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A COMPARISON OF THE CHROMATOGRAPHIC PROPERTIES OF VARIOUS POLYADENYLATE BINDING MATERIALS

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SUMMARY

The suitability of various materials for messenger RNA isolation and possible fractionation was examined by comparing the chromatographic behaviour of synthetic poly(riboadenylic acid) [poly(A)] and ribosomal RNA on each support. The ratios of poly(A) to ribosomal RNA capacities of the various materials under different chromatographic conditions were used to estimate the potential selectivity of the columns for messenger RNA isolation. Using this criterion, benzoylated cellulose and oligo(deoxythymidylate)-cellulose should be the most selective materials of those studied. The elution conditions (temperature or salt gradient) used to obtain bound poly(A) were investigated to determine the optimum conditions for possible messenger RNA fractionation. It is concluded that oligo(deoxythymidylate)-cellulose appears to be the most suitable support of those investigated for messenger RNA isolation and possible fractionation.

INTRODUCTION

The covalent linkage of poly(A)** sequences to the majority of mRNA molecules of various organisms has permitted separation of these molecules from rRNA and tRNA, using relatively simple methods of affinity chromatography[1–4]. Most procedures described involve addition of the RNA under conditions which promote base pairing between the poly(A) in the RNA and the complementary nucleotides attached to the column. The column is then washed to remove non-bound RNA and the poly(A) containing RNA is eluted usually in one step, either by lowering the ionic strength of the eluent[1,4], including formamide in the eluent[5], raising the temperature of the column[6], or a combination of these[4].

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** Abbreviations used throughout the article: Bz-cellulose = benzoylated cellulose; oligo (dT) = oligo(deoxythymidylate); poly(A) = poly(riboadenylic acid); poly(U) = poly(ribouridylic acid); RNA = ribonucleic acid; mRNA = messenger RNA; rRNA = ribosomal RNA; SDS = sodium dodecyl sulphate; tRNA = transfer RNA.

The aim of this work was to compare the binding and elution behaviour of synthetic poly(A) and rRNA to different poly(A) binding supports in order to select conditions where poly(A) binding was high and rRNA binding low, and to generally compare the characteristics of the different materials. In addition, it is considered that the variable length of poly(A) attached to natural mRNA[7] and the length of the non-poly(A) region may affect the binding or elution behaviour of mRNA on these columns sufficiently to permit some type of fractionation. Thus, we have studied the elution of poly(A) to select optimum conditions under which this may occur. A preliminary report of some of this work has appeared elsewhere[8].

Most commonly used poly(A) binding materials were studied, with the exception of millipore filters and poly(U)-glass filters[10], since these did not seem readily applicable to the procedures we were using. Benzoylated cellulose is a new poly(A) binding material.

EXPERIMENTAL

Chemicals

Poly(A) was purchased from Miles Laboratory (Elkhart, Ind., U.S.A.), Poly(U) and Sigmacell type 38 cellulose from Sigma (St. Louis, Mo., U.S.A.), CF 11 cellulose from Whatman. Sepharose 4B and poly(U)-Sepharose are products of Pharmacia (Uppsala, Sweden). Poly(U)-Sepharose was also prepared as described below.

General column procedures

The cellulose powder, substituted cellulose and poly(U)-Sepharose were packed under air pressure into glass columns (I.D. 1 cm) in 0.5 *M* salt solution buffered with 0.01 *M* Tris–HCl, pH 7.4 (20°). The columns were washed until the optical density (257 nm*) of the effluent was 0.01 or less. At this point, the columns were re-equilibrated if necessary with the required experimental buffer solution. All salt solutions used in this report were buffered with 0.01 *M* Tris–HCl, pH 7.4 (20°).

To determine the poly(A) binding capacity of the various materials, poly(A) (2 mg/ml in the same salt solution as the column buffer) was added in portions (usually 5 A_{257} units) to a column containing 0.5 or 1 g of the material. The column was washed with 10–15 ml of the salt buffer after each addition to remove the non-bound poly(A). The amount bound by the column was obtained from the difference between the amount added and the amount eluted. The additions were repeated until more than 90% of added poly(A) failed to bind to the column. The bound material was then eluted with buffer (0.01 *M* Tris–HCl, pH 7.4) and the elution was repeated with this buffer after 18 h at 20°. With poly(U)-cellulose, the 0.01 *M* Tris–HCl wash was performed at 45°. The binding capacities are expressed as A_{257} units of poly(A) retained by 1 g or 1 ml of column material. The capacity of each material for rRNA was determined by addition of 10 A_{257} units of rRNA to a column equilibrated in the indicated buffer solution. The RNA was allowed to bind for 10 min at 20°, then the column was washed with buffer to elute the non-bound RNA followed by 0.01 *M* Tris–HCl (pH 7.4) to obtain the bound RNA. The recovery was usually complete. A control column was used to correct for the elution of any non-nucleotide material.

* The absorbance at 257 nm was used since this is λ_{max} of poly(A).

