A Sequential Extraction Method for RNA from Rabbit Liver

BENGT AXELSSON,a ALLAN NORGREN,b LENNART HOLMQQUIST c and ADAM DEUTSCH *†

a Wallenberg Laboratory, Nucleic Acid Biochemistry, University of Lund, Fack, S-220 07 Lund, Sweden, b Konung Gustav V Jubileumsstiftelse, University Hospital, S-221 85 Lund, Sweden and c Department of Clinical Chemistry, University Hospital, S-221 85 Lund, Sweden

When naphthalene-1,5-disulfonate (NDS) and phenol-cresol were used for extraction of rRNA from rabbit liver approximately 80% of the extractable RNA was recovered. By subsequent treatment of the resulting insoluble interface with 4-aminosalicylate and trisopropyl-naphthalenesulfonate (PAS-TIPS) the remaining RNA was extracted. Subcellular fractionation and polyacrylamide gel electrophoresis or sucrose density gradient analysis showed that this last fraction contained 28s RNA from membrane-bound polysomes. Applying the sequential extraction method for isolation of poly(A)-containing mRNA showed that only a minor part of that RNA was extracted with NDS while the rest was recovered by PAS-TIPS treatment of the interface. Thus, a purification of poly(A)-containing mRNA was possible when the sequential extraction method was used.

Earlier studies have demonstrated a differential release of nucleic acids from mammalian tissues depending on the nature of the extraction agents used in the aqueous phase of the two phase phenol-water extraction system. Thus, the extraction of rat liver in disodium naphthalene-1,5-disulfonate (NDS) and a phenol-cresol mixture yields cytoplasmic nucleic acids, i.e. ribosomal and transfer RNA in the aqueous phase. On the other hand, all nucleic acids including DNA and rapidly labelled RNA are extracted from the resulting insoluble interface with 4-aminosalicylate and trisopropyl-naphthalenesulfonate.

When we applied this sequential extraction procedure for the isolation of rRNA we found contrary to earlier reports that a significant amount of rRNA was not extracted by NDS and phenol. This rRNA remained in the insoluble interface of the extraction system and could be released by a subsequent treatment with PAS-TIPS.

Here we have examined more closely the extraction conditions for rabbit liver RNA, especially rRNA and poly(A)-containing RNA using the sequential extraction procedure. A significant part of 28s RNA remains insoluble upon NDS extraction and can be released by a subsequent PAS-TIPS treatment. Furthermore, a considerable part of poly(A)-containing RNA remains in the phenol phase upon NDS treatment and is extracted subsequently into the aqueous phase with PAS-TIPS.

EXPERIMENTAL

Materials

[3H] Adenosine and [35S] methionine were purchased from The Radiochemical Centre, Amersham. Sodium trisopropyl-naphthalenesulfonate and disodium naphthalene-1,5-disulfonate were obtained from Eastman Kodak Co, Rochester, New York and poly(U)Sepharose from Pharmacia Fine Chemicals, Uppsala. ATP, GTP, creatine phosphate, creatinephosphokinase, HEPES, and spermine were from Sigma Chemical Co., St. Louis.
**Methods**

*Fractionation of tissue.* Albinos rabbits of mixed strain, starved for 24 h, were killed by cervical dislocation and exsanguinated. Livers were removed immediately and rinsed at 0° C in buffer (0.05 M Tris-HCl, pH 7.5, 0.025 M KCl, 0.005 M MgCl₂, and 0.25 M sucrose). They were minced and homogenized in a Potter-Elvehjem homogenizer in two ml of buffer per g of tissue. The homogenate was centrifuged at 10 000 g for 10 min to sediment cell fragments, nuclei and mitochondria. The postmitochondrial supernatant was centrifuged in a Beckman 60 Ti rotor to sediment microsomes, either at 105 000 g for 3 h or over a discontinuous sucrose gradient (10 ml 2.0 M and 10 ml 0.5 M sucrose in buffer and 13 ml postmitochondrial supernatant) for 4 h at 250 000 g. The pellet obtained at 105 000 g, the material collected between 0.5 M and 2.0 M sucrose in the gradient (containing membrane-bound polysomes) and the resulting pellet (free polysomes) were used for subsequent experiments.

