Characterization and use of the potent ribonuclease inhibitor aurintricarboxylic acid for the isolation of RNA from animal tissues

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Inclusion of aurintricarboxylic acid (ATA) in extraction buffers for the isolation of RNA from animal tissues resulted in high yields (0.5–2.0 mg/g of tissue) of undegraded material as judged by agarose-gel-electrophoretic analyses and Northern-blotting experiments. However, ATA bound to nucleic acids, forming stable complexes, and so we have established methods for spectrophotometric quantification of RNA in these coloured complexes, and for easy removal of sufficient ATA to leave RNA in a consistently hybridizable condition at the end of a purification. The use and subsequent removal of ATA was straightforward and gave satisfactory results for all rat tissues tested.

INTRODUCTION

Isolation of intact RNA is an essential step in many types of biochemical investigations, including the cloning of reverse-transcribed mRNAs and, more frequently these days, hybridization analysis of mRNA abundance. In our studies on the regulation of lactate dehydrogenase gene expression it was necessary to obtain substantial quantities of RNA from multiple samples of various mammalian tissues, a task for which conventional procedures have not proved well suited. Guanidinium isothiocyanate-based methods (e.g. [1]) were unsatisfactory with tissues because, unlike the situation with cells, large amounts of debris consistently caused trapping losses of RNA at interfaces during CsCl centrifugation. Heparin can be included in extraction buffers, but is expensive in large quantity and was not always effective in our hands.

It has previously been reported that aurintricarboxylic acid (ATA) is a potent inhibitor of a number of nucleic acid-binding enzymes, including RNAases [2,3]. A subsequence study [4] showed that, of a series of RNAase inhibitors investigated, ATA gave the least degradation, in terms of the yield and size range of the RNA isolated, from rat and *Xenopus* liver when isolation was carried out at 25 °C or below. We therefore set out to investigate the value of ATA in the isolation of RNA from animal tissues.

The mode of action of ATA as an RNAase inhibitor is thought to be via direct competition with the RNA for the RNAase active site [5]. Our studies have shown that the ATA also binds strongly to RNA and DNA. Therefore RNA isolated in the presence of ATA is always complexed with ATA. This raised important questions about the routine use of this inhibitor. For example, can the RNA be quantified in a complex with ATA? Does bound ATA interfere with hybridization of RNA, and, if so, can ATA be removed from RNA after it has served its purpose during the isolation procedure? The present paper reports studies of these problems and describes the

conditions under which ATA can be used to isolate intact RNA for hybridization analyses.

MATERIALS AND METHODS

Materials

All common reagents (AnalaR grade) were obtained from BDH Chemicals, Poole, Dorset, U.K. ATA (ammonium salt), glyoxal, 8-hydroxyquinoline, yeast tRNA, salmon sperm DNA, diethyl pyrocarbonate and Dowex MR3 mixed-bed ion-exchange resin were obtained from the Sigma Chemical Co., Poole, Dorset, U.K. Agarose (SEAKEM ME) was obtained from the FMC Corporation, Rockland, U.S.A. Sephadex G-50 (medium grade) was obtained from Pharmacia, Milton Keynes, Bucks., U.K.

Preparation of cytoplasmic RNA from rat tissues

Before use, all glassware was baked overnight, all plasticware was autoclaved at 0.1 mPa (15 lbf/in²) (gauge) for 20 min, and polypropylene centrifuge tubes were soaked overnight in 0.1% diethyl pyrocarbonate followed by autoclaving as above.

Tissues were ground to a fine powder in liquid N₂, and then suspended in about 7 vol. of diethyl pyrocarbonate-treated 25 mM-Tris/HCl buffer, pH 8, containing 25 mM-NaCl, 5 mM-MgCl₂ and 2% (v/v) Triton X-100. Where present, ATA was added to 0.5 mM before use. The homogenate was centrifuged at 35000 *g* for 10 min and the supernatant was decanted carefully to avoid disrupting the pellet, which contained nuclear material. SDS was added to the supernatant to 0.5% and swirled briefly before the addition of an equal volume of chloroform/water-saturated phenol (1:1, v/v). After low-speed centrifugation the aqueous phase was extracted up to two more times with the chloroform/phenol mixture or with chloroform alone, until deproteinization was complete. RNA was then precipitated overnight at –20 °C with 2 vol. of ethanol,

Abbreviation used: ATA, aurintricarboxylic acid.

