Polyribosome Isolation in the Presence of Diethyl Pyrocarbonate[1]

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Abstract. Isolation of polyribosomes from wheat embryos and corn root tips in the presence of diethyl pyrocarbonate showed this reagent to have a protective effect on polyribosome structure. In addition, the use of diethyl pyrocarbonate allowed initial homogenization to be performed under less stringent conditions than those normally employed for polyribosome isolation. The use of the reagent is however limited, in that it is deleterious to *in vitro* ribosomal amino acid incorporation.

Numerous studies have been reported in which the size distribution of polyribosomes has been correlated with a particular physiological state (1, 2, 4, 9, 11, 12, 18, 22, 23, 25). An implicit requirement for such studies has been a method for obtaining accurate representation of polyribosome size distribution. The widespread occurrence of ribonuclease (RNAase), particularly in plant tissues, has hampered efforts to develop such a method. The inclusion of the ribonuclease inhibitors, bentonite (24) or polyvinyl sulfate (2), in the homogenizing medium is not always effective (2, 6, 16) and, in addition, may result in considerable loss of polyribosomes (6, 20). Recent reports (5, 17) describing a more promising nuclease inhibitor, diethyl pyrocarbonate (DEP), prompted an examination of its use in polyribosome isolation. This report describes the results of those studies.

Materials and Methods

Plant Materials and Germination Conditions. Wheat embryos (*Triticum vulgare*) were isolated by a modification of the method described by Johnston and Stern (7). Samples of 250 mg were imbibed in water for 5 hr at 26°. Corn seeds (*Zea mays*) were germinated for 40 hr at 26° in a solution of 0.1 mM $CaCl_2$.

Polyribosome Isolation. Imbibed wheat embryos were blotted dry, frozen on dry ice, and ground in a mortar and pestle packed in dry ice. Corn root tip sections (3 mm) were cut, immediately frozen on dry ice, and ground similarly. The ground wheat embryos (250 mg) or corn root tips (40 tips) were homogenized in a Duall homogenizer in 7 ml of a solution containing 0.25 M sucrose, 20 mM KCl, 5 mM $MgAc_2$, 50 mM tris-Cl (pH 7.7), 1 mM Cleland's reagent. If no diethyl pyrocarbonate (DEP) was to be added during homogenization, the final tris-Cl concentration was brought to 0.1 M with 1.0 M tris-Cl (pH 7.7). If DEP was included, the tris-Cl concentration was brought to 0.1 M by adding 1.0 M untitrated tris. Diethyl pyrocarbonate has a short half-life in water (5 hr at 0°), and was therefore added (0.1 ml per 10 ml grinding solution) immediately before homogenization.

The remainder of the isolation procedure was similar to the procedure described by Lin, Key, and Bracker (10). The homogenates were cleared by centrifugation for 10 min at 17,000*g*. They were then layered over 3 ml of 1.66 M sucrose containing 20 mM KCl, 5 mM $MgAc_2$, 50 mM tris-Cl (pH 7.7), 1.0 mM Cleland's reagent and centrifuged for 90 min at 225,000*g*. Microsomal pellets were resuspended in 0.5 ml of 20 mM KCl, 5 mM $MgAc_2$, 50 mM tris-Cl (pH 7.7), 1.0 mM Cleland's reagent. Approximately 1 mg of ribosomes was layered on a 10 to 34 % sucrose density gradient containing 20 mM KCl, 5 mM $MgAc_2$, 50 mM tris-Cl (pH 7.7), and 1.0 mM Cleland's reagent and centrifuged 2 hr at 90,000*g* in a SW25 rotor. The gradients were fractionated using a modified ISCO density-gradient fractionator with continual monitoring of absorbance at 254 mμ.

In Vitro *Amino Acid Incorporation.* Polyribosomes were obtained from microsomal pellets prepared as described above. Supernatant (S-23) was obtained from dry wheat embryos (13) and added to

the incorporation medium in saturating amounts. The final incubation medium contained, in addition to polyribosomes and S-23, 44 mM KCl, 3.5 mM MgAc$_2$, 1 mM ATP, 8 mM creatine phosphate, 16 μg creatine phosphate kinase, 25 mM tris-Cl (pH 8.1), and 0.125 μc ^{14}C-leucine in a total volume of 0.4 ml. After incubation at 30° for 30 min, trichloroacetic acid insoluble precipitates were collected and counted for radioactivity (12).

