A Comparison of Three Phenol Methods for Extraction of Rat Liver RNA

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Three commonly used phenol methods for extraction of rat liver total RNA, requiring the use of 4-aminosalicylate, naphthalene-1,5-disulfonate, and heat, respectively, have been compared.

Different amounts of giant heterogeneous nucleoplasmic RNA were isolated by the three methods. The amounts isolated, being dependent upon the ionic strength of the extraction liquid, were inversely related to the amounts of rapidly labeled RNA present in the 4–28 S region. No method was able to extract additional rapidly labeled RNA after extraction by any of the other methods, suggesting that the differences observed in the patterns were caused by aggregation/disaggregation during extraction.

A previously undescribed, extremely rapidly labeled, small molecular fraction was isolated, more abundantly by the 4-aminosalicylate method than by the others. It was tentatively identified as growing polynucleotide chains. The amounts isolated were partly dependent upon the ionic strength of the aqueous phase used for extraction.

Isolated rat liver total RNA may give different sedimentation and electrophoretic patterns according to the method of extraction (*e.g.*, the methods of Parish and Kirby,¹ Attardi *et al.*,² Warner *et al.*³). The differences are found both for rapidly labeled RNA (rRNA) including giant heterogeneous nucleoplasmic RNA (giant hnRNA), and for ribosomal RNA (rRNA). This may be explained by differential extraction, configurational changes, aggregation/disaggregation phenomena, or by splitting of covalent bonds during the extraction.

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The present comparison of the above-mentioned methods was performed as part of an attempt to determine which of the isolated RNA structures represented *in vivo* existing molecules, and which were caused by the extraction procedures.

MATERIALS AND METHODS

Animals. Male 250-g CD*F inbred rats (Charles River Breeding Laboratories, Wilmington, Mass.), fed *ad libitum*, were killed at intervals varying from 1 min to 48 h after intraperitoneal injection of radioactive precursors.

Chemicals. Orotic acid-5-³H (22.3 Ci/mmol) and orotic acid-6-¹⁴C (60.8 mCi/mmol), The Radiochemical Centre, Amersham, United Kingdom. Pronase (B-grade), Calbiochem. Ribonuclease II-A and Deoxyribonuclease (DN-C), Sigma Chemical Company. The latter was freed of RNAase by isoelectric focusing in a 4–6 pH-gradient, and stored frozen. (Both Worthington RNAase-free DNAase and Sigma electrophoretically purified DNAase were found to contain traces of RNAase.) Purified human salivary α -amylase, gift from Dr. A. Hensten Pettersen, Department of Microbiology, Dental Faculty, University of Oslo.

Extraction procedures. The extraction procedures require the use of 4-aminosalicylate, naphthalene-1,5-disulfonate, and heat (60 °C), respectively, for the release of rRNA. To minimize the possibility of degradation by RNAase, of aggregation due to salting-out procedures⁴ or to high temperature,⁵ DNA and glycogen were removed by specially purified enzymes, and heat extraction was performed at 55 °C. Bentonite (treated according to Fraenkel-Conrat *et al.*⁶), 1 mg/ml, and dextran sulfate, 0.1 mg/ml, were added to all aqueous solutions except to the TKM-buffer (Tris 0.05 M, KCl 0.025 M, MgCl₂ 2.5 mM; pH 7.0) used for incubation with DNAase and amylase, where

bentonite was omitted. All glassware was cleaned with chromic-sulfuric acid, 0.1 M sodium hydroxide, or 2 % w/v SDS prior to rinsing in doubly distilled water. To avoid selective loss of low-molecular-weight RNA, all collections of precipitates were performed by centrifugation of ethanolic solutions at not less than 10 000 *g* for 1½ h. Phenol and *m*-cresol were distilled under reduced pressure before use.

Method 1 was based upon the 4-amino-salicylate method of Parish and Kirby.¹ However, the extraction was performed at 4 °C, and DNA and glycogen were removed by exposure to 250 µg DNAase and 500 µg amylase

in 25 ml TKM-buffer at 25 °C for 1 h. The enzymic treatment was terminated by shaking with 2 % w/v SDS and ½ volume phenol reagent at 4 °C for 15 min, and the nucleic acids recovered from the aqueous phase by ethanol precipitation.

