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A Sequential Extraction Method for RNA from Rabbit Liver

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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 triisopropylnaphthalenesulfonate (PAS-TIPNS) 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-TIPNS 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 released into the aqueous phase if the extraction is carried out in a mixture of sodium 4-aminosalicylate (PAS), triisopropylnaphthalenesulfonate (TIPNS) and pheno – cresol.[1,2] A combination of the two extraction procedures yields rRNA and tRNA in a first step using naphthalenesulfonate, while DNA and rapidly labelled RNA are extracted from the resulting insoluble interface with 4-aminosalicylate and triisopropylnaphthalenesulfonate.[2]

When we applied this sequential extraction procedure for the isolation of rRNA we found contrary to earlier reports [2] 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-TIPNS.

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-TIPNS 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-TIPNS.

EXPERIMENTAL

Materials

[^{3}H] Adenosine and [^{35}S] methionine were purchased from The Radiochemical Centre, Amersham. Sodium triisopropylnaphthalenesulfonate 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.

† Deceased May 30, 1976.

Methods

Fractionation of tissue. Albino 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 $MgCl_2$ 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 triisopropylnaphthalenesulfonate 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 % triiso-

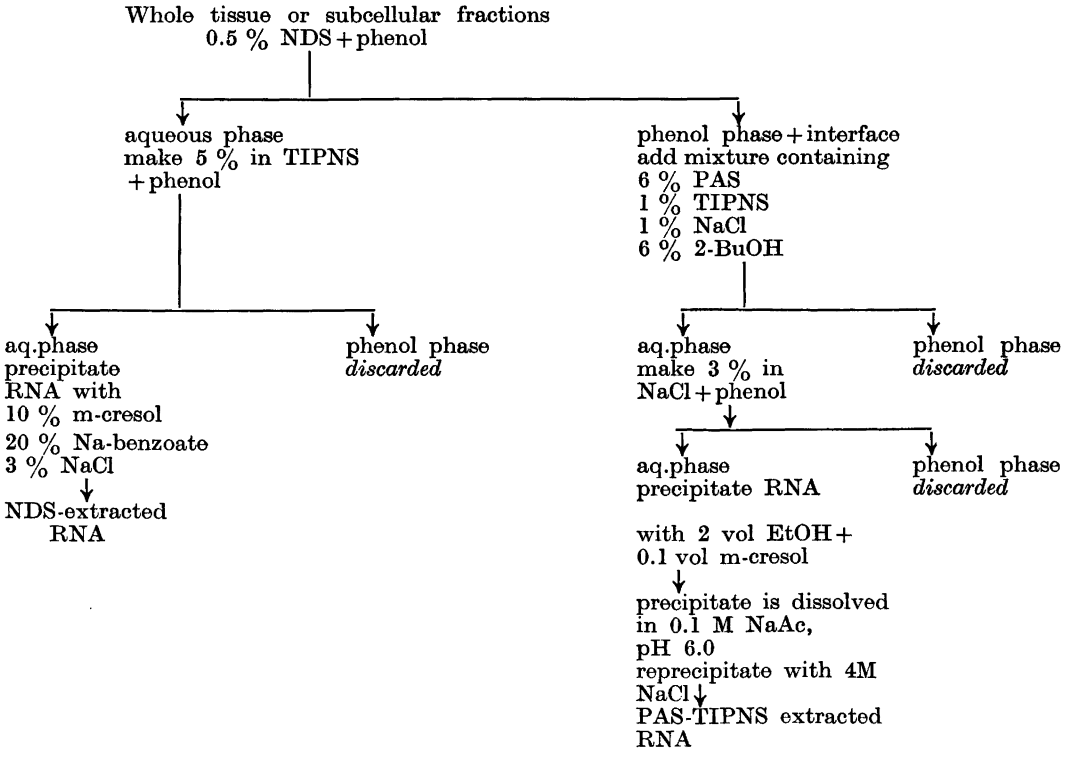

Fig. 1. Sequential extraction procedure for RNA.