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pelleted at 12000 *g* for 20 min, dried *in vacuo* and redissolved in a minimum quantity of diethyl pyrocarbonate-treated water. Residual insoluble material was removed by centrifugation, and the RNA was divided into small portions and stored in liquid N₂.

Removal of ATA from RNA

Sephadex G-50 was suspended in 1×load buffer (20 mM-Tris/HCl buffer, pH 7.6, containing 0.5 M-LiCl, 1 mM-EDTA and 0.1% SDS). Spun columns were prepared by filling 5 ml syringes with the Sephadex G-50 suspension, and these were then centrifuged in Corex centrifuge tubes in an MSE Minor centrifuge at 2000 rev./min for 3 min, eluates being collected in microcentrifuge tubes. RNA samples, prepared as above, were mixed with equal volumes of 2×load buffer, and put through these columns (2000 rev./min for 3 min). Amounts of 1–20 mg of RNA could be processed on a single column. One such treatment was usually sufficient, but, if colour was still visible in the eluate, it was re-spun through fresh columns. The RNA was precipitated, harvested and stored as described in the previous subsection. The Sephadex G-50 was re-used until it became saturated with ATA, at which time it was recycled by suspension in 1×load buffer followed by thorough washing with 1% (w/v) SDS. The Sephadex G-50 was then dried, resuspended in 1×load buffer and autoclaved at 0.1 MPa (gauge) for 20 min. Any residual SDS was removed by allowing the Sephadex G-50 to settle, aspirating off any excess buffer, adding 100 ml of 1×load buffer and swirling for a few minutes. This washing process was repeated a further four times.

Agarose-gel analysis of RNA

A 20–25 µg portion of RNA (or 0.75 µg of bacteriophage λ *Hind*III-digested DNA marker), was made up to at least 10 µl in 0.01 M-phosphate buffer, pH 6.6, and to this was added 3 µl of RNA load buffer [50% (v/v) glycerol in 10 mM-phosphate buffer, pH 6.6, containing 4 mg of Bromophenol Blue/ml]. The samples were loaded on to a 1% agarose gel made in, and immersed in, 0.01 M-phosphate buffer, pH 6.6. Constant buffer recirculation was achieved with a peristaltic pump. Electrophoresis was for 4–6 h at 45 V, and RNA was detected under u.v. light after staining with 1 µg of ethidium bromide/ml.

In experiments where RNA was subsequently blotted on to Genescreen Plus, a different running buffer (12 mM-Tris/6 mM-sodium acetate/0.3 mM-EDTA buffer, pH 7.0) was employed.

Preparation of ATA–nucleic acid complexes

A 25 µg portion of yeast tRNA was mixed with ATA, made up to a total volume of 100 µl in the homogenization buffer described above, and left at room temperature for 10 min. Then 2.5 vol. of ethanol was added, and the RNA was precipitated on ice for 1 h. The samples were centrifuged at 13000 rev./min for 10 min in the microcentrifuge and washed a variable number of times with 250 µl of 70% (v/v) ethanol. The final pellets were dried *in vacuo* and redissolved in 1 ml of sterile water.

Complexes with DNA (calf thymus) were prepared as for ATA–tRNA complexes, except that DNA to be denatured was heated in a boiling-water bath for 5 min and chilled on ice before use. A 50 µl portion of DNA

(1 mg/ml) was mixed with ATA as above, and made up to a total volume of 150 µl in standard homogenization buffer.

Northern-blot and slot-blot analysis of RNA

RNA and DNA markers were denatured with glyoxal before electrophoresis and blotted on to Genescreen Plus membranes by standard techniques. Hybridization was at 42 °C in a solution containing 1 M-NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulphate, 100 µg of denatured salmon sperm DNA/ml and 50% (v/v) formamide; probe DNA was 100 ng of pRLD42 [6], a clone of part of rat lactate dehydrogenase A mRNA, together with 375 pg of bacteriophage λ DNA markers all labelled together by using the oligonucleotide-primed labelling technique [7,8]. Washes were, for liver RNA, twice in 2×SSC/1% SDS for 10 min at room temperature, once or twice (as required) in 2×SSC/1% SDS for 30 min at 30 °C and once in 2×SSC for 30 min at room temperature (1×SSC is 0.15 M-NaCl/0.015 M-sodium citrate buffer, pH 7.5), followed by autoradiography. For testes RNA, the 30 °C wash was replaced with one at 50 °C (also in 2×SSC/1% SDS).

