Chromatography of Synthetic Polyribonucleotides on Columns of Benzoylated Diethylaminoethylcellulose*

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ABSTRACT: The properties of columns of benzoylated diethylaminoethylcellulose in resolving different types of ribonucleic acids were investigated in a systematic manner using synthetic polymers of ribonucleotides. It was observed that sequential gradients of sodium chloride and of urea removed most polymers from the column. The sodium chloride gradient eluted the base-paired replicative form of viral ribonucleic acid, the base-paired polymer duplexes formed between polyriboinosinic and polyribocytidylic acids and between polyriboadenylic and polyribouridylic acids. The order of elution of these ribonucleic acids from the column was a function of secondary structure and was independent of molecular size. The polymer polyribocytidylic acid was also eluted by this gradient. The gradient of urea eluted polymers of polyriboinosinic acid and polyribouridylic acid and the mixed copolymer composed of adenylic, cytidylic, and uridylic acids (1:1:2) from the column. This gradient, when base composition remained constant, eluted ribonucleic acids as a function of relative molecular size. Homopolymers of adenylic acid and of guanylic were not eluted by any of the gradients examined at 24°. However polyadenylic acid could be eluted by the sodium chloride gradient by either heating the column to 65°, or eluting in the presence of dilute calcium, and by the gradient of urea following formaldehyde treatment of the poly(A).

Protein fractionation is an advanced highly technical field, while the fractionation of nucleic acids remains largely underdeveloped. A solution to the problem of the purification of mRNA[1] from single-celled organisms and of mRNA and hnRNA from eukaryotic cells is an example of a major problem in the field of nucleic acid chromatography which is of critical interest in biology and which remains unsolved.

The BD-cellulose column appears to be a promising technique toward this goal. The column is used successfully for the separation of tRNAs (Gillam *et al.*, 1967), for the partial resolution of complex mixtures of RNAs obtained from bacterial cell extracts (Sedat *et al.*, 1969), and for the separation of the various nucleic acid intermediates in the replicative cycle of RNA (Stern and Friedman, 1969) and DNA (Kelly and Sinsheimer, 1967) viruses. The BD-cellulose column is known to resolve RNAs in part on the basis of secondary structure (Gillam *et al.*, 1967; Sedat *et al.*, 1967; Stern and Friedman, 1969). In this communication the resolving properties of the BD-cellulose column were examined using synthetic polymers of ribonucleotides. The secondary structure of many of these structures can be inferred from physical studies (Felsenfeld and Miles, 1967). From the systematic examination of these polymers on the column it is hoped that an empirical method can be developed for the separation of ribonucleic acids from animal cells, particularly for the resolution of the rapidly labeled mRNA and hnRNA from the other RNAs of the animal cell.

Materials and Methods

DEAE-cellulose (capacity 0.83 mequiv/g) was from Schleicher und Schuell; pyridine, reagent grade, from J. T. Baker Chemical Co.; and benzoyl chloride, reagent grade, from Eastman Organics Chemicals. The latter was not redistilled. Me_2SO was from Fisher Chemical Corp. Urea, from Baker Chemical Co., was deionized with the mixed-ion-exchange resin, Rexyn I-300 (Fisher Corp.). The urea was then treated with acid-washed Norit and passed through a filter of diatomaceous earth (Celite-545, Johns-Mansville Corp.). Bio-Gel P-100 was the product of the Bio-Rad Laboratories.

Preparation of the BD-cellulose. BD-cellulose was prepared according to the procedure of Gillam *et al.* (1967); 2.7 moles of benzoyl residues/mole of anhydroglucose were calculated for the two preparations of material. Commercially available products (Schwarz BioResearch and Regis Chemical Co.) gave identical chromatographic results.

Chromatographic Methods. BD-cellulose chromatography was performed in columns (5 × 1.4 cm inside diameter) at room temperature except where indicated. In each case RNA was dissolved in 10 ml of 0.2 M NaCl, 10 mM $MgCl_2$ in 25 mM potassium phosphate buffer (pH 6.8), and placed on the column which was then washed with 100 ml of the same buffer. The series of sequential gradients described below eluted the majority of the RNAs extracted from animal cells (Stern and Cooper, 1968). Fractions of 4.5 ml were collected at room temperature at a rate of 1 ml/min and stored at −10° until processed.