Temperature-controlled columns

For the temperature of elution experiments, a water-jacketed column was used, attached to a Haake FT water bath, which in turn was controlled by a Haake PG11 temperature programmer. The optical density of the effluent was monitored with an ISCO UA-4 absorbance monitor.

The temperature of elution of poly(A) from the various materials was determined usually by application of a linear increasing temperature gradient (18°/h) to the columns. Slower rates (12°/h) gave comparable results; faster rates of heating (30°/h) gave a 1–2° wider temperature range of elution. In most cases, the poly(A) was added to the column equilibrated in the required salt solution at 20° unless elution was expected below this temperature; in the latter case binding was performed at 2–4°. Pre-binding in 0.5 *M* salt buffer did not alter the subsequent temperature of elution in a lower ionic strength buffer. Stepwise heating (5° intervals) gave the same temperature range of elution as the continuous heating experiments.

Preparation of column materials

Oligo(dT)-cellulose was prepared by the method of Gilham[11] using Whatman CF 11 cellulose, which had been washed according to the method of Perrin *et al.*[12] and dried *in vacuo* over phosphorus pentoxide at 45°. Poly(U)-cellulose was prepared as described by Sheldon *et al.*[10]. Poly(U)-Sepharose was prepared by coupling poly(U) to cyanogen bromide activated Sepharose[5,13]. Alkaline hydrolysis in 0.1 *M* NaOH for 12 h at 20° released 7.5 A_{260} units of UMP per ml of packed Sepharose. All buffer solutions used with this material and with poly(U)-cellulose were autoclaved. Benzoylated cellulose was prepared according to the procedure described for the preparation of benzoylated DEAE-cellulose[14].

Poly(A) size distribution

Poly(A) was analysed by sedimentation on a 5–40% linear sucrose gradient in 0.14 *M* NaCl, 0.01 *M* Tris–HCl (pH 7.4) for 3.5 h at 48,000 rpm in a Beckman SW 50L rotor on a Beckman L2-65B centrifuge at 4°. The size distribution of poly(A) on 2.4% polyacrylamide gels was determined according to Loening[15], using *E. coli* rRNA as marker. The *E. coli* rRNA was isolated by phenol–chloroform extraction[16] of ribosomes prepared according to Staehelin and Maglott[17].

The poly(A) was found to sediment between about 3 and 12 S on a 5–40% sucrose gradient, with a maximum at 7 to 8 S. When analyzed on 2.4% polyacrylamide gels, the poly(A) was distributed between the 4 and 23 S *E. coli* RNA markers. The relationship between sedimentation coefficient[18], or electrophoretic mobility[19] and molecular weight of poly(A) indicated that the size range of this material was about 50–2,500 nucleotides, with a modal size of about 1,300 nucleotides. Thus, the synthetic material was considerably larger than the natural poly(A) (50–200 nucleotides[7]), but conclusions obtained concerning column capacity and salt effects should be the same for both. There may be minor differences in the temperature of elution and fractionation possibilities for the two size classes (see Discussion).

RESULTS

Factors affecting the binding of poly(A) and rRNA to unmodified cellulose powder

Unmodified cellulose powder has been found to bind poly(A)[20,21] and poly(A)

containing RNA[3,22]. Since it is the simplest available material for this purpose, we have examined its poly(A) binding properties in some detail. The capacity of cellulose powder for poly(A) and rRNA was found to depend upon salt concentration, salt cation type and the presence of detergents in the column buffer. Also, the poly(A) binding capacities of various types of cellulose were found to be different in agreement with other reports[3,21]. Fig. 1A shows the effect of salt cation on the poly(A) capacity of Sigmacell. The cellulose bound about the same amount of poly(A) in NH_4^+ and K^+ buffers, and the amount bound was about 10-fold higher than that in Na^+ and Li^+ buffers. The capacity of CF 11 cellulose for poly(A) (Fig. 1B) was about one tenth that of Sigmacell and a similar cation dependence was evident.

With experiments involving repeated additions of small portions of poly(A) to the columns, a point is reached where not all the added poly(A) binds to the material (O in Fig. 1A on KCl curve). For convenience, this point is termed the operational capacity, since it gives an idea of the actual capacity one would use in practice. Some of the added poly(A) continues to bind until a point is reached where no more poly(A) can bind to the column (M in Fig. 1A on NaCl curve); this point is referred to as the maximum capacity. The operational capacity might be expected to depend upon such factors as the amount of poly(A) in each addition and the time allowed for binding, but in these experiments we obtained reasonably constant values.