For slot blots, testes RNA samples were denatured and bound to Genescreen Plus (always in triplicate). Hybridization, washing and autoradiography employed the same conditions optimized by using Northern-blot analyses ([6,9–12]; and see above). Autoradiographs were scanned with an LKB laser-beam Ultrosan scanner.

RESULTS

ATA as an RNAase inhibitor

Fig. 1(a) shows typical cytoplasmic RNA preparations isolated from rat liver in the presence or in the absence of ATA after detection on agarose-gel electrophoretograms. Cytoplasmic RNA made in the presence of ATA consistently showed sharp 18 S and 28 S rRNA bands, whereas samples made without ATA did not. The condition of rat liver RNA made in the presence and in the absence of ATA was also tested on a Northern blot, with a probe for lactate dehydrogenase A (Fig. 1b). This clearly showed a strong band at about 1.75 kb (the expected size for the mature mRNA) when ATA was present, but only a smear of degraded material in its absence. Extents of degradation in the absence of ATA were variable, but that shown in Fig. 1 was by no means unusual. Lactate dehydrogenase A mRNA was present at very low abundance in liver, necessitating use of relatively low-stringency washes to detect the mRNA at all. Fig. 1 therefore shows that the use of ATA allowed the isolation of intact RNA, whereas its absence usually resulted in preparations that were substantially degraded. It was routinely possible to obtain 0.5–2.0 mg of RNA/g of original tissue in experiments with heart, liver, testes and kidney, whereas in our hands the guanidinium method yielded less than 50 µg of RNA/g of tissue.

Characterization of ATA–RNA complexes

It was evident during RNA isolation that ATA bound to nucleic acids to form coloured complexes, and it was important to investigate the implications of complex-formation for routine use of this inhibitor.

Fig. 2 shows that absorption spectra of ATA and RNA were simply additive in such a complex. Fig. 2(a) shows typical ATA and RNA absorption spectra. Figs.

Fig. 1. State of RNA isolated in the presence and in the absence of ATA

Liver cytoplasmic RNA was isolated as described in the Materials and methods section. (a) Duplicate samples made without (lane 1) or with (lane 2) 0.5 mM-ATA and electrophoresed through agarose gels, and RNA was detected with ethidium bromide. (b) Autoradiographs of RNA made with (lane 1) or without (lane 2) 0.5 mM-ATA after electrophoresis, blotting on to Genescreen Plus and probing with pRLD42 for lactate dehydrogenase A mRNA. Lane 3 shows bacteriophage λ DNA markers. All gel lanes contained about 25 μ g of native RNA (a) or denatured RNA (b).

2(b) and 2(c) show actual and theoretical curves for the ATA-RNA complex. By using a spectrum of ATA alone, absorbance was quantified at 327 nm (a region where RNA did not absorb significantly) and at 10 nm intervals between 200 and 350 nm. Each interval value was divided by the A_{327} value to yield a total of 15 ratios. Similarly, from the spectrum of RNA alone, absorbance was quantified at 260 nm and at 10 nm intervals between 200 and 350 nm. Each interval value was divided by the A_{260} value to yield a second set of 15 ratios. If absorbances were simply additive, the A_{260} minus the A_{327} of the curve for a complex should be due solely to the RNA present, since ATA absorbance was approximately equal at 260 nm, 280 nm and 327 nm. The $A_{260} - A_{327}$ values of actual complexes were then multiplied by the ratios calculated from the curve for pure RNA. Similarly, the A_{327} value of the curve for the complex (which was due solely to the ATA present) was quantified and multiplied by the ratios calculated from the curve for pure ATA. These two sets of data were then added together to give a theoretical curve for the complex. The theoretical and observed curves for the complex were not significantly different at any ATA/RNA ratio examined [two examples are shown in Figs. 2(b) and 2(c)]. Thus the absorbances of ATA and RNA in the complexes were

