EFFECTS OF DIETHYL PYROCARBONATE ON THE ISOLATION AND RECOVERY OF BOUND POLYSOMES FROM A PARTICULATE FRACTION OF RAT LIVER

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1. Introduction

The conventional treatment of rat liver postmitochondrial supernatant with detergents permits the recovery of a roughly equal mixture of undegraded free and bound polysomes [1], whereas similar treatment of the pellet containing mitochondria, lysosomes and the bulk of bound polysomes leads to the recovery of largely degraded polysomes [2, 3] in spite of the use of naturally-occurring nuclease inhibitor [4]. This presumably is due to detergent-mediated activation or release of nucleases that are refractory to nuclease inhibitor [5]. Intact bound polysomes can be prepared from purified rough membrane fractions; however, the whole procedure requires about 2 days of centrifugation and polysome recovery is low [1].

Recently, diethyl pyrocarbonate (DEP), a potent inhibitor of nucleases, has been employed for the preparation of undegraded polysomes and RNA from higher plants and bacteria [6–8]. The inactivation of pancreatic ribonuclease by DEP *in vitro* is believed to result from the formation of intra- and intermolecular peptide-like bonds which cause conformational changes and yield polymeric proteins [9]. It was, therefore, of interest to determine whether mitochondrial and lysosomal nucleases could be inactivated by DEP while still in their latent state, and thereby permit isolation of undegraded bound polysomes from a crude particulate fraction of rat liver.

In this report we provide evidence that the nuclease activities of the more rapidly sedimenting fraction of liver are poorly inhibited by DEP and further that the rapid decomposition of DEP may significantly alter the separation and recovery of subcellular constituents unless precautions are taken to prevent acidification of the preparation.

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2. Materials and methods

Male Sprague-Dawley rats, 150 to 200 g, were fasted for 16 to 20 hr before sacrifice. Livers were perfused under light ether anesthesia with ice-cold 0.25 M sucrose containing 1 mM $MgCl_2$. All subsequent operations were carried out in a cold room at 1–2° unless otherwise stated. The homogenizing medium contained 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$ and 50 mM Tris-HCl buffer, pH 7.4 at 25° (STKM); in some experiments the concentration of Tris buffer was 300 mM (high-Tris medium). Liver tissue was homogenized in 2 vol of medium in a glass homogenizer with 10 strokes of a motor-driven Teflon pestle (1,750 rpm) to obtain a minimum of 90% cell breakage.

To separate the bound polysome fraction from free polysomes, homogenate was diluted to 10% and centrifuged at 740 g_{max} for 2 min (SW 27.1 rotor, Beckman) and then the centrifugal force was increased and maintained at 135,000 g_{max} for 12 min (1.6×10^6 *g*-min). Under these conditions, about 90% of the bound polysome fraction is sedimented in the 1.6×10^6 *g*-min pellet, whereas at least 90% of the free polysome fraction remained in the supernatant [10, 11]. The pellet was gently suspended in STKM containing various amounts of DEP, mixed with 1/9 vol of 10% Trition X-100 and spun at 1,470 g_{max}

