Biochem. J. (1979) **183**, 181–184
Printed in Great Britain

An Improved Method for the Rapid Isolation of Brain Ribonucleic Acid

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(*Received* 16 *July* 1979)

An improved one-step method for the extraction of RNA from rat brain is described. Fresh or frozen tissue is disrupted in the powerful protein denaturant guanidine thiocyanate, and RNA isolated by ultracentrifugation through CsCl. The procedure is advantageous in that it is relatively simple, is rapid and does not expose the sample to enzyme treatments or repeated organic extractions.

Techniques of nucleic acid hybridization have provided a powerful approach to understanding the control of eukaryote gene expression (Lewin, 1975). This experimental approach usually requires relatively large amounts of high-molecular-weight RNA devoid of DNA and protein contamination. Generally, RNA is obtained by repeated phenol extraction of detergent-treated tissue homogenates, whole cells or subcellular organelles (Hastings & Kirby, 1966). To obtain quantitative recovery of polyadenylated RNA, phenol extractions are normally conducted in the presence of chloroform (Perry *et al.*, 1972) or alkaline buffers (Brawerman *et al.*, 1972). Since phenol cannot be relied upon to totally inhibit ribonuclease activity, RNA is often prepared in the presence of a variety of ribonuclease inhibitors (see, e.g., Mendelsohn & Young, 1978). Residual protein and DNA are subsequently removed from the crude nucleic acid extract by treatment with proteolytic enzymes and deoxyribonuclease. In the latter instance, special precautions are required to remove ribonuclease from commercial preparations of deoxyribonuclease I (Zimmerman & Sandeem, 1966; Brison & Chambon, 1976).

Glisin *et al.* (1974) described a rapid RNA isolation procedure based on ultracentrifugation of whole-cell homogenates in CsCl. The CsCl centrifugation procedure was reported to be superior to phenol extraction in terms of simplicity, processing time, yield and biological activity of the resulting RNA. Our early attempts to isolate RNA from whole brain by this method resulted in the recovery of partially degraded RNA in low yield. Ostensibly, this difficulty arose from incomplete inhibition of the relatively high ribonuclease activity present in brain. In the present paper we describe modifications in the CsCl procedure that facilitate the isolation of brain RNA with minimal degradation. The method described should prove especially useful in obtaining RNA from small amounts of starting material or from tissues containing high ribonuclease activities.

Materials and Methods

Chemicals

All chemicals used were of reagent grade or the highest quality available. CsCl and dimethyl sulphoxide were purchased from Schwarz BioResearch (Orangeburg, NY, U.S.A.). Guanidine thiocyanate was obtained from Tridom Chemical (Haupauge, NY, U.S.A.). Diethyl pyrocarbonate and *N*-lauroylsarcosine were the products of Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hydroxyapatite (DNA grade, HTP) and Chelex 100 were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). ^{3}H-labelled *Escherichia coli* rRNA was purchased from Miles Biochemicals (Elkhart, IN, U.S.A.) and [5,6-^{3}H]uridine (50Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.).

Extraction of total RNA

Fresh or frozen whole brain from male Fisher 344 rats was homogenized in 4vol. (w/v) of 5.0M-guanidine thiocyanate/50mM-Tris/HCl (pH7.6)/10mM-EDTA/5% 2-mercaptoethanol (see Ullrich *et al.*, 1977; footnote 6). The homogenization buffer was previously filtered (Nalgene, 0.2μm pore diam.) and treated with 0.05% diethyl pyrocarbonate. The homogenate was made 4% (w/v) with respect to *N*-lauroylsarcosine, and solid CsCl was added to 0.15g/ml. After gentle mixing, the suspension was layered over a 2.0ml cushion of 5.7M-CsCl (density 1.705g/cm^3)/0.1M-EDTA (Glisin *et al.*, 1974) and centrifuged in a Beckman SW-41 rotor at 36000rev./min for 18–24h at 20°C. Under these conditions RNA will sediment through the 5.7M-CsCl cushion and appear as a clear pellet at the bottom of the tube. Addition of CsCl to the homogenate in amounts

greater than 0.25g/ml resulted in the incomplete sedimentation of RNA and a subsequent lower recovery. After centrifugation, the homogenate was removed by aspiration, and the polyallomer tubes were rinsed with 0.05% diethyl pyrocarbonate followed by two washes with water. After removal of the CsCl cushion, the RNA pellets were suspended in 0.25% sodium dodecyl sulphate and precipitated by addition of 6M-ammonium acetate to 4% (v/v) and 2vol. of cold ethanol (Osterburg *et al.*, 1975).