Fig. 2. Absorption spectra of RNA and ATA

(a) Typical absorption spectra of (A) ATA (0.05 mM) and (B) tRNA (50 μ g/ml). (b) Duplicate absorption spectra of ATA-tRNA complex, made with ATA at 0.5 mM. $\odot$ symbols show the path of a theoretical curve based on simple addition of ATA and RNA absorbances (see the text). (c) As in (b) except that ATA was at 5 mM during complex-formation. RNA was at 25 μ g per 100 μ l during binding, after which complexes were washed twice by ethanol precipitation and dissolved in 1 ml of water for spectrophotometry.

Table 1. Spectrophotometric analysis of ATA–RNA interactions

(a) Quantification of tRNA in the presence of ATA. A 25 μ g portion of yeast tRNA was used in each sample. The numbers shown are arbitrary units, based on direct absorbance measurements, and are the averages for two to six samples with standard deviations in parentheses. (A), Total absorbance at wavelength shown; (B), absorbance after subtraction of that at 327 nm. (b) Stability of ATA–tRNA binding. Complexes were made with the use of 25 μ g of yeast tRNA and 5 mM-ATA. (c) Binding of 25 μ g of yeast tRNA to different concentrations of ATA.

(a) Quantification of tRNA in the presence of ATA

Sample		A_{260}	A_{280}	A_{327}	A_{260}/A_{280} ratio
tRNA only	(A)	30.5 (± 3.8)	14.7 (± 2.2)	0.8 (± 1.8)	2.1:1
	(B)	27.9	13.9	0	2.1:1
50 mM-ATA	(A)	22.5 (± 1.5)	22.5 (± 1.5)	22.5 (± 1.5)	1.0:1
	(B)	0	0	0	
tRNA + 0.5 mM-ATA	(A)	33.8 (± 0.75)	17.5 (± 0.5)	4.25 (± 0.25)	1.9:1
	(B)	29.6	13.25	0	2.2:1
tRNA + 5 mM-ATA	(A)	39.0 (± 3.5)	23.25 (± 1.75)	9.75 (± 0.25)	1.7:1
	(B)	29.25	13.5	0	2.2:1

(b) Stability of ATA–tRNA binding

	No. of washes	A_{215}	A_{260}	A_{215}/A_{260} ratio
	1	118.50 (± 1.50)	50.75 (± 1.25)	2.33:1
	2	103.25 (± 5.25)	45.75 (± 3.75)	2.26:1
	3	99.75 (± 3.75)	45.25 (± 1.75)	2.20:1
	4	98.00 (± 2.00)	43.50 (± 1.50)	2.25:1
	5	95.50 (± 1.50)	43.50 (± 0.00)	2.20:1
	6	94.50 (± 0.50)	42.00 (± 0.00)	2.25:1

(c) Binding of tRNA to different concentrations of ATA

	Concn. of ATA (mM)	A_{215}	A_{260}	A_{215}/A_{260} ratio
	0	32.50 (± 3.20)	32.40 (± 4.00)	1.0:1
	0.05	28.50 (± 4.50)	27.00 (± 4.00)	1.1:1
	0.25	45.25 (± 2.25)	35.00 (± 3.00)	1.3:1
	0.50	52.50 (± 2.50)	33.75 (± 0.75)	1.6:1
	2.50	59.25 (± 1.75)	35.50 (± 0.50)	1.7:1
	5.00	99.75 (± 3.75)	45.25 (± 1.75)	2.2:1

essentially additive, permitting simple quantification of the amount of RNA present as described below.

Table 1(a) shows how RNA could be accurately quantified in a complex. The A_{327} value was subtracted from the A_{260} value and the A_{280} value. This removed the effect of the ATA at these wavelengths, as illustrated by examining the A_{260}/A_{280} ratio. Pure tRNA had an A_{260}/A_{280} ratio of 2.1–2.2:1, whereas pure ATA had a ratio of 1:1. This means that the A_{260}/A_{280} ratio of RNA containing ATA was lowered as a function of the amount of ATA present. However, if the subtractions outlined above were made, the A_{260}/A_{280} ratio of the complex returned to the 2.1–2.2:1 ratio shown by pure RNA, thus eliminating the effects of the ATA. These values could

then be used to calculate the RNA concentration in the usual manner. This was found to hold true over a 100-fold concentration range of ATA (two ATA concentrations are shown in Table 1).