Method 2 (modified from Attardi *et al.*²). The aqueous phase, used for homogenization and extraction, consisted of 2 % w/v SDS and 0.5 % w/v sodium naphthalene-1,5-disulfonate in TKM-buffer. Otherwise, the procedure was identical to Method 1.

Method 3 (from Warner *et al.*³). This method was also identical to Method 1, except for the

METHOD 1

GRADIENT	38400 dpm
PELLET	19700 "
TUBE	1400 "
TOTAL	59500 dpm

METHOD 2

GRADIENT	38300 dpm
PELLET	1100 "
TUBE	0 "
TOTAL	39400 dpm

METHOD 3

GRADIENT	37100 dpm
PELLET	2750 "
TUBE	0 "
TOTAL	39850 dpm

Fig. 1a-c. Ultracentrifugation of rat liver total RNA isolated by the three methods. 1 mg RNA, extracted 20 min after administration of 50 µCi ¹⁴C-orotic acid, was dissolved in 0.1 M sodium acetate pH 6.0 and applied directly on 30 ml 10-40 % w/w linear sucrose gradients made up in the same buffer. (a) RNA₁; (b) RNA₂; (c) RNA₃. Spinco SW 25.1 rotor, 24 000 rpm, 5 °C, 14 h. — OD₂₆₀; - - - Radioactivity (dpm × 5 × 10⁻⁴).

Fig. 2a-f. Gel electrophoresis of 300 μ g rat liver total RNA labeled for 20 min, in magnesium-buffer (a-c), and in EDTA-buffer (d-f). (a,d) RNA₁; (b,e) RNA₂; (c,f) RNA₃. 2.6 % w/v acrylamide, 12 mA/tube, 20 °C, 2 $\frac{3}{4}$ h. — Densitometer tracing (265 nm); ● Radioactivity (dpm/mg gel).

following: The aqueous phase consisted of 1 % w/v SDS and 10 mM EDTA in 0.05 M sodium acetate pH 5.1. Extraction was performed by heating at 55 °C (true temperature of the solution) for 3 min before chilling. After re-extraction at 55 °C, the combined aqueous phases were treated with $\frac{1}{2}$ volume phenol at 4 °C.

In the exhaustive extractions, temperatures gradually increasing up to 100 °C were used for all methods.

Ultracentrifugation. Routinely 1 mg RNA, dissolved in 1 ml gradient buffer, was applied on top of linear 30 ml 10–40 % w/w sucrose gradients containing 0.1 M sodium acetate pH 6.0, and run at 5 °C and 24 000 rpm for 14 h in a Spinco SW 25.1 rotor. Fractions of 0.8 ml were collected, diluted to 2 ml, and read spectrophotometrically. Liquid scintillation

counting was performed as described previously.⁷ *s* values were approximated by the method of Martin and Ames.⁸

Gel electrophoresis. Homogeneous 2.6 % acrylamide gels with a diameter of 10 mm and a length of 8 cm were run and scanned as described.⁹ As the electrophoretic conditions were found to be disaggregating, electrophoresis was performed both with magnesium in the buffer for stabilization, and in EDTA-buffer. The migration of the individual fractions was affected to a slightly different degree. The buffers consisted of 2 % v/v glycerol in 0.05 M sodium phosphate with 1 mM EDTA (EDTA-buffer) or with 5 mM magnesium acetate (magnesium-buffer) pH 6.25. After scanning, the gels were frozen, and cut into 1.5 mm slices. These were weighed, hydrolyzed,¹⁰ and counted.

RESULTS AND DISCUSSION

*Characteristics of RNA₁, RNA₂ and RNA₃.** By all methods 40–50 mg RNA was extracted per liver. The specific activity of RNA₁ was some 50 % higher than that of the others (Fig. 1a–c).