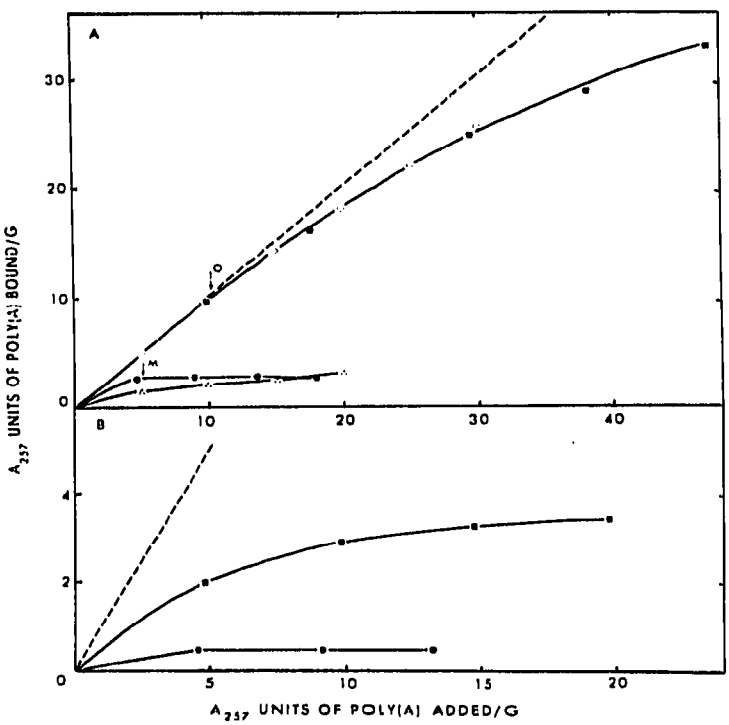

Fig. 1. Effect of salt cation type on poly(A) capacity of (A) Sigmacell and (B) CF 11 cellulose. Poly(A) binding capacity of the cellulose equilibrated in 0.01 *M* Tris–HCl (pH 7.4) containing the indicated salt at a concentration of 0.5 *M* was determined by addition of portions of poly(A) as described in Experimental. ■—■, KCl; ○—○, NH_4Cl; △—△, LiCl; ●—●, NaCl; — — —, complete binding.

The effect of KCl concentration on the operational and maximum capacities of Sigmacell is shown in Fig. 2 (top two lines). Also included is the effect of salt concentration (KCl or NaCl) upon the binding of rRNA to Sigmacell (lower two lines). There is a linear relationship between the log of maximum and operational capacities and salt concentration, although only three points were obtained for the operational capacity.

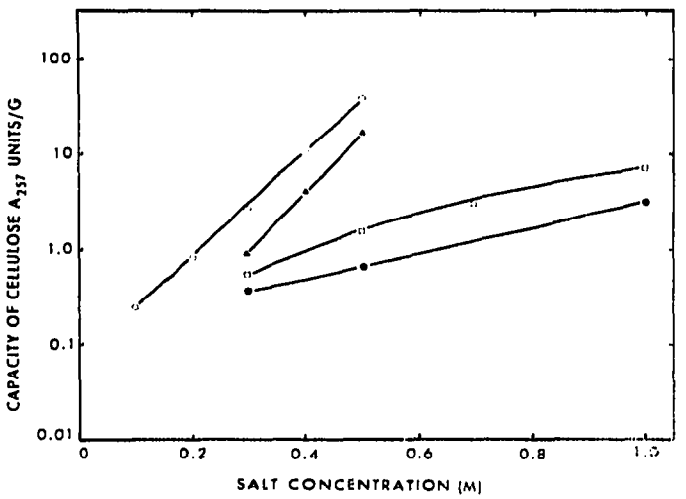

Fig. 2. Effect of salt concentration on capacity of Sigmacell for poly(A) and rRNA. Poly(A) and rRNA binding capacities of the cellulose in 0.01 *M* Tris–HCl containing the indicated salt concentration were determined as described in Experimental. □—□, rRNA capacity in KCl; ●—●, rRNA capacity in NaCl; ▲—▲, operational capacity for poly(A); ○—○, maximum capacity for poly(A).

The binding of *E. coli* rRNA to Sigmacell also increased in a roughly logarithmic manner with salt concentration, although the rate of increase was lower than that observed with poly(A). The binding of rRNA was higher in KCl than in NaCl buffers, which is an interesting parallel with poly(A), although the effect was not as pronounced. The capacity of Sigmacell for rRNA was considerably higher than that reported by Sullivan and Roberts[3] for Sigma α cellulose; it is possible that different batches of cellulose differ in their rRNA as well as poly(A) capacities. Similar extents of binding were observed with plant rRNA[23] so this is not a phenomenon of a particular type of rRNA. In accord with Sullivan and Roberts[3], we found the binding of rRNA decreased at higher temperatures. The following capacities (A_{257} units/g) of Sigmacell for rRNA in 0.5 *M* KCl buffer at the indicated temperatures were obtained: 4°, 3.4; 23°, 1.4; 30°, 0.66; 45°, 0.44. Even at 45°, however, the capacity of this batch of cellulose for rRNA was high enough to cause considerable contamination of mRNA unless it was reduced further by some other means.

Detergents in the binding buffer were found either to decrease or eliminate the poly(A) binding to Sigmacell, perhaps by washing out the lignins responsible for poly(A) binding[21], or by preventing the interaction between the poly(A) and the lignins. Extensive washing of the Sigmacell with 0.5 *M* NaCl buffer saturated with SDS at 20° reduced the poly(A) capacity of the cellulose from 3 to 0.8 A_{257} units/g. No

poly(A) bound to Sigmacell when added to a column equilibrated in 0.5 *M* KCl buffer containing 6% *p*-aminosalicylate.