Table 1(b) demonstrates the stability of the ATA–RNA complex. An A_{215}/A_{260} ratio was found to be most useful in characterizing ATA–nucleic acid complexes; this was 1:1 for pure RNA and 6:1 for pure ATA, with complexes having ratios between these values depending on the amounts of ATA and RNA present. The A_{215}/A_{260} ratio could be measured more accurately than the A_{260}/A_{327} ratio owing to higher absolute values. Essentially no further ATA could be removed after two washes, as no significant decrease in the A_{215}/A_{260} ratio was observed

after this point. The first two washes presumably removed excess weakly bound ATA.

Table 1(c) reports studies on the formation of ATA-RNA complex over a range of ATA concentrations, including that used during RNA isolation from rat tissues. A 25 μ g portion of yeast tRNA was treated with various concentrations of ATA, precipitated in ethanol and washed twice with 70% (v/v) ethanol (by the procedure indicated in the Materials and methods section). Over the 100-fold concentration range used, the RNA was not saturated with ATA, as the A_{215}/A_{260} ratios did not plateau. ATA in the homogenization buffer during preparation of RNA from rat tissues was routinely at 0.5 mM, evidently a sub-saturative concentration with respect to complex-formation.

ATA also binds to DNA

Table 2 shows the results of the reaction of native or denatured salmon sperm DNA with various concentrations of ATA. Both states of DNA were equally capable of complexing the inhibitor. DNA binding was further investigated by analysing the electrophoretic mobilities of a supercoiled plasmid on agarose gels, with ATA or ethidium bromide in the running buffer at various concentrations. The ethidium bromide was used as a control, as it is a well-known intercalating agent. Such compounds progressively retard plasmid mobility on agarose gels, as supercoiling is unwound as a result of intercalation, but at higher concentrations mobility increased again owing to supercoiling in the opposite direction. Fig. 3(a) shows significant effects of ATA on plasmid mobility, but not as much as seen with ethidium bromide. The concentration of ethidium bromide required for maximum plasmid retardation was 130 nM, whereas the concentration of ATA required was 150 μ M. Retardation of the plasmid by ATA did not prove that ATA was intercalating, though the pattern of retardation and subsequent acceleration indicated some effect on supercoiling. However, when the mobility of RNA made in the presence of various concentrations of ATA was compared with that of RNA prepared in its absence, none of which contained any added ATA in the gel buffers (Fig. 3b), there were no differences in the electrophoretic mobilities of any of the samples. Testis

RNA was used for these experiments because, although usually degraded in the absence of ATA (lane 9), RNAase problems were less than with other tissues and it was occasionally possible to obtain material showing 18 S and 28 S bands after isolation without inhibitors (duplicate lanes 10 and 11). ATA apparently dissociated from RNA during electrophoresis, and moved as a visible band of colour just ahead of tRNA (result not shown).

Hybridization efficiency of RNA made in the presence of ATA

We also investigated the effects of ATA on the hybridization efficiency of RNA. Rat testis RNA made in the presence of ATA was probed for lactate dehydrogenase A mRNA with the use of cDNA probe pRLD42 [6]. As shown in Fig. 4(a), this probe highlighted a single band of about 1.85 kb (as in liver) under wash temperatures ($2 \times$ SSC/1% SDS) of 40 °C or higher; at 30 °C in this tissue (and sometimes in liver) a band of larger size was also seen, but above 50 °C the signal intensity was sharply diminished. RNA made in the absence of ATA rarely yielded any discrete banding pattern on Northern blots; material made with ATA but from which most ATA was subsequently removed by Sephadex G-50 spun columns (see the Materials and methods section) still yielded the 1.85 kb bands (see lanes 5 and 6 of Fig. 4a) but usually showed at least some signs of degradation. Quantification of ATA effects on hybridization efficiency of RNA was investigated by using slot blots, with wash stringencies always giving a single sharp band on Northern blots (i.e. the 50 °C wash of Fig. 4a). Autoradiography and densitometry showed that RNA made in the presence of ATA, and with the inhibitor still bound, always gave a lower hybridization signal than RNA prepared in its absence (exemplified in Fig. 4b). However, removing the ATA from RNA with the use of Sephadex G-50 resulted in some recovery of hybridization relative to blots of unprocessed ATA-RNA complexes (Fig. 4c). Hybridization to ATA-RNA complexes in Fig. 4(c) was lower than in Fig. 4(b) because those in the former had higher ATA/RNA ratios than the latter (results not shown).