Sucrose solutions were treated with DEP at a concentration of 0.2 ml per 100 ml. Excess reagent was hydrolyzed by heating in a boiling water bath for 10 min. After cooling, the appropriate salts, buffer, and Cleland's reagent were added.

Diethyl pyrocarbonate was obtained from Fisher Scientific Company (diethyl oxydiformate).

Results

Most reported preparations of polyribosomes from plant tissues give sucrose density gradient patterns similar to the one presented in Fig. 1A. The inclusion of diethyl pyrocarbonate in the homogenizing medium yields a pattern (Fig. 1B) which contains a high percentage of large polyribosomes and relatively few monoribosomes. Since DEP is known to inactivate ribonuclease (5), it seems likely that the difference between the patterns (Fig. 1A and 1B) is due to protection of root tip polyribosomes by DEP. This idea was further supported by experiments (not shown) in which exogenous ribonuclease was added during homogenization. In the presence of DEP, complete protection of polyribosomes was obtained. DEP had no effect on total microsomal yield.[2]

In addition to the protective effect, the incorporation of DEP into the homogenizing medium allows some deviation from the strict control of temperature below 4°. Thus, if homogenates without DEP are incubated for 3 min at 30°, considerable polyribosome breakdown results (Fig. 1C). Similar treatment of DEP containing homogenates results in no detectable change in polyribosome distribution (Fig. 1D). It should be noted, however, that some temperature precautions must be maintained. DEP hydrolyzes to ethanol and carbon dioxide (1.5 hr half-life at 20°, 5 hr half-life at 0°) and thereby causes a decrease in pH. If allowed to proceed too far, the buffering capacity of the grinding solution is exceeded and acid precipitation of ribosomes results (Fig. 1E).

The extent of DEP protection of polyribosomes is examined in Fig. 1F. If microsomes are isolated from a grinding solution containing DEP and resuspended in a solution free of DEP, subsequent

[2] The differences in total O.D. in Fig. 1A and 1C vs. 1B and 1D reflect unequal amounts of microsomes applied to the gradients (see the legend to table I).
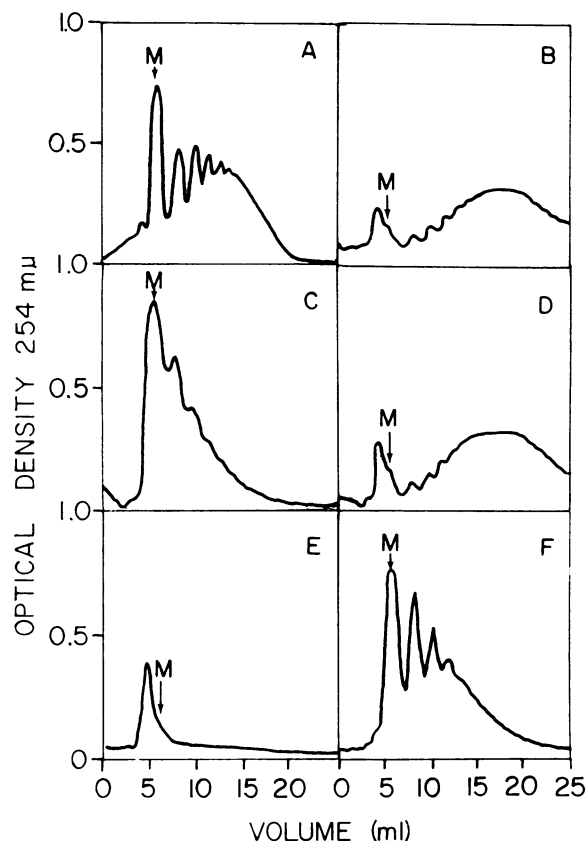

Fig. 1 Sucrose density gradient centrifugation of polyribosomes from corn root tips. A) Control — No DEP in grinding solution. B) Polyribosomes prepared with DEP in grinding solution. C) Same as (A) — root tip homogenate brought to 30° for 3 min. D) Same as (B) — root tip homogenate brought to 30° for 3 min. E) Same as (B), except all grinding and homogenization steps were performed at room temperature. F) Resuspended microsome pellet from (B) brought to 30° for 3 min. (M = momoribosomes.) (Approx. 1.2 mg of ribosomes layered on gradients A, C, and F; 1.0 mg of ribosomes layered on gradients B and D; ribosomes in gradient E from an amount of tissue normally yielding 1.2 mg of ribosomes.)

incubation for 3 min at 30° results in considerable polyribosome breakdown (Fig. 1F). Complete protection at 30°, therefore, appears to require the continuous presence of DEP.