Elution of poly(A) from cellulose

Bound poly(A) can be eluted from cellulose powder with low-ionic-strength buffers[20]. We found that the recovery of bound poly(A) was only 75 ± 10% with a single wash of 0.01 *M* Tris–HCl or distilled water (pH 7). After keeping the column in the elution buffer for 18 h at 20°, most of the remaining poly(A) was obtained. A decreasing salt gradient (0.5–0.0 *M* KCl) was next investigated to determine the elution range of poly(A) and whether any fractionation was detectable. The salt concentration range over which poly(A) eluted was dependent upon the amount bound and this appeared to be a consequence of the logarithmic dependence of capacity upon salt concentration (Fig. 2). At a load of 4 A_{257} units of poly(A) per g, the poly(A) eluted from 0.4 *M* KCl to the end of the gradient. At a load of 0.5 A_{257} units/g, elution did not begin until below 0.3 *M* salt. The wider elution range in the first case was not due to fractionation of the poly(A), since the fraction originally eluting between 0.3 and 0.2 *M*, when reapplied to the column, eluted below 0.2 *M*, which was consistent with the behaviour expected if the elution range was dependent upon column load. This result indicates that if fractionation of poly(A) or mRNA is being investigated with cellulose powder, low column loads should be used.

Elution of bound poly(A) by increasing the temperature of the column was not satisfactory in view of the high temperatures necessary. In 0.5 *M* KCl buffer, poly(A) eluted only above 70° and in 0.3 *M* KCl, between 60° and 80°.

Capacity of oligo(dT)-cellulose for poly(A) and rRNA

In 0.5 *M* NaCl buffer, freshly prepared oligo(dT)-cellulose was found to have an operational capacity of 54 A_{257} units/g and a maximum capacity of 75 A_{257} units/g was estimated by extrapolation. Thus, when freshly prepared, the oligo(dT)-cellulose had about 150-fold higher binding capacity than cellulose under the same conditions (Fig. 1B), however, the capacity gradually decreased with use.

The effect of salt concentration on the capacity of oligo(dT)-cellulose for poly(A) is shown in Fig. 3. Up to 0.3 *M* both the operational and maximum capacities were almost proportional to salt concentration. At higher salt concentrations, both seemed to approach a limit. This was in marked contrast to the logarithmic increase of poly(A) binding capacity with increasing salt concentration observed with cellulose (Fig. 2) and indicates the oligo(dT)-cellulose could be quite successfully used for mRNA isolation at lower ionic strengths. In this way, poly(A) binding by the cellulose itself can be minimized, which may be desirable in view of the unfavourable elution characteristics of poly(A) from cellulose. The capacity of the oligo(dT)-cellulose for poly(A) in 0.3 *M* KCl containing buffer was about 10% higher than the capacity in 0.3 *M* NaCl buffer[23]. If poly(A) was pre-bound in 0.5 *M* NaCl buffer, followed by equilibration in a lower-ionic-strength solution, more poly(A) bound than expected from Fig. 3. Under these conditions, the column was found to retain 37 A_{257} units of poly(A)/g in 0.1 *M* NaCl, compared with 14 A_{257} units/g when the binding was performed in 0.1 *M* NaCl buffer.

The capacity of oligo(dT)-cellulose for rRNA was determined as for Sigmacell (Fig. 2). In both 0.5 *M* and 1 *M* NaCl buffers, less than 0.1 A_{257} units of *E. coli*

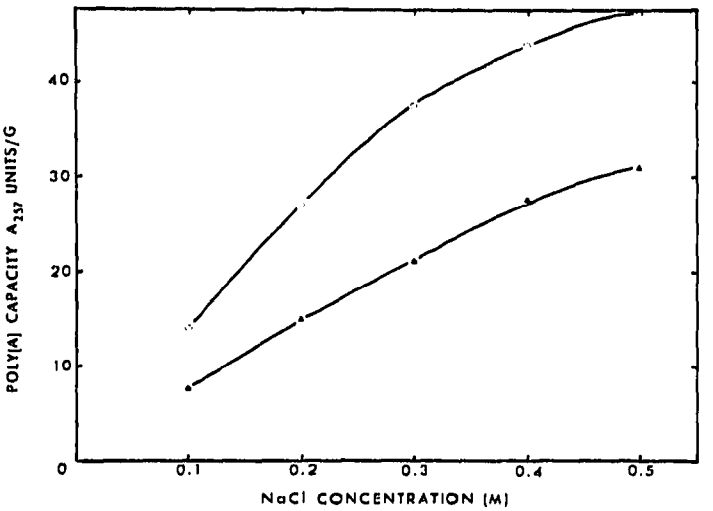

Fig. 3. Effect of NaCl concentration on the capacity of oligo(dT)-cellulose for poly(A). The poly(A) binding capacities of oligo(dT)-cellulose in 0.01 *M* Tris–HCl (pH 7.4) containing the indicated concentrations of NaCl were determined by addition of portions of poly(A) as described in Experimental. ▲—▲, Operational capacity; ○—○, maximum capacity of the oligo(dT)-cellulose.

rRNA bound to 1 g of the oligo(dT)-cellulose compared with 0.8 A_{257} units/g with Sigmacell. The low amount of rRNA binding to the oligo(dT)-cellulose indicates that this material should be a very discriminating support for mRNA. This would not be the case if the cellulose used to prepare the oligo(dT)-cellulose had a high rRNA capacity (*e.g.*, Sigmacell).