UV-patterns. All materials had UV-absorbing peaks in the region > 28 S, representing aggregates of rRNA (Fig. 2a–c). The aggregates of RNA₁ and RNA₂ were to a large extent dissociated by electrophoresis in EDTA-buffer at room temperature (Fig. 2d,e), while the more abundant aggregates of the hot-phenol-extracted RNA₃ were harder to disaggregate (Fig. 2f).

When run in magnesium-buffer, more peaks were visible between the ribosomal peaks and in the 4–18 S region in RNA₃ than in the cold-extracted RNAs (Fig. 2a–c). These extra peaks were quite reproducible on careful electrophoretic examination. Traces of several of them could be seen when RNA₁ and RNA₂ were run under more dissociating conditions (Fig. 2d,e). Ultracentrifugation demonstrated the same differences between the materials as gel electrophoresis, although the resolution was less (Fig. 1a–c). Furthermore, the 28 S RNA₃ peak then appeared lower and broader than the other 28 S peaks, and it often sedimented more slowly.

Dissociation of 5 S RNA from nuclear 28 S RNA,¹¹ and formation of rRNA aggregates during heating are probably responsible for part of the reduction of the 28 S RNA₃ peak. Its broadening and reduction in sedimentation rate indicate that conformational changes may be caused by the heating. The increased amount of UV-absorbing material between the ribosomal peaks and in the 4–18 S region might partly be derived from ribosomal RNA. This is supported by the release of corresponding material in RNA₁ and RNA₂ upon heating.¹²

The 3 S peak was not labeled using orotic acid as radioactive precursor, and probably represents DNA breakdown products from the DNAase treatment.

Radioactivity patterns. Varying amounts of rapid label remained confined to the top slices of the gels (> 60–70 S) (Fig. 2a–c). After

labeling for 20 min, only about 50 % of rRNA₁ entered the gels. Almost all rRNA₂ entered, while 10–15 % of rRNA₃ was excluded. Except for the material in the 1–3 S rRNA peak (see below), the excluded material was the most rapidly labeled. Upon ultracentrifugation, all RNA sedimenting faster than 65 S had the same labeling kinetics, suggesting that these molecules all belong to the same group. rRNA₁ tended to adhere to the nitro-cellulose tube walls when centrifuged. Because of the great differences in the patterns, at least two – if not all three methods – must give distorted pictures of the *in vivo* state of rRNA.

The radioactivity in the 4–28 S region was quantitatively inversely related to the amount of the apparent giant rRNA. In doubly labeled RNA, the short label: long label ratio in the 4–28 S region was slightly lower than in the rapidly sedimenting RNA. This could indicate that the label in the 4–28 S region represents the same molecular groups as the giant structures, in addition to some other, more slowly labeled molecules.

In RNA₁ an additional peak of radioactivity, 25–30 % of the total, migrated in the 6–8 S region or in the 1–3 S region, depending on the electrophoresis being performed in magnesium- or EDTA-buffer. It always sedimented as a 1–3 S peak. It was inconstantly present in RNA₂ and RNA₃. Only traces were regained after ethanol precipitation from EDTA-buffer. After thorough DNAase degradation during extraction, so that no 3 S UV-absorbing material was left, it was absent. It was the first fraction to become labeled, with significant amounts of label already after 2 min, and with maximal labeling after 10 min. For all labeling times it retained a strikingly solitary character, never merging with the RNA of higher molecular weight.

Chromatin associated 3 S RNA has been described.^{13–16} The existence of this RNA as a separate entity has recently been questioned.^{17–19} The smaller size of our 1–3 S rRNA and its extremely rapid labeling do not indicate similarity between these fractions. According to these parameters our fraction may represent growing polynucleotide chains. During the isolation, it is possibly precipitated bound to DNA or to other macromolecular structures. The electrophoretic migration at 6–8 S in

* Subscript 1, 2, or 3: That which pertains to Extraction method 1, 2, or 3.

magnesium-buffer could be caused by charge differences, as it sedimented at 1–3 S, irrespective of EDTA or magnesium being present in the buffer.