Polyribosomes from imbibing wheat embryos can be isolated with apparently little RNAase degradation (Fig. 2A). However, even in this situation, addition of DEP decreases the proportion of single ribosomes and shifts the distribution to larger polyribosomes (Fig. 2B). To eliminate the possibility that DEP might simply be forming ribosome aggregates, polyribosomes prepared in the presence of DEP were exposed to exogenous ribonuclease for 1 min at 30°. The rapid conversion to monoribosomes (Fig. 2C) indicates that the material was
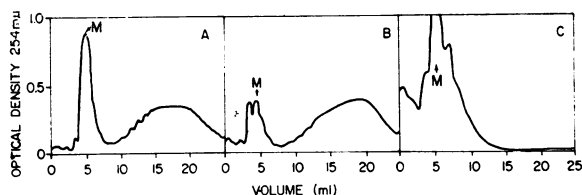
FIG. 2. Sucrose density gradient centrifugation of polyribosomes from 5 hr imbibed wheat embryos. A) Polyribosomes prepared with grinding solution containing no DEP. B) Same as (A), but with DEP in grinding solution. C) Resuspended microsome pellet from (B) exposed to 1.0 μg RNAase/ml for 1 min at 30°. (M = monoribosomes.) (Approx. 600 μg ribosomes/gradient.)

indeed polyribosomal and that DEP functions primarily as a protecting agent.

Polyribosomes prepared with DEP lose much of their capacity for *in vitro* amino acid incorporation (table I). This is not unexpected in view of the known ability of DEP to inactivate enzymes (15). Presumably, ribosome associated protein, essential for peptide synthesis (14, 21), would not be immune to DEP inactivation.

Discussion

The accuracy of conclusions drawn from experiments which seek to use polyribosome content and size distribution as an analytical method is dependent on obtaining preparations which reflect the true distribution *in situ*. From the results presented above, and a comparative survey of much of the relevant literature, it is apparent that several procedures heretofore used do not yield intact polyribosomes. The use of diethyl pyrocarbonate appears to offer a more precise method for observing polyribosome distributions. Thus, inclusion of DEP in the grinding solution for corn root tip tissue produces much improved polyribosome patterns as compared to control tissue (Fig. 1A and 1B). Even in tissues where endogenous ribonuclease presents less of a problem, such as wheat embryos, the beneficial effect of DEP is still evident (Fig. 2A and 2B). In addition, when DEP is present, strict control of temperature below 4° during polyribosome isolation is not obligatory (Fig. 1C and 1D). It should be

noted, however, that deleterious effects may occur if extensive hydrolysis of DEP is allowed (Fig. 1E).

In experiments designed to ascertain the capacity of DEP for RNAase control, exogenous ribonuclease (1 μg/ml) was added to the homogenization medium. It was found that the level of DEP used in the above experiments (0.1 ml/10 ml homogenization solution) was adequate to completely protect the polyribosomes. In the absence of DEP, complete breakdown to monoribosomes occurred. On the basis of these observations, as well as other studies describing the inactivation of proteins by DEP (5, 15), it might have been expected that presence of DEP during initial homogenization would afford permanent protection against ribonuclease. The datum of Fig. 1F, however, indicates that some ribonuclease activity is still functional in the microsomal pellet. One possible explanation is that latent ribonuclease, associated with ribosomes (6), is initially protected from DEP inactivation and is activated subsequently during incubation at 30°.

Diethyl pyrocarbonate preparations of polyribosomes appear to contain increased amounts of ribosomal subparticles as compared to control preparations. It is not known if this reflects the true situation in the intact tissue or is an effect of DEP. If plant ribosomes normally dissociate and reassociate in a manner similar to bacterial ribosomes (3, 8, 19), the possibility exists that DEP may interact with the protein of dissociated ribosomal subunits to prevent reassociation, resulting in an apparent net increase of subunit concentration.

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Table I. In Vitro *Amino Acid Incorporation by Polyribosomes*

Treatment of grinding solution		Microsomal RNA (μg)/Incubation		
Sucrose pretreated with DEP	DEP added to grinding soln.	28	56	112
		^{14}C-Leucine incorporated (cpm)		
—	—	3824	7831	15,121
+	—	3876	7994	16,358
+	+	734	1213	1484

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