propylnaphthalenesulfonate (TIPNS). After stirring for 30 min at room temperature the phases were separated by centrifugation (10 000 *g* for 10 min). The aqueous phase was made 3 % in NaCl. Half a volume of the phenol solution was added and the mixture was stirred for 10 min. The aqueous phase was collected and two volumes of ethanol containing 10 % *m*-cresol (v/v) were added. RNA and DNA were allowed to precipitate at −20 °C for 1 h. The precipitate was washed twice at +4 °C with 2 % sodium acetate in 75 % ethanol and was then dissolved in 0.1 M sodium acetate, pH 6.0. The solution was made 4 M in NaCl and RNA was allowed to precipitate overnight at +4 °C. After centrifugation the RNA pellet was washed with 4 M NaCl in 0.1 M sodium acetate and dissolved in water. In some cases the pellet was further washed with 75 % ethanol, ethanol and finally dried in a vacuum. RNA obtained in this manner will be called PAS-TIPNS extracted RNA.

Sucrose density gradient centrifugation. Approximately 150 μg of RNA in 100 μl sodium acetate buffer was applied on top of 4 ml linear 5−20 % (w/v) sucrose gradients prepared in 0.01 M sodium acetate, pH 5.2. Centrifugation was for 4 h at +4 °C at 35 000 rpm in a Beckman SW-39 rotor. The gradients were eluted from the bottom of the tubes and monitored continuously at 254 nm. Fractions containing 4 drops were collected for radioactivity analyses.

Electrophoresis. (a) *RNA.* Mixed agarose-polyacrylamide gels (0.5 % agarose, 2 % total acrylamide monomer concentration, with a 5 % proportion of *N,N'*-methylene-bisacrylamide) were prepared in a buffer consisting of 0.04 M Tris-HCl, pH 7.8, 0.02 M sodium acetate and 0.001 M EDTA.[3] Five cm gels were polymerized in glass tubes, inner diameter 0.5 cm. Electrophoresis was for 35 min at 3−4 mA per tube at +4 °C using the gel buffer in the electrode vessels. Samples containing 0.5 ODU_{260} of RNA were layered over the gel. After the run, gels were placed in 1 M acetic acid for 15 min before staining for 1 h in 0.2 % methylene blue dissolved in 0.2 M sodium acetate and 0.2 M acetic acid. Excess stain was removed by rinsing in several changes of water. (b) *Proteins.* Sodium dodecylsulfate-polyacrylamide gel electrophoresis was performed in slab gels according to Neville[4] in a modified (R. Ohlsson, B. Jergil, personal communication) Pharmacia GR 4 apparatus. Radioactive material from *in vitro* protein synthesis (1.5×10^6 cpm) was applied to the gel. Electrophoresis was run at 20 mA per gel and was discontinued when the front reached the bottom of the gel. Gels were stained in 0.1 % Coomassie Brilliant Blue dissolved in 50 % methanol and 7 % acetic acid and destained in 25 % methanol and 7 % acetic acid. Gels were dried under vacuum at +100 °C and autoradiographed. The exposure was for 3 days using Osray T4 films.

Chromatography on poly(U)-Sepharose. Isolation of poly(A)-containing mRNA on poly(U)-Sepharose was performed according to Lindberg and Persson[5] in a 1 × 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.[6] 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) 5μCi [^{35}S]methionine (200−300 Ci/mmol), 30 μg/ml spermine,[7] 20 μl S-30 extract[6] 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 00 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.

Analysis of poly(A)-containing mRNA. RNA samples (2−5 ODU_{260}) labelled with [^{3}H]-adenosine were dissolved in icecold 0.01 M Tris-HCl, pH 7.6, 0.5 M KCl and 0.001 M $MgCl_2$ and filtered slowly through Millipore filters (HA 0.45 μm, Millipore Filter Corp., Bedford, Mass.), previously soaked in the same solution.[8] The filters were heatdried before counting.