Gradient Elution System. Two sequential gradients were suggested in part from the work of Kelly and Sinsheimer (1964, 1967). Preliminary experiments demonstrated that these gradients successfully eluted 85% of animal cell RNA applied to the column. These gradients were the following. A linear NaCl gradient was comprised of 140 ml each of 0.2 and 1.8 M

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[1] Abbreviations used are: mRNA, messenger ribonucleic acid; hnRNA, heterogeneous nuclear RNA; BD-cellulose, benzoylated DEAE-cellulose; RI, replicative intermediate; RF, replicative form; SFV, the Semliki Forest virus, an RNA containing animal virus which is a group A arbovirus; poly(A), polymer of riboadenylic acid; poly(G), polymer of riboguanylic acid; poly(U), polymer of ribouridylic; poly(I), polymer of riboinosinic acid; poly(C), polymer of ribocytidylic acid; poly(I)·poly(C), a hydrogen-bonded base-paired duplex formed between polyriboinosinic and polyribocytidylic acids; poly(A)·poly(U), a hydrogen-bonded base-paired duplex formed between polyriboadenylic and polyribouridylic acids; poly(A,U,C), a mixed copolymer composed of riboadenylic, ribouridylic, ribocytidylic acids; AMP, adenosine 5′-monophosphate; Me_2SO, dimethyl sulfoxide.

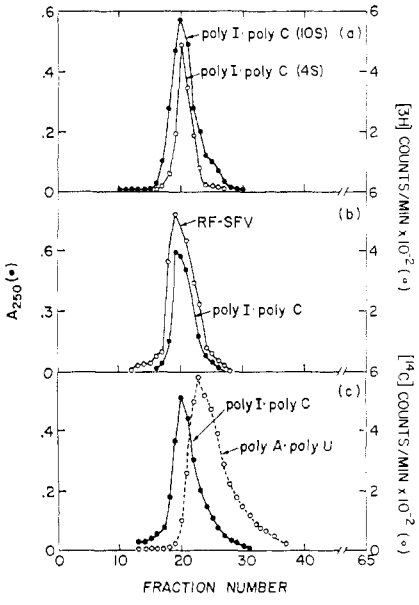

FIGURE 1: BD-cellulose column chromatography of synthetic and naturally occurring double-stranded RNAs. (a) The chromatographic profile of two preparations of poly(I)·poly(C) differing in relative molecular size; 1500 cpm of 4S [^{3}H]poly(I)·poly(C) and 0.5 mg of unlabeled 10S poly(I)·poly(C) were dissolved in 10 ml of 25 mM potassium phosphate buffer (pH 6.8) containing 0.2 M NaCl and 10 mM $MgCl_2$ and placed on a BD-cellulose column (5 × 1.4 cm inside diameter) at room temperature. The column was washed with 100 ml of the same buffer. The RNA was eluted with a linear gradient formed from 140 ml each of 25 mM potassium phosphate buffer (pH 6.8), 10 mM $MgCl_2$, containing 0.2 and 1.8 M NaCl. Fractions (4.5 ml) were collected with a flow rate of 1 ml/min. The A_{250} of each fraction was determined. For the measurement of acid-precipitable [^{3}H]poly(I)·poly(C) each fraction was chilled to 0° and one-tenth volume of 50% trichloroacetic acid added. The washed precipitate which had been collected on a Millipore filter was dried and counted. A_{250} (●); ^{3}H counts per minute (○). (b) The chromatographic profile of a synthetic and a naturally occurring double-stranded RNA. The naturally occurring double-stranded RF from chick cells infected with the RNA virus SFV and the synthetic duplex poly(I)·poly(C); 1000 cpm of [^{3}H]FR RNA and 0.5 mg, respectively, were mixed in 10 ml of 20 mM potassium phosphate buffer (pH 6.8) containing 0.2 M NaCl–10 mM $MgCl_2$, and placed on a BD-cellulose column. Chromatography was carried out as described under part a. A_{250} (●); ^{3}H counts per minute (○). (c) The chromatographic profile of two different synthetic double-stranded homopolymer complexes; 0.5 mg of poly(I)·poly(C) and 2000 cpm of [^{14}C]poly(A)·poly(U) mixed in 10 ml of 20 mM potassium phosphate buffer containing 0.2 M NaCl and 10 mM $MgCl_2$ were placed on a BD-cellulose column and chromatography was carried out as described under part a. A_{250} (●); ^{14}C counts per minute (○). In each case, the contribution of the labeled polymer to the total A_{250} was negligible.