Elution of poly(A) from oligo(dT)-cellulose

Poly(A) bound to oligo(dT)-cellulose can be eluted by raising the temperature of the column above the melting temperature of the oligo(dT)–poly(A) complex or by decreasing the salt concentration of the buffer[24]. The temperature at which elution begins and ceases is related to the salt concentration of the eluting buffer. In fact, the temperature of elution was found to be proportional to the log of the salt concentration. Such a relationship has been previously reported for the interaction of poly(A) and poly(U)[25]. The results are shown in Fig. 4. The lower line represents the temperature at which poly(A) commenced eluting from the oligo(dT)-cellulose and the upper line the temperature at which elution ceased. As can be seen from the divergence of the two lines with decreasing salt concentration, the range over which poly(A) eluted increased at lower ionic strengths. Thus, the poly(A) eluted over a range of 9° in 0.3 *M* salt and 19° in 0.03 *M* salt.

Another batch of oligo(dT)-cellulose showed different temperature of elution characteristics. The elution temperatures were about 10° lower and the temperature ranges of elution were wider than those shown in Fig. 4. These effects could have been due to differences in the lengths of the dT-oligomers attached to each batch of oligo(dT)-cellulose.

The elution range of poly(A) from oligo(dT)-cellulose was found to depend upon the amount of poly(A) bound, as is shown in Table I. At low column loads

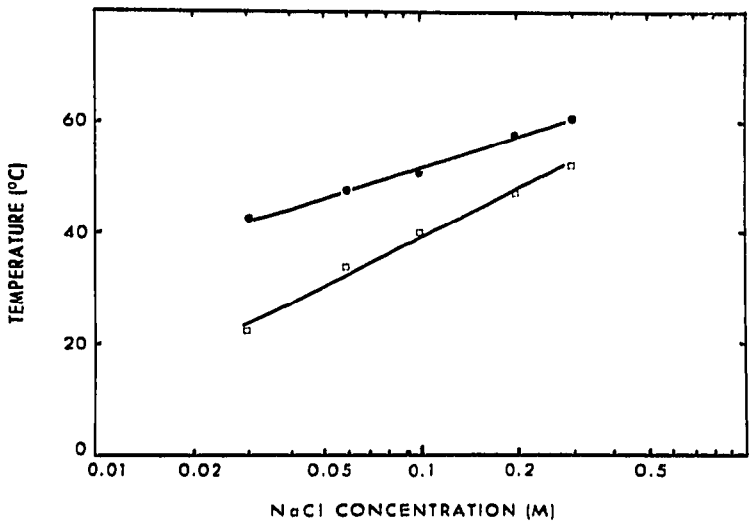

Fig. 4. Variation of temperature of elution of poly(A) from oligo(dT)-cellulose with salt concentration. Oligo(dT)-cellulose (1.25 g dry weight) in a jacketed column was equilibrated in the indicated salt concentration buffered with 0.01 *M* Tris–HCl (pH 7.4), and the temperature of the column adjusted below the expected elution range. Poly(A) (4.7 A_{257} units in 0.5 *M* NaCl containing buffer) was added and the column washed with the indicated buffer to remove any non-bound poly(A). The temperature of the column was raised at 18°/h with a column flow-rate of 0.5 ml/min. The A_{254} of the effluent was monitored continuously and the temperature range of elution determined from this; □—□, temperature at which poly(A) first began eluting; ●—●, temperature at which poly(A) ceased eluting.

(less than 6% saturated), the temperature range of elution was 8°. At loads approaching saturation level of the column, the range was 19°. The temperature range of elution in 0.03 *M* NaCl buffer was also reduced by lowering the column saturation, but at comparable levels with 0.1 *M* NaCl buffer, the elution range was still somewhat greater. The temperature range of elution in any of these experiments was not altered by binding the poly(A) to a column equilibrated with 0.5 *M* salt followed by re-equilibration with the various lower salt concentrations.

The relatively broad temperature range (8°) of elution of poly(A) at low column loads could have either been due to some fractionation of the poly(A) or simply to the range of melting temperatures of the oligo(dT)–poly(A) complex. To investigate the possibility of fractionation, a larger column (18.5 × 1 cm) of oligo(dT)-cellulose was used and 5 A_{257} units of poly(A) were bound in 0.1 *M* NaCl containing buffer and eluted by a temperature gradient (18°/h). The poly(A) eluted from 46°–54° and the effluent was divided into three portions, 46°–48.5° (A), 48.5°–51.5° (B), and 51.5°–54° (C). Fraction A was re-applied to the column and eluted by the temperature gradient. It appeared over the range 46°–53°, *i.e.* practically the same as the original sample. Fraction B was found to re-elute from 47°–54°. Thus, very little fractionation of the poly(A) occurred under these conditions.

