibitor. In addition, the ratios involving large polyribosomes (LP/P or LP/T) as well as the polyribosome size class of maximum absorption were far better indicators of the degree of degradation of isolated polyribosomes than ratios involving total polyribosomes (P/T).

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