NaCl in 25 mM potassium phosphate buffer (pH 6.8), containing 10 mM $MgCl_2$. Magnesium was included in the initial gradient to give partial protection of RNA from ribonucleases which might contaminate RNA preparations. Magnesium-containing buffers separate ribonucleases from RNA during DEAE-cellulose chromatography (Muench and Berg, 1966) and are used routinely in resolution of tRNA on BD-cellulose columns (Gillam *et al.*, 1967).

The second gradient contained simultaneous gradients of urea and NaCl. One-hundred and forty milliliters of each of the following solutions were used: 0.2 M NaCl in 25 mM potassium phosphate buffer (pH 6.8) and 1.8 M NaCl in 25 mM potassium phosphate buffer (pH 6.8) containing 8 M urea. Gradients of urea at constant concentrations of NaCl at either 0.2 or 2.5 M were less successful in resolving peaks of RNA than simultaneous gradient of NaCl and urea. The column was then washed routinely with a solution of 10% ethanol containing 1.8 M NaCl.

Measurement of Acid-Precipitable Radioactive Material. Solutions to be counted were chilled to 0° and one-tenth volume of 50% trichloroacetic acid was added. This was incubated for 10 min at 0° and then poured onto a Millipore filter and suction applied. The filter was washed three times with 10-ml aliquots of 5% trichloroacetic acid at 0°. The filter was dried in an oven at 55° for 30 min, and then placed in counting vials containing 4 ml of toluene with 5% Liquifluor (Nuclear-Chicago Corp.) and counted in a Packard Tri-Carb liquid scintillation counter.

Sucrose Gradient Centrifugation. Sucrose density gradient centrifugation was carried out with a gradient of sucrose (4.5 ml) from 6 to 30% in 0.02 M Tris-HCl (pH 7.4), and 0.2 M NaCl using the SW-65 rotor at 300,000*g* at 3°. Thirty fractions were collected.

Synthetic Polynucleotides. Synthetic polyribonucleotides were purchased from Miles Laboratories, Inc., BioPolymers, Inc., P-L Laboratories, and Schwarz BioResearch, Inc. Relative *s* values of all synthetic polymers were established by density gradient centrifugation in sucrose using a cytoplasmic extract of RNA from rat liver for optical density markers. This RNA extract contained 28S, 18S, and 4S RNA peaks corresponding to the RNA from the large and small subunit of the animal cell ribosome and tRNA, respectively.

Viral RNAs. The growth of SFV and extraction and preparation of viral RNA were described previously (Friedman, 1968; Friedman *et al.*, 1966).

Results

Chromatography of Double-Stranded RNAs. Inosine is a base-pairing analog of guanine. The base-paired duplex molecule formed by poly(I) and poly(C) is the simplest double-stranded RNA which can be formed from synthetic homopolymer pairs. There is minimal interference of duplex structure from highly stable helix formations or from multistrand complexing with these polymers (Chamberlin and Patterson, 1965). Two preparations of poly(I)·poly(C) of widely differing molecular size were compared on the BD-cellulose. The Bio-Polymers and Miles Laboratories preparations of poly(I)·poly(C) had relative *s* values of 4 and 10 S, respectively, as determined by density gradient centrifugation in sucrose. Figure 1a demonstrates that these two preparations were eluted from the column in the same position using the NaCl gradient. This result indicated that the BD-cellulose column chromatographed RNAs independently of molecular size during the course of NaCl gradient elution.

A preparation of poly(I)·poly(C) was chromatographed on the BD-cellulose column together with a naturally occurring double-stranded RNA, the RF from the replicative cycle of an RNA-containing animal virus, the SFV (Figure 1b). Using the NaCl gradient, these two RNAs were also eluted at the same position from the column, as single homogeneous peaks.