Isolation of polyadenylated RNA

Poly(A)-containing RNA was isolated by binding to oligo(dT)–cellulose as described previously (Kaplan *et al.*, 1978). Since the RNA obtained by CsCl centrifugation was highly aggregated, the sample was suspended (1mg/ml) in 10mM-Tris/HCl (pH7.5)/2mM-EDTA/100mM-NaCl/0.5% sodium dodecyl sulphate and heated at 65°C for 5min before chromatography. To minimize contamination by rRNA, poly(A)-containing RNA was recycled over the column four times. The final poly(A)-containing RNA obtained was heterogeneous in size, with most of it sedimenting between 18S and 28S on dimethyl sulphoxide/sucrose gradients (see below).

Preparation of DNA

DNA was isolated from purified rat liver nuclei as previously described (Kaplan *et al.*, 1978). Total DNA was sheared to 450-nucleotide (nt) fragments in 0.2M-ammonium acetate/66% glycerol in a VirTis 60 homogenizer (Britten *et al.*, 1974). Non-repetitive DNA was isolated from total sheared DNA by two cycles of reassociation to an equivalent C_0t of 500 in 0.41M-sodium phosphate buffer (pH6.8) at 70°C; C_0t is the DNA concentration in mol of nucleotide·litre^{-1}·s. Unreassociated DNA was separated from duplex structures by chromatography on hydroxyapatite in 0.12M-sodium phosphate buffer, pH6.8, at 60°C.

^{3}H-labelled non-repetitive DNA was prepared *in vitro* from a double-stranded non-repetitive DNA template by 'gap translation' with *Escherichia coli* DNA polymerase I (Galau *et al.*, 1976). Details of the procedure for labelling *in vitro* were previously reported (Kaplan *et al.*, 1978). The [^{3}H]DNA tracer obtained had a specific radioactivity of approx. 6.0×10^6c.p.m./μg and was 200–250 nucleotides in length. The non-repetitive [^{3}H]DNA reassociated with a second-order rate constant of 4.4×10^{-4}mol^{-1}·s^{-1} and reached a limit of 82% hydroxyapatite-bound duplexes. The DNA tracer reassociated to 2–3% at C_0t values $\leq$50 and was thus essentially free of foldback and repetitive DNA sequences. The [^{3}H]DNA tracer was similar to DNA probes ^{3}H-labelled *in vivo* with regard to kinetic complexity, reactivity and repetitive DNA sequence content (Kaplan *et al.*, 1978; Pearson *et al.*, 1978). The mean 'melting' temperature (T_m) of hybrids of the [^{3}H]DNA and total DNA was 83°C as monitored by thermal elution from hydroxyapatite.

RNA–DNA hybridization reactions

Non-repetitive [^{3}H]DNA was mixed with poly(A)-containing RNA in 0.41M-sodium phosphate buffer/1mM-EDTA/0.1% sodium dodecyl sulphate at RNA concentrations of 3–6mg/ml and RNA/DNA mass ratios of 3000–6000:1. Samples of the reaction mixture (1μl) were sealed in glass capillaries, denatured (100°C for 3min) and treated as previously described (Kaplan *et al.*, 1978). Hybrid formation was determined by chromatography on hydroxyapatite (Britten & Kohne, 1968). The content of [^{3}H]DNA duplexes in RNA-driven reactions was estimated after digestion of RNA–DNA hybrids with 10μg of ribonuclease A/ml (Galau *et al.*, 1974).

Sucrose-density-gradient analysis

RNA samples were disaggregated before analysis by incubation at 37°C for 5min in 80% (v/v) dimethyl sulphoxide in buffer A [10mM-Tris/HCl (pH6.5)/0.1M-LiCl/5mM-EDTA]. The sample (200μl) was subsequently diluted with an equal volume of buffer A and applied to linear 5–20% (w/v) sucrose gradients containing 50% dimethyl sulphoxide in buffer A (Bantle *et al.*, 1976). Samples were centrifuged in a Beckman SW-41 rotor at 32000rev./min for 16–19h at 20°C, and u.v. absorbance of the gradients was monitored continuously at 254nm.