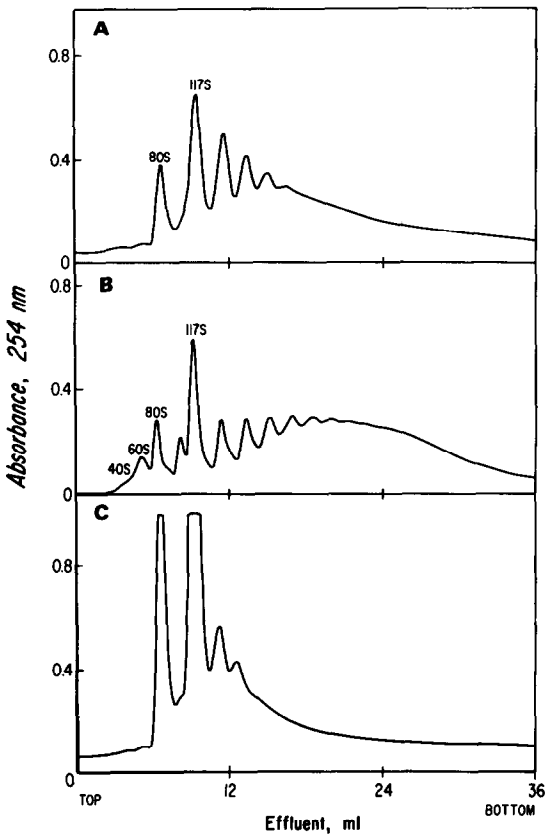

Fig. 1. Sedimentation profiles of polysomes on 15–27.8% (w/w) isokinetic sucrose gradients containing 75 mM KCl–5 mM $MgCl_2$–10 mM Tris-HCl (pH 7.4 at 25°) centrifuged at 131,000 g_{max} for 105 min in SW 27 rotor. A) Bound polysomes from 1.6×10^6 g-min pellet isolated in the presence of S_3. B) Free polysomes isolated from the post-mitochondrial supernatant. C) Bound polysomes isolated in the presence of 0.1% DEP in STKM. Input on gradient was 10 A_{260} units for free and 8 units for bound polysomes. A_{254} was continuously monitored with an automatic density gradient fractionator (ISCO). Approximate sedimentation coefficients [12] are shown above the peaks.

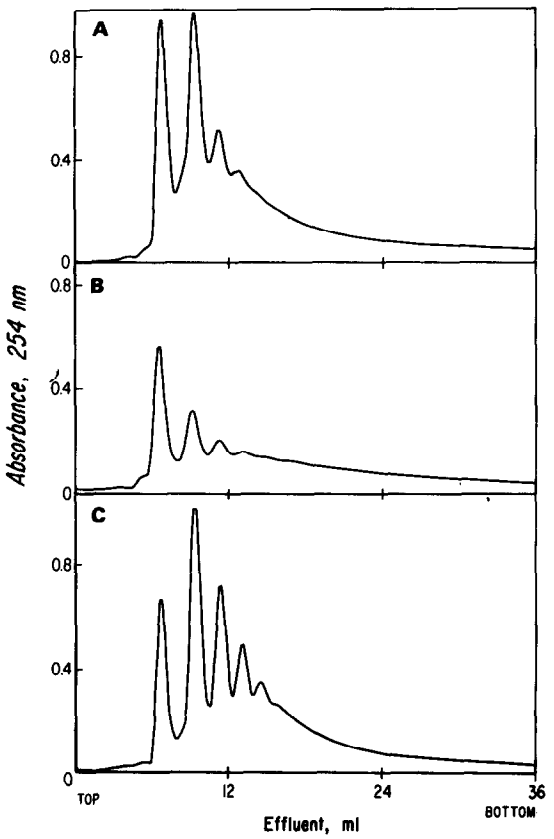

Fig. 2. Sedimentation profiles of bound polysomes from 1.6×10^6 g-min pellet. Liver was homogenized in STKM. A) Pellet in STKM–0.25% DEP incubated at 30° for 5 min. B) Pellet in STKM–0.5% DEP incubated at 30° for 5 min; similar distribution seen when supplemented with 1/3 vol of S_3 before addition of Triton or when Triton was added before incubation. C) Pellet suspended in high-Tris (0.3 M) medium containing 0.5% DEP and maintained at 1° before supplementation with 1/3 vol of S_3. Similar pattern obtained when incubated at 30° or 37° for 5 min before the addition of S_3. See fig. 1 for other details.

for 5 min (HB-4 rotor, Sorvall) to sediment nuclei. In some experiments the 1.6×10^6 g-min pellets were suspended in S_3 fraction (diluted 1:1 with STKM) to study the effect of the natural nuclease inhibitor (see below). Sodium deoxycholate (1/9 vol of 13%) was added to the supernatant to insure complete disruption of the rough endoplasmic reticulum membranes, and aliquots of the mixture (2.5 ml) were layered over discontinuous gradients comprised of 3 ml 1.38 M sucrose–TKM and 3 ml 2 M sucrose–TKM and centrifuged at 144,900 g_{max} for 24 hr (50 Ti or 65 rotor, Beckman) to pellet the polysomes. The pellet was gently suspended in TKM buffer and clarified by low-speed centrifugation prior to sedimentation analysis [12].