*Extraction of RNA.* An outline of the extraction procedure is presented in Fig. 1. Whole tissue or subcellular fractions were homogenized with 10 ml of 0.5 % (w/v) naphthalene-1,5-disulfonate (NDS) per g wet tissue. Then 6.6 ml of a phenol solution (500 g phenol, 70 ml m-cresol, 0.5 g 8-hydroxyquinoline and 55 ml water) was added. The mixture was stirred for 30 min at room temperature before centrifugation at 10 000 g for 10 min. The upper aqueous phase was removed and the phenol phase and the interface were washed twice with 0.5 % naphthalene-1,5-disulfonate. The aqueous phases were combined and solid sodium trisopropyl-naphthalenesulfonate was added to a concentration of 5 % followed by half a volume of the phenol solution described above. After stirring for 10 min the phases were separated by centrifugation at 10 000 g for 10 min. The aqueous phase was made 10 % in m-cresol, 3 % in NaCl and 20 % in sodium benzoate (m-cresol mixture) to precipitate the RNA except 4S and 5S RNA which remain in solution. The precipitate was washed with the m-cresol mixture followed by 1 % NaCl in 75 % ethanol, 75 % ethanol and ethanol. RNA obtained in this manner will be called NDS-extracted RNA.

The phenol phase and the interface from the naphthalene-1,5-disulfonate extraction were treated with 10 ml per g wet tissue of solution containing 6 % sodium 4-aminosalicylate (PAS), 6 % butanol (v/v), 1 % NaCl and 1 % trisos-
Sequential Extraction of RNA

Chromatography on poly(U)-Sepharose. Isolation of poly(A)-containing mRNA on poly(U)-Sepharose was performed according to Lindberg and Persson in a 1 x 3.5 cm column [the binding capacity was 2.5 mg poly(A)]. RNA (5-15 mg) was dissolved in water and diluted with 10-15 vol of 0.05 M Tris HCl, pH 7.5, 0.7 M NaCl, 0.01 M EDTA and 25 % formamide before application to the column. Poly(A)-binding material was eluted with 0.01 M Tris HCl, pH 7.5, 0.01 M EDTA, 0.2 % sarcosyl and 90 % formamide. The material was precipitated with 2 vol of ethanol and 0.1 vol of 20 % potassium acetate, washed in ethanol, dried in a vacuum and dissolved in water.

In vitro protein synthesis. Translation experiments were carried out in a wheat embryo extract. The incubation mixture contained 1 mM ATP, 0.2 mM GTP, 8.8 mM creatine phosphate, 80 µg/ml creatine phosphokinase, 2 mM dithioerythritol, 20 mM HEPES, pH 7.6, 64 mM KCl, 3.2 mM magnesium acetate, 16 µM each of 19 amino acids (except methionine) 60 Ci [35S]methionine (200-300 Ci/mmol), 30 µg/ml spermine, 20 µl S-30 extract and mRNA purified from 50-500 µg RNA in a total volume of 50 µl. After incubation for 60 min at +30 °C 5 µl aliquots were put on Whatman 30 filters and labelled proteins were precipitated in cold 10 % trichloroacetic acid. The filters were washed by boiling for 10 min in the same solution, followed by 10 min in 5 % trichloroacetic acid and 5 min each in ethanol and ether. The filters were dried and counted in a liquid scintillation counter.

RESULTS AND DISCUSSION

Extraction of rRNA with NDS and PASTIPNS. In a preliminary experiment we found that the recovery of RNA using the NDS extraction method was lower than with another method utilizing a pH 7.6 buffer in the presence of SDS and phenol. This indicates that an incomplete extraction of RNA occurs when NDS is used. Furthermore, treating the tissue directly with PASTIPNS gave a much higher recovery of rRNA than using NDS. These results encouraged us to study the sequential extraction method of Kirby in more detail. The method was first applied on unfractionated rabbit liver tissue (Table 1).