To investigate salt gradient elution, poly(A) (5 A_{257} units) was bound to a 1-g column of oligo(dT)-cellulose, and the column re-equilibrated in 0.2 *M* NaCl buffer. A decreasing linear salt gradient at 25° was applied and elution of ultraviolet (UV)

TABLE I

LOAD DEPENDENCE OF ELUTION RANGE OF POLY(A) FROM OLIGO(dT)-CELLULOSE

Various amounts of poly(A) in 0.5 *M* NaCl buffer were added to a jacketed column of oligo(dT)-cellulose (1.25 g dry weight) equilibrated in buffer containing the indicated salt concentration. The temperature of the column was kept below the expected elution temperature during the addition and was then raised at 18°/h. The elution of poly(A) was monitored continuously.

Salt concentration (M)	*Load (A_{257} units/g)*	*Saturation (%)*	*Elution temperature (°C)*	*Temperature range (°C)*
0.1	0.4	2.9	46–54	8
0.1	0.79	5.6	44–52	8
0.1	1.98	14	42–52	10
0.1	3.74	27	41–52	11
0.1	11.7	84	35–51	16
0.1	15.9	110*	33–52	19
0.03	0.79	13	28–42	14
0.03	3.74	62	22.5–42.5	20

* The apparent 110% saturation was presumably a result of adding poly(A) in 0.5 *M* NaCl buffer.

absorbing material was monitored. Elution commenced at about 0.11 *M* NaCl and continued until the salt concentration dropped to zero. This result was predicted from the dependence of elution temperature on salt concentration for this batch of oligo(dT)-cellulose. Presumably the range of salt concentrations over which poly(A) eluted would be reduced at lower column loads.

Capacity of poly(U)-cellulose for poly(A)

One gram of freshly prepared poly(U)-cellulose was found to completely retain 2.5 A_{257} units of poly(A) (operational capacity) and had a maximum capacity of 6 A_{257} units in 0.1 *M* NaCl buffer. After a few cycles of use, including natural RNA chromatography, its maximum capacity dropped to about 2 A_{257} units of poly(A) per g (ref. 23). The poly(A) binding capacity of poly(U)-cellulose was dependent upon salt concentration. The maximum capacities at 20° were found to be as follows: 0.01 *M* Tris–HCl (pH 7.4), 0.04 A_{257} units/g; 0.05 *M* NaCl buffer, 1.0 A_{257} units/g; 0.1 *M* NaCl buffer, 2.0 A_{257} units/g. As observed with oligo(dT)-cellulose, the poly(A) capacity in lower-ionic-strength buffers was increased if the poly(A) was pre-bound in 0.5 *M* NaCl buffer, followed by re-equilibration with the lower salt buffer.

Elution of poly(A) from poly(U)-cellulose

As with oligo(dT)-cellulose, the temperature of elution of poly(A) from poly-(U)-cellulose was dependent upon the ionic strength of the elution buffer. Poly(A) (5 A_{257} units) was added to a column of poly(U)-cellulose equilibrated with 0.1 *M* NaCl containing buffer. After washing to remove non-bound material the column was re-equilibrated with buffer containing the required concentration of NaCl. The temperature of the column was increased in 5° steps. The temperatures over which poly(A) eluted at the various salt concentrations were as follows: no salt, 20°–44°; 0.05 *M* NaCl, 39°–55°; 0.01 *M* NaCl, 56°–62°. The wide elution ranges at the two lower salt concentrations were in part due to the saturation of the column, since, as

was observed with oligo(dT)-cellulose (Table I), the temperature range of elution of poly(A) increased at higher column saturations. Elution of poly(A) with distilled water at room temperature caused continuous leaching of fine cellulose particles containing poly(U) from the column; this did not occur to the same extent with 0.01 *M* Tris–HCl elution.

Characterization of poly(U)-Sepharose

Poly(U)-Sepharose was prepared by the Lindberg and Persson[5] modification of the original method of Wagner *et al.*[26] for coupling polynucleotides to CNBr-activated Sepharose. The maximum poly(A) capacity of the Sepharose was estimated to be 13 A_{257} units per ml packed volume in 0.5 *M* NaCl buffer. While bound poly(A) was completely eluted from poly(U)-cellulose with 0.01 *M* Tris–HCl at 45°, only a small fraction (10%) of the poly(A) could be eluted from poly(U)-Sepharose (both commercial and prepared) under the same conditions or at 75°. With 0.5 *M* NaCl buffer, however, the majority of the bound poly(A) was released at 75°. The temperatures used in these experiments did not appear to affect the subsequent binding of poly(A) to the poly(U)-Sepharose, but above 75° some UV material eluted from the column (presumably poly(U)). Almost quantitative recovery of bound poly(A) was obtained when the column was washed with 90% formamide, 0.01 *M* Tris–HCl at 20°, as expected from the results of Lindberg and Persson[5].