RESULTS AND DISCUSSION

Extraction of rRNA with NDS and PAS-TIPNS. In a preliminary experiment we found that the recovery of RNA using the NDS extraction method[1] was lower than with another method utilizing a pH 7.6 buffer in the presence of SDS and phenol.[9] This indicates that an incomplete extraction of RNA occurs when NDS is used. Furthermore, treating the tissue directly with PAS-TIPNS 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). In the first NDS

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

Fraction	RNA extracted	Incorporation time 20 min		11 h	
	ODU_{260}/g tissue	dpm/ODU_{260}	ODU_{260}/g tissue	dpm/ODU_{260}	ODU_{260}/g tissue
Whole tissue					
NDS	84 ± 12				
PAS-TIPNS	10 ± 2				
10 000 *g* pellet					
NDS	55 ± 20	316	50	926	74
PAS-TIPNS	4 ± 3	1987	4	3190	4
105 000 *g* pellet					
NDS	27 ± 8	65	35	1390	19
PAS-TIPNS	5 ± 2	191	3	2200	4

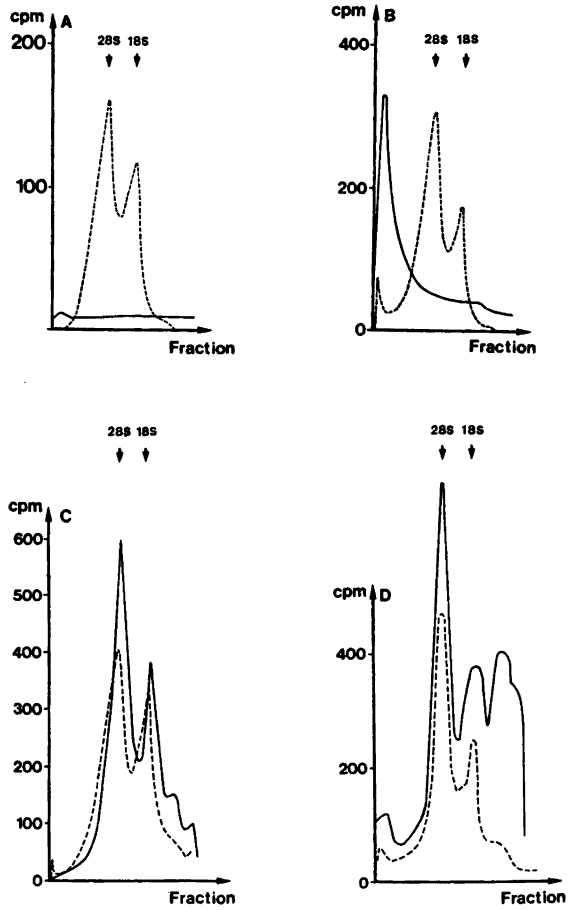

Fig. 2. Sucrose density gradients of RNA extracted by the sequential method. Rabbits were injected with 0.5 mCi [^{3}]-adenosine 20 min (nuclear-containing pellet) and 11 h (microsomes) before sacrifice. The nuclear-containing (10 000 *g*) pellet extracted with (A) NDS, (B) PAS-TIPNS and microsomes extracted 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_{234}.

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.[2]

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 [^{3}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 [2] 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