Table 1. Extraction of RNA from whole tissue and subcellular fractions with NDS and PAS-TIPNS.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>RNA extracted</th>
<th>Incorporation time</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>ODU$_{500}$/g tissue</td>
<td>20 min dpm/ODU$_{500}$</td>
<td>ODU$_{500}$/g tissue</td>
<td>11 h dpm/ODU$_{500}$</td>
</tr>
<tr>
<td>Whole tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDS</td>
<td>84 ± 12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAS-TIPNS</td>
<td>10 ± 2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>10 000 g pellet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDS</td>
<td>55 ± 20</td>
<td>316</td>
<td>50</td>
<td>926</td>
</tr>
<tr>
<td>PAS-TIPNS</td>
<td>4 ± 3</td>
<td>1987</td>
<td>4</td>
<td>3190</td>
</tr>
<tr>
<td>105 000 g pellet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDS</td>
<td>27 ± 8</td>
<td>65</td>
<td>35</td>
<td>1390</td>
</tr>
<tr>
<td>PAS-TIPNS</td>
<td>5 ± 2</td>
<td>191</td>
<td>3</td>
<td>2200</td>
</tr>
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</table>

Fig. 2. Sucrose density gradients of RNA extracted by the sequential method. Rabbits were injected with 0.5 mCi [4]$^3$-adenosine 20 min (nuclear-containing pellet) and 11 h (microsomes) before sacrifice. The nuclear-containing (10 000 g) pellet extracted with (A) NDS, (B) PAS-TIPNS and microsomes extracted with (C) NDS and (D) PAS-TIPNS were run in 5–20 % gradients for 4 h at 35 000 rpm in a Beckman SW 39 rotor. Fractions of 4 drops were collected and counted in a liquid scintillation counter. ———, cpm; - - -, $A_{260}$.
step, 85% of the total extractable RNA was recovered, while the remaining RNA was extracted in a second step by PAS-TIPNS. In order to study the subcellular localization of the RNA extracted by PAS-TIPNS a fractionation of the liver cell in fractions containing nuclei (10,000 g pellet) and microsomes was made prior to extraction. Of the total RNA extracted from these two fractions together approximately 40% originated in the microsomes. Both in the 10,000 g pellet and in the microsomes 10–15% of the RNA remained insoluble upon NDS treatment and was extracted subsequently by PAS-TIPNS. Thus a significant proportion of the microsomal RNA remains unextracted by NDS and is recovered by PAS-TIPNS treatment contrary to the findings of Parish and Kirby.

We have also examined the distribution of radioactively labelled RNA between the 10,000 g pellet and the microsomes, 20 min and 11 h after intraperitoneal injections of [H] adenosine (Table 1). After 20 min of labelling the specific radioactivity of RNA extracted by NDS was low in both fractions, while it was several times higher in the RNA extracted subsequently by PAS-TIPNS. Most of the label was found in the 10,000 g pellet. After 11 h the label was equally distributed between the fractions. The difference in specific radioactivity between the RNA extracted by PAS-TIPNS and NDS was then less pronounced than after 20 min of incorporation.

In order to identify the RNA extracted by the sequential procedure the material was examined by sucrose density gradient centrifugation and polyacrylamide gel electrophoresis. Fig. 2 shows the sucrose density gradient profiles of RNA extracted from the 10,000 g pellet and microsomes which have been labelled for 20 min and 11 h. After 20 min of labelling no distinct RNA peak was seen in the material extracted with NDS from the 10,000 g pellet while radioactive material much heavier than 28s RNA was extracted by the subsequent treatment with PAS-TIPNS. Since the RNA extracted with PAS-TIPNS should be composed mainly of nuclear RNA, this is in accordance with earlier investigations. The amount of radioactive material incorporated into the microsomal fraction was not sufficient for a centrifugation analysis. After 11 h of labelling

Fig. 3. Polyacrylamide-agarose gel electrophoresis of microsomal RNA sequentially extracted with (A) NDS and (B) PAS-TIPNS and (C) direct extraction with PAS-TIPNS. Electrophoresis was performed as described in Experimental. The gels were photographed and the negative film was scanned in a densitometer.

the gradient profiles in both the 10,000 g pellet and the microsomes showed radioactive peaks corresponding to 28s and 18s RNA in both the NDS and the PAS-TIPNS extracts. In addition, radioactive material of a lower sedimentation rate than 18s was particularly conspicuous in the PAS-TIPNS extract of the microsomal fraction. This material which has a high specific radioactivity has not yet been identified but might be degraded mRNA. The material of the PAS-TIPNS extract sedimenting around 18s appears to contain labelled RNA other than 18s RNA. The absorbancy ratio of 28s:18s RNA in the PAS-TIPNS extract is considerably higher than the expected 2.6:1 ratio seen for instance in the NDS extracts.