For ATA to be useful in the isolation of RNA for hybridization analysis, it was therefore necessary to

Table 2. Binding of ATA to native and denatured salmon sperm DNA

Batches (50 μ g) of DNA were treated with ATA and washed twice by ethanol precipitation before spectrophotometric analysis. Data are shown as averages of arbitrary units, with standard deviations in parentheses. (N), Native DNA; (D), denatured DNA.

Concn. of ATA (mM)		A_{215}	A_{260}	A_{215}/A_{260} ratio
0	(N)	51.25 (± 8.25)	56.50 (± 11.5)	0.91:1
	(D)	71.00 (± 0)	75.50 (± 0)	0.94:1
0.05	(N)	55.70 (± 3.1)	57.70 (± 4.50)	0.97:1
	(D)	58.00 (± 0)	55.00 (± 0)	1.05:1
0.25	(N)	80.30 (± 6.8)	63.70 (± 2.10)	1.26:1
	(D)	105.8 (± 4.5)	83.30 (± 8.40)	1.27:1
0.50	(N)	100.2 (± 9.6)	73.00 (± 5.40)	1.37:1
	(D)	120.3 (± 1.2)	90.70 (± 14.7)	1.33:1
5.00	(N)	147.0 (± 9.9)	116.7 (± 23.4)	1.26:1
	(D)	156.3 (± 2.4)	150.3 (± 16.5)	1.04:1

Fig. 3. Effects of ATA on electrophoretic mobilities of RNA and DNA

(a) Electrophoretic mobilities of 200 ng batches of supercoiled plasmid DNA in 0.8% agarose gels with various concentrations of ethidium bromide ($\odot$) or ATA ($\square$) in running buffers. DNA was in all cases detected with ethidium bromide at the end of electrophoresis. Extent of retardation is expressed as apparent plasmid DNA size, in kbp, relative to relaxed marker DNAs run simultaneously. (b) Electrophoretic mobilities of testis RNA samples isolated with various concentrations of ATA in extraction buffers but without ATA in gel running buffers. Lanes 1 and 12, bacteriophage λ DNA markers; lanes 2-8: RNA isolated in the presence of 5 mM-, 2.5 mM-, 0.5 mM-, 0.25 mM-, 0.15 mM-, 0.05 mM- and 0.025 mM-ATA respectively; lanes 9-11, RNA isolated without ATA (see the text). All lanes contained 8-20 μg of RNA, and after electrophoresis the RNA was detected with ethidium bromide.

establish the consistency with which ATA could be removed before the blotting. ATA-RNA complexes isolated from a variety of tissues generally had A_{215}/A_{260} ratios in the range 4.0-5.4:1; after passage through Sephadex G-50 this could be decreased to around 1.55:1 (Table 3). The slight differences between tissues were not significant (comparing liver and skeletal muscle: $t = 0.114$, degrees of freedom = 21, $P = 2.080$). Evidently ATA was never completely removed; pure RNA had an A_{215}/A_{260} ratio of about 1:1 (Table 1). Nevertheless, removal was sufficiently reliable for different samples of RNA from particular tissues to yield consistent estimates of lactate dehydrogenase A mRNA abundance. Slot blots of testis RNA made in the presence of ATA from three rats of identical ages, and cleaned by Sephadex G-50 chromatography, yielded hybridization regression slopes all within 10% of their mean value (average = 67, S.D. = 6.4).