Exhaustive extraction. To investigate whether the differences in the rapid label patterns were caused by selective extraction of rRNAs, the extraction according to each of the methods was repeated until no more radioactivity was released into the aqueous phase. No additional radioactivity could then be extracted by any of the methods, even if the temperatures were gradually increased to 100 °C. Any residual interphase contained only negligible amounts of radioactivity when solubilized and counted directly. These studies, as well as the labeling kinetics, and the conversion studies performed by heating RNA,¹² indicate that the extra amount of label obtained with Method 1 is due to 1–3 S rRNA not being precipitated by the other methods. The other major differences in the radioactivity patterns could be due to different aggregational or conformational states of the isolated RNAs.

Effects of the composition of the aqueous phase. The presence of the large amounts of rapidly sedimenting rRNA₁ and the rapidly labeled 1–3 S peak was investigated based on the difference in composition between the aqueous solutions of Methods 1 and 2. The patterns

were unaffected by an exchange of SDS for tri-isopropylphenylthallene sulfonate, by addition of 6 % v/v butan-2-ol to extraction buffer₂, by addition of EDTA to the extraction buffers, or by omission of *m*-cresol from the phenol reagent. Addition of potassium chloride to increase the ionic strength of extraction buffer₂ up to that of aqueous phase₁ resulted in a radioactive recovery of about 80 % of the usual. A strong increase in radioactivity was seen in the 1–3 S region, while the distribution in the rest of the gradient was intermediate between RNA₁ and RNA₂ (Fig. 3). The ionic strength of the extraction liquid therefore seems to be essential. A specific effect of the extracting agents on the droplet size in the water-phenol emulsion could possibly also influence the patterns.

REFERENCES

1. Parish, J. H. and Kirby, K. S. *Biochim. Biophys. Acta* 129 (1966) 554.
2. Attardi, G., Parnas, H., Hwang, M.-I. H. and Attardi, B. J. *Mol. Biol.* 20 (1966) 145.
3. Warner, J. R., Soeiro, R., Birnboim, H. C., Girard, M. and Darnell, J. E. *J. Mol. Biol.* 19 (1966) 349.
4. Hastings, J. R. B., Parish, J. H., Kirby, K. S. and Cook, E. A. *Biochim. Biophys. Acta* 155 (1968) 603.
5. Wagner, E. K., Katz, L. and Penman, S. *Biochem. Biophys. Res. Commun.* 28 (1967) 152.
6. Fraenkel-Conrat, H., Singer, B. and Tsugita, A. *Virology* 14 (1961) 54.
7. Paus, P. N. In *Liquid Scintillation Counting*, Heyden & Son, London, New York, Rheine 1972, Vol. 2, pp. 205–212.
8. Martin, R. G. and Ames, B. N. *J. Biol. Chem.* 236 (1961) 1372.
9. Paus, P. N. and Alfheim, I. *Anal. Biochem.* 50 (1972) 430.
10. Paus, P. N. *Anal. Biochem.* 42 (1971) 372.
11. Pène, J. J., Knight, E., Jr. and Darnell, J. E., Jr. *J. Mol. Biol.* 33 (1968) 609.
12. Paus, P. N. *Acta Chem. Scand. B. In press.*
13. Huang, R.-C. C. and Bonner, J. *Proc. Nat. Acad. Sci. U. S.* 54 (1965) 960.
14. Bonner, J. and Widholm, J. *Proc. Nat. Acad. Sci. U.S.* 57 (1967) 1379.
15. Dahmus, M. E. and McConnell, D. J. *Biochemistry* 8 (1969) 1524.
16. Arnold, E. A. and Young, K. E. *Biochim. Biophys. Acta* 269 (1972) 252.
17. Artman, M. and Roth, J. S. *J. Mol. Biol.* 60 (1971) 291.
18. Heyden, H. W. v. and Zachau, H. G. *Biochim. Biophys. Acta* 232 (1971) 651.
19. Szeszák, F. and Pihl, A. *FEBS Lett.* 20 (1972) 177.

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Fig. 3. Ultracentrifugation of 1 mg rat liver total RNA, isolated by Method 2 with 0.1 M KCl added to the extraction buffer, 30 min after administration of 50 μ Ci 14 C-orotic acid. Legend otherwise as in Fig. 1.