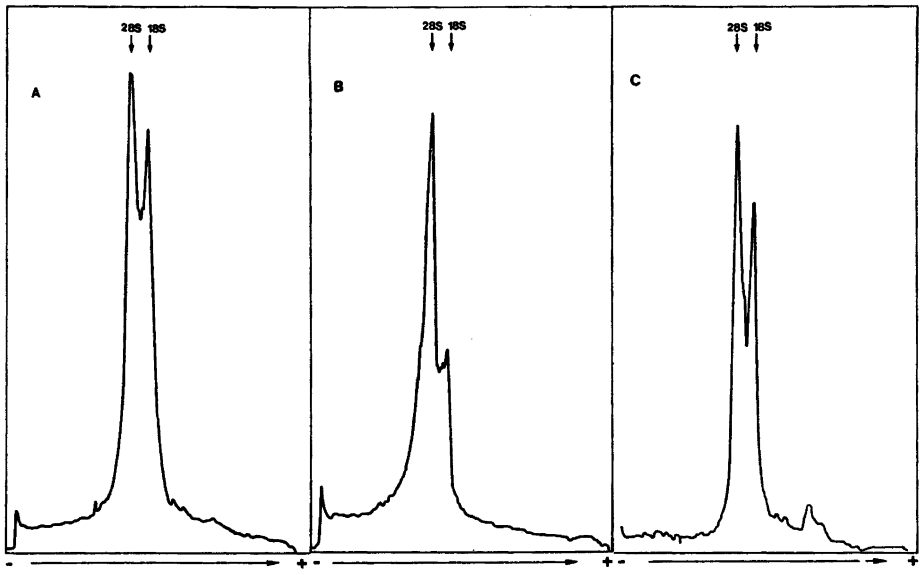

Fig. 3. Polyacrylamide-agarose gel electrophoresis of microsomal RNA sequentially extracted with (A) NDS and (B) PAS-TIPNS and (C) direct extraction with PAS-TIPNS. Electrophoresis was performed as described in Experimental. The gels were photographed and the negative film was scanned in a densitometer.

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 2.5:1 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 2.5:1 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 th esucrose 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.

Fraction	RNA extracted (ODU_{260}/g tissue) *Experiment A* [a]		*Experiment B* [b]	
	NDS	PAS-TIPNS	NDS	PAS-TIPNS
Free polysomes	7.0	0.1	4.0	0.1
Membrane-bound polysomes	5.0	1.7	2.6	1.1
Membrane-bound polysomes after deoxycholate treatment			2.7	0.0

[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 [^{3}H]-adenosine 22 h before sacrifice. RNA was extracted by the sequential method. RNA samples (2 – 5 ODU_{260}) 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 $MgCl_2$. The filters were washed in the same solution, heatdried and counted.

Fraction	Extraction agent	RNA extracted [a]	Poly(A)-containing [a] RNA
Whole tissue	NDS	102 672	1233
	PAS-TIPNS	37 956	2204
Microsomes	NDS	31 864	192
	PAS-TIPNS	13 243	410

[a] 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 rRNA 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 [18,11] 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).[16,17] The proteins associated to the poly(A)-moiety of the mRNA or poly(A)-sequency alone, have been reported to cause an incomplete extraction of poly(A)-containing mRNA [8,18] 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 subfractionation of this RNA could be achieved by the sequential method. To follow the extraction, RNA was labelled radioactively by an injection of [^{3}H] adenosine 22 h before sacri-

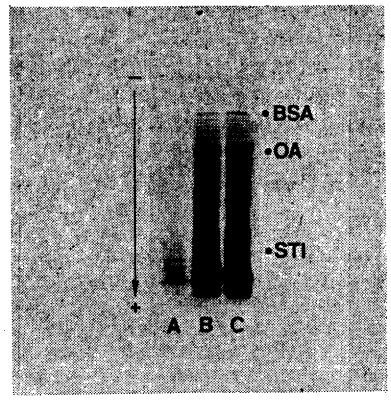

Fig. 4. SDS-Polyacrylamide gel electrophoresis of polypeptides synthesized from microsomal mRNA extracted by the sequential method. After extraction the RNA was further purified by poly(U)-Sepharose chromatography. The polypeptides were labelled with ^{35}S-methionine. Electrophoresis was run as described 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.

fice of the animal. Unfractionated liver tissue and microsomes were extracted by the sequential method and poly(A)-containing RNA was selectively isolated by adsorbtion 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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