The base-paired duplex molecule poly(A)·poly(U) has a less stable helical structure compared to other double-stranded RNAs as judged by its lower melting temperature (Chamberlin, 1966; Michelson *et al.*, 1967). Poly(A)·poly(U) was chromatographed together with a preparation of poly(I)·poly(C) on the BD-cellulose column using the NaCl gradient to determine if the difference in helical structure would be reflected in the chromatographic profile. The poly(A)·poly(U) was

retained on the column longer than the poly(I)·poly(C) which correlated with the decreased helical structure (Figure 1c). This result confirmed previous observations (Stern and Friedman, 1969) that the lower the degree of secondary structure in a RNA molecule, the greater the opportunity for interaction with the column material and the more retarded is that RNA in its elution from the column.[2]

Magnesium is included in the NaCl gradients used to resolve tRNAs from each other on the BD-cellulose column (Gillam *et al.*, 1967) as well as in the NaCl gradient of the present experiments. The reproducible differences in the elution profiles of poly(A)·poly(U), poly(I)·poly(C), and the viral RF from the column were utilized in an attempt to examine the effect of magnesium on the elution patterns. Magnesium was omitted from the gradient. However no changes were observed in the subsequent elution profiles of the three RNA preparations. It was concluded that magnesium had no effect on the elution of these RNAs from the BD-cellulose column during the course of NaCl gradient elution. The magnesium was nevertheless retained in the NaCl gradient to protect RNAs from the ribonuclease which frequently contaminates RNA preparations.

Double-stranded RNAs are relatively resistant to digestion by ribonuclease A in the presence of 0.2 M NaCl with 20 μg of enzyme/ml at 37° for 30 min. However in the preparation of the SFV RF following the ribonuclease A digestion of the viral nucleic acid, it was necessary to inactivate the ribonuclease A with Pronase digestion (1.5 mg/ml for 60 min at 37° in 0.2 M NaCl and 20 mM Tris HCl, pH 7.4) before placing the sample on the column. No acid-precipitable RNA was recovered from the column when this step was omitted. This observation suggested that RNAs were better substrates for ribonuclease when adsorbed to the column material than in solution.[3]

Chromatography of Poly(C). Among the homopolymers examined, only poly(C) was eluted from the BD-cellulose column by the gradient of NaCl. At 25° the poly(C) was eluted at the same position as the duplex poly(I)·poly(C) (Figure 2a). This observation suggested an experiment which could test the hypothesis that the BD-cellulose column resolves RNAs on the basis of secondary structure. Poly(I)·poly(C) should continue to be eluted from the column in the same position as the temperature is increased, as long as the temperature remains below the T_m of poly(I)·poly(C), 66° in 0.2 M NaCl (Chamberlin and Patterson, 1965). In contrast, poly(C) should be more retarded by the column with increasing temperature as the polymer loses secondary structure and as the bases become more exposed to the column material. This prediction was correct (Figure 2b–d). As the temperature was elevated binding increased between poly(C) and the resin and the poly(C) became increasingly retarded in its elution from the column. In addition, as temperature increased, the peak of poly(I)·poly(C) became progressively broadened though the center of the peak remained in the same position. The profiles of poly(C) and poly(I)·poly(C) though illustrated in the same chromatogram represent separate column runs.[4] Poly(C) is postulated to have a highly ordered secondary structure (Brahms, 1963; Fasman *et al.*, 1964) compatible with the present experiments.

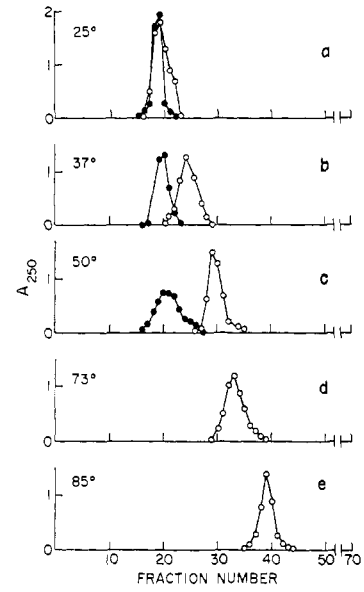

FIGURE 2: BD-cellulose column chromatography of poly(C) and poly(I)·poly(C) at different temperatures. In each case 1 mg of poly(C) and 1 mg of poly(I)·poly(C) were dissolved in 10 ml of 20 mM potassium phosphate buffer (pH 6.8) containing 0.2 M NaCl and 10 mM $MgCl_2$ and placed on a jacketed column with circulating heated water being pumped at the indicated temperature. Chromatography was carried out as described under Figure 1a. A_{250} of poly(C) (●); A_{250} of poly(I)·poly(C) (○). The profiles at each temperature represent a composite of separate chromatograms of the poly(C) and the poly(I)·poly(C).