A particle-free supernatant fraction of 33% homogenate (S_3, 3.5×10^7 g-min) was prepared [1] for each experiment and used as a source of nuclease inhibitor [4].

Diethyl pyrocarbonate (Calbiochem) was dissolved

in cold homogenizing medium immediately before use.

3. Resuts

3.1. *Bound versus free polysomes*

Fig. 1A shows a representative sedimentation profile of the bound polysome fraction isolated from the 1.6×10^6 *g*-min pellet in the presence of S_3 as described above; for comparison, a profile of the free polysome fraction isolated from the same liver is presented in fig. 1B. The bound fraction is largely comprised of monomers through hexamers, whereas the free fraction contains a large proportion of rapidly sedimenting polysomes. Analysis of the profiles with an electronic curve analyzer (Model 310, DuPont) showed that the percentage of the total absorbance encompassed by the subunit, monosome and disome peaks was greater for the bound fraction (33%) than for the free fraction (23%), suggesting disaggregation of bound polysomes by nucleases as a cause for their small size. Accordingly we attempted to inactivate the nucleases of 1.6×10^6 *g*-min pellet by treatment with DEP both before and after the addition of detergents.

3.2. *Studies with DEP in standard-Tris medium*

In initial studies, livers were homogenized in STKM containing 0.1%, 1% and 5% DEP and the 1.6×10^6 *g*-min pellets were suspended in the corresponding medium. When high DEP concentrations (1% to 5%) were used, both polysome and nuclear fractions were aggregated and heavily contaminated with extraneous cellular materials and the yield of bound polysomes was low. These effects were less pronounced with 0.1% DEP, but sedimentation analysis of bound polysomes (fig. 1C) revealed an increase in monomer through tetramer peaks as compared to polysomes isolated in the presence of S_3 (fig. 1A), suggesting a lower extent of nuclease inhibition by DEP.

To determine the effect of temperature on the DEP reaction with bound nucleases, 1.6×10^6 *g*-min pellets were suspended in STKM containing 0.25% or 0.5% DEP and incubated at 30° for 5 or 10 min or at 37° for 5 min before the addition of Triton. As noted earlier, 0.5% DEP-treated fractions were aggregated and contaminated with extraneous materials. Sedimentation analysis of bound polysome fractions showed that polysome breakdown was extensive with either 0.25% DEP (fig. 2A) or 0.5% (fig. 2B) at 30° or 37°; with 0.5% DEP, the total absorbance on the gradient was low compared to the input indicating the presence of contaminants. This was also shown by the ratios of A_{260} to A_{280} which were 1.70–1.75 for polysomes derived from preparations treated with either S_3 or 0.25% DEP and 1.54–1.60 for polysomes obtained with 0.5% DEP.

To ascertain whether breakdown of bound polysomes occurred because of the absence of nuclease inhibitor in the 1.6×10^6 *g*-min pellets during Triton treatment, the latter were suspended in STKM containing 0.5% DEP, incubated at 30° for 5 min and then supplemented with 1/3 vol of S_3 before the addition of Triton. The size distribution of polysomes was similar to that obtained in the absence of S_3 (fig. 2B) suggesting that polysome breakdown occurred during incubation with DEP. Similar results were obtained when Triton was added to the suspension containing 0.5% DEP prior to incubation at 30° (not shown), suggesting that "activated or released" nucleases may be protected from reacting with DEP by other cellular materials.