The incomplete elution of poly(A) in buffers of low ionic strengths at temperatures above the melting point of the poly(A)–poly(U) helix, and the effectiveness of buffers of higher ionic strengths in completing the elution, indicate that the poly(A) must have been interacting with some other groups on the Sepharose. This interaction was eliminated at higher ionic strengths (consistent with charged groups), permitting elution when the poly(A)–poly(U) helix was disrupted. At lower ionic strengths, although the helix was disrupted, the poly(A) was retained by the secondary binding forces. Formamide successfully eluted bound poly(A) at low ionic strengths and hence this solvent must also eliminate the secondary binding forces. This binding was relatively non-specific, since rRNA bound to poly(U)–Sepharose in 0.01 *M* Tris–HCl (capacity of Sepharose 0.75 A_{257} units/ml) and could be eluted by washing with 0.5 *M* NaCl buffer. In 0.01 *M* Tris–HCl, rRNA did not bind to unsubstituted Sepharose. As expected, poly(U) did not bind to poly(U)-Sepharose in 0.5 *M* salt, since the non-specific interactions were eliminated by the high salt concentration.

Characterization of poly(A) binding to benzoylated cellulose

The benzoylated cellulose (Bz-cellulose) prepared in the same manner as benzoylated DEAE-cellulose[14] was found to be a very effective poly(A) binding support. Once bound, the poly(A) was not eluted by washing the column with 0.01 *M* Tris–HCl at 20°, but could be eluted by inclusion of 25% absolute alcohol in the Tris buffer. Use of temperature or formamide elution has not been investigated. The capacity of this material for poly(A) was quite high. In 0.1 *M* NaCl containing buffer, 57 A_{257} units were completely retained (operational capacity) by one gram and the Bz-cellulose had maximum capacity of 95 A_{257} units/g. This capacity is estimated to be 2,800-fold greater than cellulose powder in 0.1 *M* NaCl containing buffer, and 8-fold higher than oligo(dT)-cellulose. In 0.01 *M* Tris-HCl at 20°, the Bz-cellulose was saturated by 6 A_{257} units of poly(A) per g.

In 0.1 *M* NaCl, less than 0.2 A_{257} units per g of rRNA appeared to bind to the Bz-cellulose, and were eluted with 0.01 *M* Tris–HCl, but accurate estimation of binding was difficult because of the elution of UV-absorbing material from the column. Even after extensive washing with 50% alcohol, or 0.5 *M* NaCl, A_{254}-absorbing material eluted from the Bz-cellulose at any buffer change and was particularly noticeable in the alcohol-containing buffers. This was reduced but not eliminated by exhaustive removal of fines by decantation. The successful application of Bz-cellulose to mRNA isolation will depend upon separation of the elution impurities from the RNA.

Selectivity factors for the various poly(A) binding materials

The rRNA and poly(A) capacities for some of the supports studied are compared in Table II. The selectivity factor (*Sf*) is defined as the ratio of maximum poly(A) capacity to rRNA capacity of each material under the same conditions, since this permits easy comparison of the potential effectiveness of the materials for separating rRNA from poly(A)-containing RNA. Clearly under the conditions used, oligo(dT)-cellulose and Bz-cellulose are superior to cellulose powder and poly(U)-cellulose. The selectivity factor of cellulose powder varied with salt concentration and cation type, since both of these alter rRNA and poly(A) capacity and the best values can be estimated from the data in Figs. 1 and 2. As noted previously, the rRNA capacity of this batch of Sigmacell was higher than that reported by Sullivan and Roberts[3], but the selectivity factor for Sigma α cellulose in 0.3 *M* NaCl at 45° calculated from their data was 31, which is not a great improvement over our highest value of 27.

TABLE II

SELECTIVITY FACTORS FOR VARIOUS POLY(A) BINDING MATERIALS

Data collated from results: Sigmacell from Figs. 1 and 2, oligo(dT)-cellulose from Fig. 3. Maximum poly(A) capacities are used (mg/g) calculated assuming 23.5 A_{257} units are equivalent to 1 mg. The RNA capacity of poly(U)-cellulose was estimated to be the same as oligo(dT)-cellulose, since CF 11 cellulose was used in both cases. The capacity in mg/g was calculated assuming 25 A_{257} units are equivalent to 1 mg. The selectivity factor (*Sf*) is defined as the ratio of maximum poly(A) capacity to rRNA capacity under the same conditions.