Chromatography of Poly(I). The homopolymer poly(I) could not be eluted by the NaCl gradient, but was quantitatively eluted by the gradient of NaCl and urea. Several commercial sources of poly(I) were obtained and compared on the BD-cellulose column and on sucrose density gradient centrifugation. This demonstrated that the relative sedimentation rates of the various poly(I) preparations were proportional to their relative mobilities on the column. Figure 3 illustrates the elution patterns of poly(I) ranging from 1 to 28 S. The poly(I) with the lowest relative sedimentation rate was the earliest to be eluted from the BD-cellulose with the NaCl and urea gradient. The poly(I) with the highest *s* value was the preparation most firmly bound to the column, eluting at the highest concentration of NaCl and urea.

Chromatography of Poly(U). Poly(U) could not be eluted

[2] Base-paired duplex RNAs are inducers of the antiviral agent interferon (Field *et al.*, 1967; Hamilton *et al.*, 1969). Figure 1b demonstrates that the homopolymer duplex poly(I)·poly(C) had a degree of secondary structure, equivalent to the secondary structure of the RF of an RNA virus by the criterion of BD-cellulose chromatography. Both of these polymers are potent inducers of interferon. Poly(A)·poly(U) is also an inducer of interferon but a far less effective one (Field *et al.*, 1967). Figure 1c offers a possible explanation for this difference in that poly(A)·poly(U) is a less rigid double-stranded molecule, by T_m criterion, and is retained more strongly to the BD-cellulose column than either poly(I)·poly(C) or the naturally occurring RNA duplex. The decreased helicity of the molecule may underly its weaker ability to induce interferon.

[3] This observation also explains why it is difficult to recover biologically active tRNA from the BD-cellulose column. Loss of tRNA amino acid acceptor activity is a sensitive assay for the presence of ribonuclease. Recovery of biological activity following BD-cellulose column chromatography is difficult unless the tRNA preparation is scrupulously free of contamination by nucleases (R. Stern and U. Z. Littauer, unpublished observations).

[4] These observations have practical application in the large-scale preparation of poly(I)·poly(C) and in its preparation for antiviral studies. Low molecular weight material is not adsorbed by the BD-cellulose in the presence of 0.2 M NaCl. At 50° the poly(I)·poly(C) product can be separated from any unannealed poly(C) with a gradient of NaCl. Unannealed poly(I) remains adsorbed.

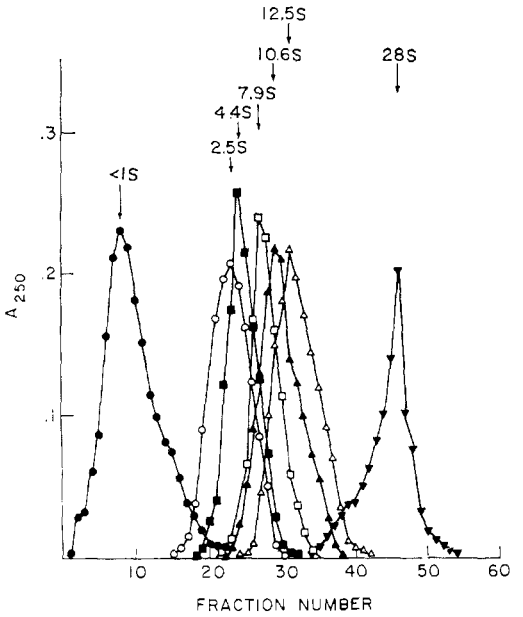

FIGURE 3: BD-cellulose column chromatography of preparations of poly(I) of differing relative molecular sizes. In each case 1.0 mg of poly(I) was dissolved in 10 ml of 25 mM potassium phosphate buffer (pH 6.8) containing 0.2 M NaCl and placed on the column. A gradient was applied formed by 140 ml each of 25 mM potassium phosphate buffer (pH 6.8) containing 0.2 M NaCl and 0.1 M sodium formate buffer (pH 3.4) containing 1.6 M NaCl and 8 M urea. Fractions were collected and A_{250} was determined. The composite profiles of seven separate chromatographs are shown. Preliminary experiments established that no A_{250}-absorbing material was eluted from the column by the gradient of NaCl described in the legend of Figure 1a.