3.3. *Studies with DEP in high-Tris medium*

DEP decomposes rapidly in aqueous media liberating ethanol and CO_2 which may significantly reduce the pH of STKM. With 0.5% DEP, the pH of homogenate prepared in STKM dropped from 7.4 to 5.8 after incubation at 30° or 37° for 5 min, whereas the pH of homogenate prepared with 0.3 M Tris buffer in the medium remained constant.

To investigate whether aggregation of subcellular components resulted from acidification of the preparation or from reaction with DEP, the 1.6×10^6 *g*-min pellets prepared in STKM were suspended in high-Tris medium containing 0.25% or 0.5% DEP. The suspensions were incubated at 0°, 30° and 37° for 5 min, cooled, supplemented with 1/3 vol of S_3 and treated with Triton. In high-Tris medium, no evidence of aggregation or contamination of either nuclear or polysome fractions was seen and the ratios of A_{260} to A_{280} for all bound polysome preparations were at least 1.70. Sedimentation analysis of these preparations, however, revealed that greater polysome breakdown occurred in the high-Tris, DEP medium (fig. 2C), especially at 30° and 37° (not shown), than with S_3 (fig. 1A). Similar results were obtained when suspen-

sions were treated with Triton before incubation, providing additional evidence that particulate nucleases are largely protected from reacting with DEP.

In contrast, recent studies [13] have shown that nucleases which degrade 28 S rRNA while still within the ribosome are appreciably inhibited by DEP in high-Tris medium but not in STKM. However, the high-Tris medium extracts RNA from nuclei, as shown by a low nuclear RNA content (4% as compared to 6% of cellular RNA when STKM is used). Presumably, nuclear proteins are also extracted, since a considerable amount of DNA was found in the nRNA preparations [13].

4. Discussion and conclusions

The present studies show that nucleases of the more rapidly-sedimenting fraction of rat liver (1.6×10^6 *g*-min pellet), in either "latent" or "free" state, are not wholly inhibited by DEP concentrations of 0.1 to 0.5% as indicated by the ability of the enzymes to degrade polysomes. High DEP concentrations (0.25 to 5%) significantly lower the pH of conventionally used concentrations of Tris buffer (0.05 M), causing aggregation of subcellular constituents, and thereby altering their sedimentation characteristics. Some side-effects of DEP treatment can be prevented by the use of higher buffer concentrations, i.e., 0.3 M Tris for 0.5% DEP; however, such high salt concentrations extract nuclear RNA.

Recent studies [14, 15] have shown that while DEP is a potent inhibitor of pancreatic ribonuclease *in vitro*, its inhibitory action on tissue nucleases is markedly reduced by the protective effect of other proteins [15]. Thus, high DEP concentrations (2.5–3%) are required to inhibit small amounts of tissue nucleases [15] and permit recovery of intact RNA from plant and animal tissues [7, 8, 16]. The present studies indicate that such high DEP concentrations cause denaturation and precipitation of microsomes and ribosomes, which agrees with observations made by other workers [6, 16]. These effects of DEP are due in part to rapid decomposition of this compound ($T_{½}$ = 1.5 hr at 20°) to CO_2 which, in sufficient concentration, lowers the pH of weakly buffered media to 6.0 or below. Weeks and Marcus [6] obviated this pH effect by titrating the CO_2 with a concentrated solution of Tris base during isolation of polysomes from plant tissue; however the isolated polysomes showed reduced ability to incorporate amino acids. The high concentration of Tris buffer (0.3 M) used in the present studies maintained constant pH during isolation, prevented acid precipitation of subcellular constituents and increased the yield of polysomes, but did not increase the degree of inhibition of tissue nucleases, as shown by the distribution of bound polysomes (fig. 2C). Thus, while high DEP concentrations have been used successfully for isolation of intact RNAs from plant and animal tissues, two factors, i.e., the extensive reaction of DEP with non-nuclease protein [9, 15] and the necessity to maintain neutral pH with biologically-relevant concentrations of buffer, would seem to make DEP less useful for isolation of polysomes, especially from mammalian tissue.

Acknowledgements

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