Material	*Salt*	*rRNA capacity (mg/g)*	*Poly(A) capacity (mg/g)*	*Sf*
Sigmacell	0.3 *M* KCl	0.02	0.113	5.6
Sigmacell	0.5 *M* KCl	0.06	1.65	27
Sigmacell	0.5 *M* NaCl	0.028	0.152	5.4
Oligo(dT)-cellulose	0.1 *M* NaCl	<0.004	0.61	>150
Oligo(dT)-cellulose	0.5 *M* NaCl	<0.004	2.04	>510
Poly(U)-cellulose	0.1 *M* NaCl	<0.004	0.087	>22
Bz-cellulose	0.1 *M* NaCl	<0.008	4.13	>520

DISCUSSION

The results presented here are relevant both to the routine use of affinity chromatography for the separation of poly(A) containing RNA from non-poly(A) RNA (principally rRNA), and to the possible use of these columns for achieving some de-

gree of fractionation of the bound molecules. In the first case, the principle concern is the selectivity of each column for poly(A) (Table II). Also the elution of bound material must be complete, under conditions which do not favour degradation of the RNA and decomposition of the column material itself, leading to contamination of eluted RNA.

For comparison of the potential selectivity of the columns for poly(A) *versus* rRNA, selectivity factors (*Sf*) were calculated for the columns and these are shown in Table II. The figures clearly show that cellulose powder and poly(U)-cellulose are less selective than oligo(dT)-cellulose and Bz-cellulose. The exact value of *Sf* depends upon the interaction of various factors which alter poly(A) and rRNA capacities, such as the salt concentration and cation type, and thus it can be optimized by judicious choice of the chromatography conditions.

Quantitative recovery of bound poly(A) was not found to be a problem with oligo(dT)-cellulose or poly(U)-cellulose. With cellulose powder, however, complete recovery of poly(A) proved difficult indicating some losses of poly(A) containing RNA may occur with this material. Considerable problems were encountered in recovering poly(A) from poly(U)-Sepharose. This appeared to be due to a binding force retaining poly(A) at low salt concentrations. Although this occurred with both batches of poly-(U)-Sepharose tested here, Ihle *et al.*[27] report complete recovery of poly(A) from poly(U)-Sepharose by temperature elution in buffers of low ionic strengths; and Firtel *et al.*[28] recovered mRNA from poly(U)-Sepharose by washing with water. There may be differences between batches of poly(U)-Sepharose, perhaps resulting from the CNBr activation step.

In most cases, the elution conditions used to release bound poly(A) would not be expected to cause mRNA degradation, however, during temperature elution, prolonged exposure to elevated temperatures (in excess of 50°) may be undesirable. The elution temperatures can be reduced by lowering the salt concentration of the eluent (Fig. 4). When using temperature elution to obtain bound poly(A) from oligo-(dT)-cellulose, conditions should be chosen to minimize the cellulose contribution to the poly(A) binding, since very high temperatures were necessary to elute poly(A) from cellulose powder. In cases where rRNA is bound to the column and would contaminate mRNA, selective elution conditions, such as temperature gradients, may be successful in removing the bound rRNA prior to elution of mRNA.

Breakdown of the column materials during elution only appeared to be a serious problem with poly(U)-cellulose and Bz-cellulose. In the former case, contamination by poly(U) could severely affect experiments with the isolated mRNA[6]; but in the latter case, the eluted impurities may be removable or inert.

A detailed study of the elution range of poly(A) from the various columns was carried out to determine the optimum conditions for possible mRNA fractionation using these materials. It is considered that the narrower the temperature (or salt concentration) range over which the poly(A) eluted, the better the potential resolving power of the column, since smaller effects on melting temperature due to differences in poly(A) size or mRNA length should be detectable under these conditions.

The chromatography conditions were found to influence greatly the elution range of poly(A) from the various columns. The elution range increased at higher column saturations; a similar effect has been reported by Astell and Smith[29,30]. To obtain reproducible elution patterns, the same column saturation should be used.

Also, at higher eluent salt concentrations, the temperature range of elution of poly(A) from oligo(dT)-cellulose was decreased, as may be expected from the effect of salt concentration on the melting range of synthetic DNAs[31]. Thus, to optimize the probability of mRNA fractionation, low column loads and higher salt concentrations in the eluent should be used. The advantage of higher salt concentrations, however, is in part offset by the increased likelihood of mRNA degradation at the higher temperatures necessary to complete the elution.

The results indicate that the limiting 8° elution temperature range of poly(A) from oligo(dT)-cellulose was not due to fractionation of the poly(A) into different molecular weight classes. This failure to fractionate was expected from the long chain length of the poly(A); above a chain length of about 200, the melting temperature of poly(A)–poly(dT) complexes essentially reaches a limiting value[32]. The melting range of complexes of complimentary polynucleotides is wider than the corresponding complexes of oligonucleotides and polynucleotides[33], hence poly(A) eluted over a 6° range from poly(U)-cellulose, indicating that poly(U) columns may be useful for fractionation studies. Poly(U)-cellulose would not be suitable because of its low capacity and instability. Poly(U)-Sepharose offers considerable advantages except for the difficulty in eluting poly(A) with some batches, although as mentioned above, Ihle *et al.*[27] successfully used temperature elution of poly(A) from poly(U)-Sepharose and their data indicate that fractionation of poly(A) up to a chain length of 200 may have been possible.

In conclusion, for mRNA isolation, oligo(dT)-cellulose seems to offer distinct advantages over the other supports studied and should also be quite suitable for fractionation investigation. Chromatography of natural mRNA on this material is currently being studied.

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