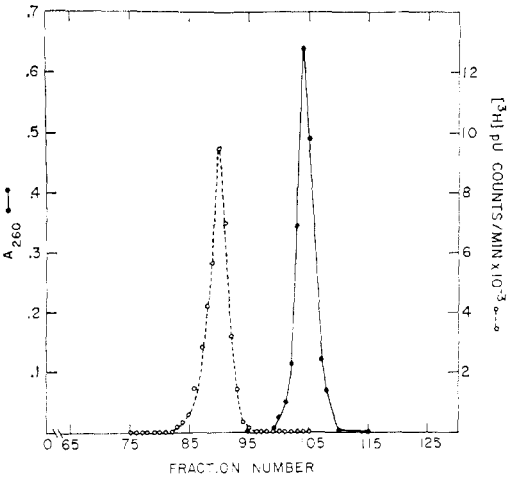

FIGURE 4: BD-cellulose column chromatography of two preparations of poly(U) of differing relative molecular size; 3500 cpm of 4S [^{3}H]poly(U) and 0.5 mg of 8S poly(U) were dissolved in 10 ml of 25 mM potassium phosphate buffer (pH 6.8) containing 0.2 M NaCl and 10 mM $MgCl_2$ and placed on the column. The gradient described in the legend of Figure 1a was applied and fractions were collected (1–64). No radioactively labeled or A_{260}-absorbing material was eluted. A second gradient (fractions 65–128) described in the legend of Figure 3 was applied. A_{260} and the level of acid-precipitable radioactively labeled material were determined for each fraction. ^{3}H counts per minute (○); A_{260} (●). The contribution of the labeled poly(U) to the total A_{260} was negligible.

from the BD-cellulose column with NaCl but was eluted from the column as a function of relative sedimentation values by the urea–NaCl gradient. Figure 4 illustrates the elution pattern of 4S [^{3}H]poly(U) and unlabeled 8S poly(U) on the BD-cellulose column. Again the RNA species with the larger relative sedimentation value was more retarded by the column. Poly(U) was more retarded on the column than a poly(I) of comparable relative sedimentation value. However it was not established which of the two polynucleotide chains was actually the larger.

Poly(U) has no apparent organized secondary structure except at temperatures below 8° (Lipsett, 1960). The chromatographic properties of poly(U) were examined at low temperatures. The polymer was applied to a jacketed column at 3° and chromatography using the NaCl gradient was carried out at that temperature. No elution of poly(U) was observed. Lowering the temperature to 3° did not convert poly(U) into a structure with sufficient secondary structure to permit elution with the NaCl gradient. However it cannot be ruled out that the chromatographic properties of the column are not altered at low temperatures.

Chromatography of Poly(A) and Poly(G). Poly(A) and poly(G) were applied to the BD-cellulose column. However they were not eluted by either of the sequential gradients or by the ethanol–NaCl solution. However, if elution was done at 65° polymers could be recovered with the NaCl gradient.

An attempt was made to elute poly(A) from the column using milder chromatographic conditions. Poly(A) in neutral solution has the hydrodynamic properties of a single-stranded flexible random coil (Felsenfeld and Miles, 1967). At an acid pH, poly(A) is converted to a rigid structure, postulated to be a highly ordered twin-stranded helix which has been characterized by X-ray crystallography (Rich *et al.*, 1961; Barszcz and Shugar, 1968). Poly(A) was placed on a BD-cellulose column in a solution containing 0.2 M NaCl and 0.1 M sodium formate (pH 3.4) and a gradient of NaCl from 0.2 to 1.8 M in 0.1 M sodium formate (pH 3.4) applied. However the poly(A) was not eluted in the course of this acidic gradient at a pH at which poly(A) has been postulated to have a high degree of secondary structure.

Poly(A) in the presence of calcium ions undergoes a marked change in structure. The primary amino groups of adenine interact with the phosphoryl groups. The calcium ions bind to the phosphoryl groups, thus blocking this interaction (Steiner and Beers, 1957). The effect of calcium ions on the chromatography of poly(A) was examined. Poly(A) was heated to 70°, 2.2 mM $CaCl_2$ added, and the solution cooled to room temperature and placed on the BD-cellulose column. A gradient from 0.2 to 1.8 M NaCl in 25 mM, Tris buffer (pH 6.8), and 2.2 mM $CaCl_2$ was applied. Tris buffer was substituted for potassium phosphate to avoid calcium, magnesium, and phosphate ion interactions. Figure 5a illustrates that the poly(A) was eluted by this gradient. As a control for the effects of heating in the presence of calcium ions, the following experiments were performed. Omission of the preliminary heating step of the poly(A) prevented the subsequent elution from occurring. Preliminary heating and cooling of the poly(A) in the absence of the $CaCl_2$ also prevented subsequent elution with the $CaCl_2$ containing gradient. However the possibility that calcium ion catalyzed the breakdown of poly(A) during the heating step cannot be ruled out, instead of facilitating elution by complexing with the amino groups of poly(A).