The RNA extracted from the microsomal fraction by NDS and consecutively by PAS-TIPNS was further analyzed by polyacrylamide-agarose gel electrophoresis (Fig. 3). As a control the microsomes were extracted with PAS-TIPNS without a prior NDS treatment. Again the PAS-TIPNS extract of the sequential procedure contained a higher proportion of 28s RNA than expected, while the NDS extract and the control extraction with PAS-TIPNS showed the expected 2.6:1 distribution between 28s and 18s RNA. Therefore the high 28s:18s ratio of the PAS-TIPNS extract obtained through the sequential procedure appears not to be due to artifacts of the preparation. More likely it is due to an incomplete extraction of 28s RNA in the NDS step leaving a significant proportion of the 28s RNA to be extracted by PAS-TIPNS. Since the amount of RNA extracted by PAS-TIPNS compared to NDS is rather low (see Table 1), this extra 18s RNA in the NDS extract will not appreciably affect the RNA pattern.

In order to further examine the localization of the PAS-TIPNS extractable RNA, free and membrane-bound polysomes were isolated and extracted by the sequential method. While most of the RNA from free polysomes could be extracted with NDS (Table 2), approximately a third of the RNA from membrane-bound polysomes was retained in the phenol phase upon NDS treatment and was extracted into the aqueous phase during the following PAS-TIPNS treatment. When ribosomes were released from membrane-bound polysomes with deoxycholate prior to extraction all of the ribosomal RNA was extracted with NDS. It therefore appears that a sizeable fraction of the 28s RNA of membrane-bound polysomes is protected from NDS extraction by the membrane. The recovery of the RNA from free and membrane-bound polysomes was about 40 % of the amount obtained in the unfractionated microsomes (cf. Table 1). This was due to losses in the sucrose cushion which retains monosomes and ribosomal subunits on gradient centrifugation. All the RNA remaining in the sucrose cushion was extracted by NDS when the sequential method was applied. This is in line with the other results presented indicating that only membrane-bound RNA is left unextracted by NDS.

Table 2. Recovery of RNA from free and membrane-bound polysomes sequentially extracted with NDS and PAS-TIPNS.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>RNA extracted (ODU\textsubscript{260}/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment A (^a)</td>
</tr>
<tr>
<td></td>
<td>NDS</td>
</tr>
<tr>
<td>Free polysomes</td>
<td>7.0</td>
</tr>
<tr>
<td>Membrane-bound polysomes</td>
<td>5.0</td>
</tr>
<tr>
<td>after deoxycholate treatment</td>
<td></td>
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</tbody>
</table>

\(^a\) A postmitochondrial supernatant was layered over a discontinuous sucrose gradient (see Materials and Methods) and centrifuged for 4 h at 250,000 g. Free and membrane-bound polysomes were collected and extracted. \(^b\) Isolated membrane-bound polysomes from a discontinuous sucrose gradient centrifugation were divided into two parts. One part was treated with 1 % deoxycholate dissolved in postmicrosomal supernatant (to inhibit RNAase activity) and recentrifuged over a discontinuous sucrose gradient, while the other part was suspended in postmicrosomal supernatant prior to centrifugation. Free polysomes were also resuspended and recentrifuged.

Table 3. Sequential extraction of poly(A)-containing RNA from whole liver tissue and microsomes with NDS. Rabbits were injected with 0.5 mCi [H]-adenosine 22 h before sacrifice. RNA was extracted by the sequential method. RNA samples (2 - 5 ODU mm) were applied on Millipore filters in the presence of 0.1 M Tris-HCl, pH 7.6, 0.5 M KCl and 0.001 M MgCl2. The filters were washed in the same solution, heat dried and counted.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Extraction agent</th>
<th>RNA extracted *</th>
<th>Poly(A)-containing * RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole tissue</td>
<td>NDS</td>
<td>102 672</td>
<td>1233</td>
</tr>
<tr>
<td></td>
<td>PAS-TIPNS</td>
<td>37 956</td>
<td>2204</td>
</tr>
<tr>
<td>Microsomes</td>
<td>NDS</td>
<td>31 864</td>
<td>192</td>
</tr>
<tr>
<td></td>
<td>PAS-TIPNS</td>
<td>13 243</td>
<td>410</td>
</tr>
</tbody>
</table>

* dpm per g tissue.