Results and Discussion

Homogenization of brain in guanidine thiocyanate and subsequent centrifugation in CsCl resulted in the recovery of 1.0mg of total RNA/g of tissue (Table 1), a value identical with that obtained by using standard alkaline phenol extraction (Kaplan *et al.*, 1978). No difference was observed in the yield of total RNA from fresh or frozen tissue (Table 1). The A_{260}/A_{280} and A_{260}/A_{230} ratios of all RNA preparations were $\geq$2.0, indicating that protein contamination was negligible. DNA contamination of the RNA preparations was estimated by including tracer quantities of ^{3}H-labelled rat liver DNA (500nt) in the tissue homogenates. In agreement with the findings obtained by Glisin *et al.* (1974), less than 0.3% of the [^{3}H]DNA was recovered in the RNA pellet (results not shown).

As shown in Table 1, approx. 4.4% of total RNA bound to oligo(dT)–cellulose after repeated passages over the column. Treatment of brain total RNA with 80% dimethyl sulphoxide to further diminish RNA aggregation before chromatography (Bantle *et al.*, 1976) did not alter the yield of poly(A)-containing RNA. The recovery of poly(A)-containing RNA by this procedure is about 60% greater than that

Table 1. *Yields and complexity of total poly(A)-containing RNA from brain*

Preparation no.	Total RNA (mg/g of tissue)	Poly(A)-containing RNA (μg/g of tissue)	Hybridization value* (mean % ± S.D.)	Corrected hybridization value† (mean %)	Complexity (nt)‡
I (frozen tissue)	0.97	46.5	10.0 ± 1.5 (4)	12.2	4.9×10^8
II (frozen tissue)	1.00	43.0	10.7 ± 0.5 (4)	13.0	5.2×10^8
III (fresh tissue)	1.03	40.1	10.0 ± 1.0 (4)	12.2	4.9×10^8
IV (phenol-extracted)§	0.89	26.4	9.5 ± 1.4 (5)	12.6	5.0×10^8

* Results obtained from reaction mixtures incubated to R_0t value ≥20000 at RNA/DNA ratios of 4000:1. Data are corrected for [^{3}H]DNA duplex content.

† Maximum value for [^{3}H]DNA reassociation in 0.41 M-phosphate buffer, pH 6.8, at 70°C in the presence of excess unlabelled DNA (450 nt) was 82% at C_0t values ≥50000. Hybridization values were corrected to 100% [^{3}H]DNA reactivity.

‡ Calculation based on the assumption that DNA transcription is asymmetric and that the rat non-repetitive genome is 2.0×10^9 nt.

§ Average values for two whole-brain preparations from 60-day-old Sprague–Dawley rats (representative data from Kaplan *et al.*, 1978).

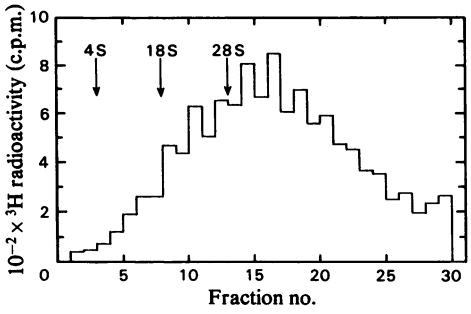

Fig. 1. *Sucrose-density-gradient profile of rapidly labelled [^{3}H]RNA from brain*

Rats (5-day-old) were killed 30 min after intraperitoneal administration of [5,6-^{3}H]uridine (0.3 mCi/animal; 50 μl of saline). Total RNA was extracted from frozen tissue and analysed by gradient centrifugation as described in the Materials and Methods section. Arrows mark the positions of tRNA, 18S rRNA and 28S rRNA present in the same gradient. The direction of migration is from left to right.