It has been postulated that calcium-treated poly(A) has a structure analogous to the formaldehyde-treated polymer (Steiner and Beers, 1961). This hypothesis was tested. Poly(A) was treated with formaldehyde as described previously (Boedtker, 1968). The formaldehyde-treated poly(A) could not be eluted by either the Tris or the phosphate containing

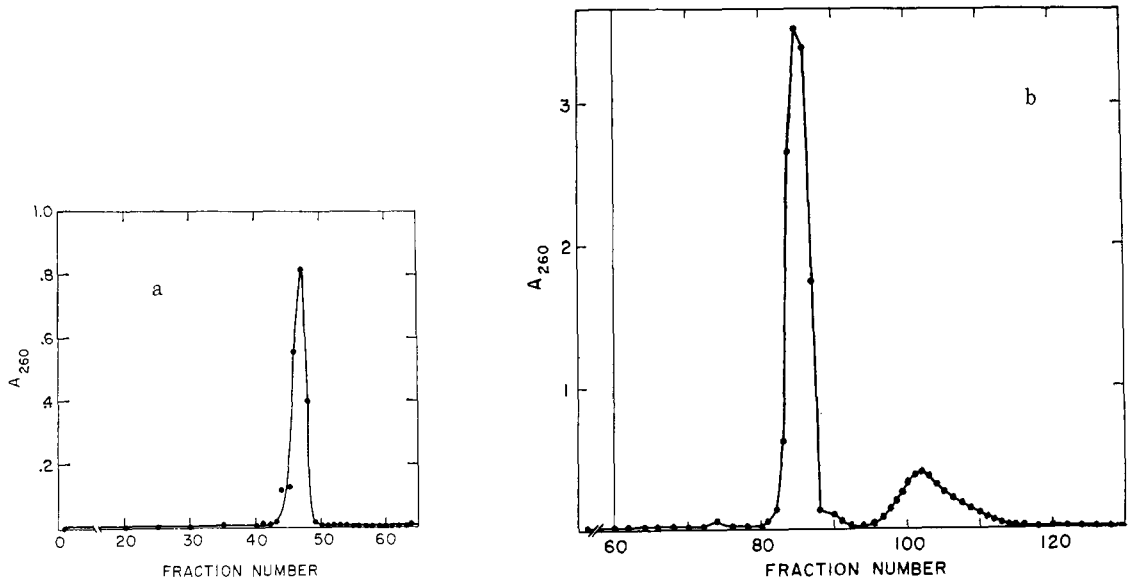

FIGURE 5: BD-cellulose column chromatography of two preparations of poly(A). (a) 0.5 mg of poly(A) was dissolved in 10 ml of 25 mM Tris-HCl buffer (pH 6.8) containing 0.2 M NaCl and 2.2 mM $CaCl_2$ and heated to 70° for 10 min. The solution was then cooled to room temperature and placed on a BD-cellulose. Chromatography was carried out as described in the legend of Figure 1 except that the NaCl gradient was in 25 mM Tris-HCl (pH 6.8) and 2.2 mM $CaCl_2$. A_{260} of each fraction was determined. (b) Chromatographic profile of poly(A) following formaldehyde treatment. Poly(A) (2 mg) was dissolved in 9 ml of 25 mM potassium phosphate buffer (pH 6.8) containing 0.2 M NaCl. 37% formaldehyde was diluted to 30% adding one-fifth the volume of 0.45 M Na_2HPO_4 and 0.05 M NaH_2PO_4. This formaldehyde solution (1 ml) was added to the poly(A), heated to 63° for 15 min, cooled rapidly in ice–water, and then placed on the column. Chromatography was carried out as described in the legend of Figure 1a. No A_{260}-absorbing material was detected in the collected fractions (no. 1–59). A second gradient containing urea was applied as described in the legend of Figure 3. A_{260}-absorbing material was determined (fractions 60–128).

NaCl gradient, but was eluted by the gradient of urea and NaCl. This suggested the structure of poly(A) in the presence of $CaCl_2$ was not analogous to formaldehyde-treated poly(A) since the denaturing solvent gradient was required to elute the latter. However, formaldehyde treatment did diminish the binding between poly(A) and the column resin. Calcium containing buffers or formaldehyde treatment may change the nature of the binding to the column in other ways than changing the structure of the polynucleotides. Therefore, the results of these experiments are inconclusive.