DISCUSSION

The value of ATA as a potent RNAase inhibitor has been apparent for some time. Because ATA is so effective, it has proved valuable in preparing RNA from mammalian tissues that are notorious for having very high endogenous RNAase activities, especially brain, kidney and, to a somewhat lesser extent, liver. The RNA from

mammalian tissues is especially at risk from degradation because of the time that it takes to dissect out and homogenize organs under study. Therefore it is essential to add a strong RNAase inhibitor as early as possible to ensure reasonable yields of high-quality RNA. In fact, it has proved impossible to make intact RNA from some tissues in the absence of ATA or other potent RNAase inhibitors, and the use of ATA gave far higher yields of intact RNA than with other inhibitors we have tried, including guanidinium isothiocyanate. Further advantages in using this inhibitor rather than any other are that it is easy to use, safe and very cheap (even to the extent that the Sephadex G-50 used to remove it from the RNA can be recycled). Removal by Sephadex G-50 spun columns was very quick, and thus preferable to electroelution, which the results shown in Fig. 3 indicated could be a possible alternative. Although we have not investigated use of RNA made in the presence of ATA by enzymes such as reverse transcriptase, DNA isolated in the presence of ATA can be digested with restriction endonucleases and can serve as a template/primer for DNA polymerase I after ATA removal by a method similar to ours [2]. However, many studies require RNA only to be intact and in good condition for hybridization analysis, and for this purpose ATA certainly seems well suited.

The protective action of ATA was reportedly due to

Fig. 4. Hybridization efficiency of RNA isolated with ATA

(a) Northern-blot analyses of rat testis RNA probed with pRLD42 (see the Materials and methods section). Lanes 1–4 each contained 15 μg of RNA isolated in the presence of 0.5 mM-ATA and run directly; lanes 5 and 6 contained similar amounts of RNA made in the same way, but with

Table 3. Efficiency of removal of ATA from ATA–RNA complexes

Four to 26 samples were measured from each tissue; absorbances at 215 nm, after ATA removal, ranged from 0.25 to 1.26 (average 0.24). Ratios are averages with standard deviations in parentheses.

Tissue source of RNA	A_{215}/A_{260} ratio of RNA–ATA complexes	
	As isolated (ATA still present)	After Sephadex G-50 wash
Testes	4.83:1 (±0.60:1)	1.61:1 (±0.32:1)
Liver	—	1.28:1 (±0.12:1)
Skeletal muscle	4.37:1 (—)	1.80:1 (±0.24:1)
Heart muscle	5.38:1 (—)	1.79:1 (±0.35:1)
Overall	4.85:1 (±0.56:1)	1.55:1 (±0.33:1)

ATA binding in the active site of RNAase [5]. It was apparent, however, that the ATA was also binding to the RNA in our preparations, since even purified samples were red in colour. Therefore ATA may not only inactivate endogenous RNAase, but also, by binding to the RNA, protect it from possible degradation by later contamination with exogenous RNAases. In a washed ATA–tRNA complex from which loosely bound ATA has been removed there were approximately four ATA monomers per nucleotide residue [calculated from Fig. 2(a) and Table 1(a)]. Commercial preparations of ATA are invariably mixtures of monomeric and polymeric material, with average unit lengths of about 5.5 ATA molecules irrespective of concentration in solution; polymeric ATA constitutes the effective RNAase inhibitor [3], so our results indicate that there was almost one molecule of functional inhibitor bound per nucleotide residue.

The conclusions of our studies on ATA therefore are that it is a very useful RNAase inhibitor, as it enables easy recovery of a high yield of RNA in good condition, even from tissues with high endogenous RNAase activities. Sufficient ATA can then be removed to yield RNA suitable for slot-blotting experiments and thus quantification of changes in abundance of specific mRNAs during growth and development.

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ATA removed by Sephadex G-50 before electrophoresis. Washes after hybridization were with $2 \times \text{SSC}/1\%$ SDS at 60 °C (lane 1), 50 °C (lanes 2, 5 and 6), 40 °C (lane 3) or 30 °C (lane 4). (b) Slot-blot analyses of RNA made in the absence (○) and in the presence (□) of 0.5 mM-ATA; (c) as in (b) except with the use of RNA made with ATA (□) or RNA made with ATA that was subsequently removed (○). Hybridization signals represent integrated values from laser scans of autoradiographs; pRLD42 was used as probe, and wash stringency was $2 \times \text{SSC}/1\%$ SDS at 50 °C (see the Materials and methods section).

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