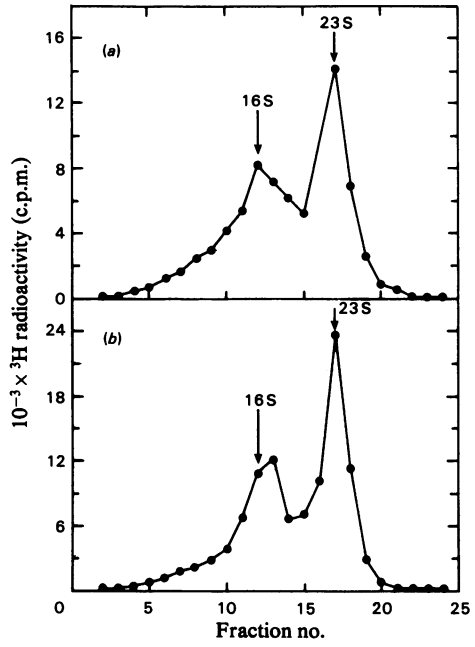

Fig. 2. *Density-gradient profile of ^{3}H-labelled E. coli 16S and 23S rRNA*

(*a*) Migration pattern of [^{3}H]rRNA recovered by the guanidine thiocyanate/CsCl isolation procedure (see the Materials and Methods section). (*b*) ^{3}H-labelled rRNA standard. The direction of migration is from left to right.

previously obtained from phenol-extracted brain RNA (Kaplan *et al.*, 1978). Since the size and sequence complexity of the poly(A)-containing RNA obtained from the guanidine thiocyanate/CsCl procedure (see below) and phenol method appear to be identical, the lower recovery obtained with phenol may be caused by a cleavage or decrease in the size of the 3′-terminal poly(A) segment, resulting in the non-quantitative binding of RNA to oligo(dT)–cellulose.

The density-gradient sedimentation profile of rapidly labelled RNA isolated from 5-day-old brain (frozen tissue) is shown in Fig. 1. The pattern of migration was heterodisperse, with most of the RNA sedimenting at more than 28S under denaturing conditions. The overall size of the RNA and absence of significant amounts of radioactivity from the 4S

region of the gradient indicates that the sample has undergone little nucleolytic degradation.

To evaluate further the efficacy of the isolation procedure, a ^{3}H-labelled RNA of known molecular weight was added to the brain homogenate and the integrity of the recovered standard was monitored. Fig. 2 shows the sedimentation profile of the ^{3}H-labelled *E. coli* 16S and 23S rRNA standard. Little alteration was observed in the pattern of migration of the isolated [^{3}H]rRNA (Fig. 2*a*) compared with the parental tracer (Fig. 2*b*). Recovery of the [^{3}H]rRNA was 81%. The remaining 15–20% of the [^{3}H]rRNA not located in the RNA pellet appeared in the DNA fraction that accumulates just above the 5.7M-CsCl cushion.

The base-sequence complexity of total poly(A)-containing RNA isolated by the guanidine thiocyanate/CsCl procedure was determined by RNA-driven hybridization reactions with non-repetitive [^{3}H]DNA. The sequence complexity of poly(A)-containing RNA was derived from the hybridization values obtained at RNA C_0t (R_0t) values $\geqslant 2.0\times10^4$ mol·litre^{-1}·s. The T_m of the RNA–DNA hybrids was 80.5°C, a value 2.5°C below that of non-repetitive [^{3}H]DNA duplexes formed under identical conditions. At saturation, a maximum of 10.2% of the [^{3}H]DNA formed RNA–DNA hybrids (Table 1, average for three preparations). After correction for the fraction of [^{3}H]DNA that could not form stable duplexes under the stringent hybridization conditions employed (Table 1, footnote †), brain RNA was complementary to 12.5% of the non-repetitive [^{3}H]DNA. Assuming asymmetric transcription, the sequence complexity of the RNA was 5.0×10^8 nt, a value identical with that previously obtained for phenol-extracted RNA (Kaplan *et al.*, 1978; see Table 1).

In summary, we have described a relatively simple single-step procedure for isolation of total RNA from brain. The RNA obtained is equal or superior to that provided by phenol extraction with regard to yield, purity, size and the content and sequence complexity of poly(A)-containing RNA. The guanidine thiocyanate/CsCl procedure is advantageous in that it is simple, is rapid, is highly reproducible and does not expose the RNA to enzymic treatments or exhaustive organic extractions. The method should prove especially useful for the isolation of RNA from small amounts of biological sample and/or from tissues with unusually high ribonuclease activity.

We thank Dr. B. S. Schachter for valuable technical advice. The preparation of this manuscript was assisted by comments from Dr. C. E. Finch (University of Southern California). This work was supported, in part, by Biomedical Research Support Grant 5S07 RR 05396.

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