The choice of extraction method for RNA is very important to obtain optimal amounts of the RNA species intended for study. The results presented indicate that extraction with NDS and phenol - cresol according to Kirby 1 yields rRNA from free polysomes and most of the RNA from small subunits of membrane-bound polysomes. On the other hand, the large ribosomal subunit of membrane-bound polysomes tends to be retained in the interface upon NDS extraction and the RNA is released by the addition of PAS-TIPNS. The reason for this retention of rRNA from membrane-bound large ribosomal subunits upon NDS treatment may have several explanations. The most likely explanation might be the tight association between the large ribosomal subunit and the endoplasmic reticulum 14,11 which appears to be mediated via specific proteins 12 or the other components which may stabilize the ribosome-membrane binding. 13 - 15 The more efficient extraction obtained with PAS-TIPNS may be due to the strong hydrophobic nature of TIPNS compared to NDS, probably resulting in a detergent-like action which should aid in the detachment of the large ribosomal subunit from the microsomal membrane.

Extraction of poly(A)-containing RNA with NDS and PAS-TIPNS. The retention in the phenol phase of rRNA upon extraction with NDS, possibly due to interactions between ribosomes and the membrane, made us extend the study of RNA extraction to include mRNA. It is well known that mRNA exists as an mRNP, (free unit or bound to the membrane). 14,15 The proteins associated to the poly(A)-moiety of the mRNA or poly(A)-sequence alone, have been reported to cause an incomplete extraction of poly(A)-containing mRNA 6,18 when an aqueous phenol mixture is used. We therefore examined whether a preferential extraction of poly(A)-containing RNA would occur with NDS and PAS-TIPNS and whether a sub-fractionation of this RNA could be achieved by the sequential method. To follow the extraction, RNA was labelled radioactively by an injection of [H]-adenosine 22 h before sacri-

Fig. 4. SDS-Polyacrylamide gel electrophoresis of polypeptides synthesized from microsomal mRNA extracted by the sequential method. After extraction the RNA was further purified by poly(U)-Sepharose chromatography. The polypeptides were labelled with 35S-methionine. Electrophoresis was run as described in Experimental and was dried and autoradiographed. (A) No mRNA added, (B) mRNA from the NDS extract and (C) mRNA from the PAS-TIPNS extract. BSA: Bovine Serum Albumin, OA: Ovalbumin and STI: Soybean Trypsin Inhibitor have been used as molecular weight markers.

office of the animal. Unfractionated liver tissue and microsomes were extracted by the sequential method and poly(A)-containing RNA was selectively isolated by adsorption to Millipore filters.8 Table 3 shows the distribution of poly(A)-containing RNA from NDS and PAS-TIPNS extracted material. Even though about 30% of the total RNA label appeared in the PAS-TIPNS fraction there was a clear enrichment of poly(A)-containing material in that extract (65% of the total poly(A)-containing RNA). Considering the total amount of RNA in the extracts a fivefold enrichment of poly(A)-containing RNA was found in the PAS-TIPNS fractions compared to the NDS ones.

We tested whether different species of poly(A)-containing mRNA were extracted by NDS and PAS-TIPNS. The extracted RNA was purified on poly(U)-Sepharose 4B and was then used to direct protein synthesis in a wheat germ in vitro system. The radioactively labelled polypeptides were separated by SDS gel electrophoresis and autoradiographed (Fig. 4). When the products of NDS and PAS-TIPNS extracted RNA were compared no significant differences were found in their polypeptide pattern. NDS treatment therefore seems insufficient for a complete extraction of the poly(A)-containing RNA rather than being a selective extraction step. However, NDS extraction prior to PAS-TIPNS appears to be a useful step to remove large amounts of RNA, primarily rRNA, which otherwise will contaminate the mRNA preparation.

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