Two peaks were observed in the chromatogram of formaldehyde-treated sample of poly(A) (Figure 5b). Treatment with formaldehyde included a heat step (60° for 15 min) which may have caused strand breaks generating several populations of poly(A)'s from an originally homogeneous material.

Chromatography of Poly(A,U,C.). The single-stranded copolymer poly(A,U,C) (A:U:C, 1:1:2) was placed on the BD-cellulose column and the systems were applied. The polymer was not eluted with the NaCl gradient but was quantitativey eluted with NaCl and urea as shown in Figure 6.

Chromatography of Complexes Formed by a Homopolymer and Mononucleotides. A source of artifact that can be invoked in using the BD-cellulose column is the interaction between polynucleotide chains base paired with small oligonucleotide segments and even nucleotides that have been generated by the action of nucleases that often contaminate RNA preparations. The formation of base-paired helices between pyrimidine homopolymers and the purine monomers has been observed (Howard *et al.*, 1964, 1966; Huang and Ts'o, 1966). Duplex interactions can be detected by optical measurements. An equimolar mixture of poly(U) and AMP applied to the column did not have the chromatographic properties of a poly(A)·poly(U) homopolymer duplex. Instead a pattern consistent with poly(U) and AMP was obtained suggesting that the base-pairing interaction of the polymer and the mononucleotides did not possess sufficient stability to survive column chromatography.

Discussion

There are several properties of BD-cellulose which must be invoked to explain the resin's ability to resolve RNAs. First, BD-cellulose acts as an anion exchange resin. The order of elution of ribonucleoside 5'-phosphates from DEAE-cellulose, CMP, UMP, AMP, is the same order of elution obtained from BD-cellulose columns (Gillam *et al.*, 1967). BD-cellulose is a

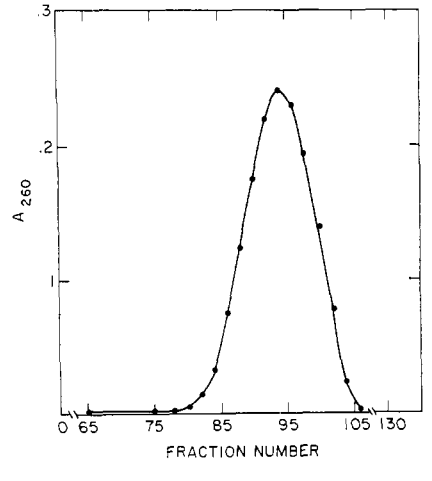

FIGURE 6: BD-cellulose column chromatography of the mixed copolymer poly(A,U,C). Poly(A,U,C) (0.5 mg) (A:U:C, 1:1:2) was dissolved in 10 ml of 25 mM potassium phosphate buffer (pH 6.8) and placed on the BD-cellulose column. A gradient of NaCl was applied as described in the legend of Figure 1a. A_{260} of each fraction was determined.

lipophilic resin as is illustrated by $tRNA^{Tyr}$ and $tRNA^{Trp}$ aminoacylated with their aromatic amino acids. These are strongly bound to the BD-cellulose but can be eluted easily from the column when deacylated (Maxwell *et al.*, 1968). However the major forces exploited in BD-cellulose chromatography are the secondary or nonionic interactions between polynucleotides and the column resin. These are nonspecific hydrophobic forces between the bases and the benzoyl groups, hydrogen-bonding, and "stacking" interactions, or intercalation of the unpaired bases of the RNA between benzoyl groups.

The ability of the column to separate species of RNA on the basis of degree of secondary structure (Gillam *et al.*, 1967; Kelly and Sinsheimer, 1967; Stern and Friedman, 1969) is a reflection of the secondary binding forces between the RNAs and the column resin. The greater the proportion of base pairing in an RNA chain, the fewer the bases available for interaction with the BD-cellulose, and the easier it is to elute such an RNA from the column.

The gradient of NaCl eluted those RNAs from the column that contained high degrees of secondary structure. The experiments with the viral RF and the preparations of poly(I)·poly(C) demonstrated that elution with the NaCl gradient was independent of relative size and was largely dependent on the proportion of secondary structure in an RNA chain. Similar observations have been made previously using the intermediates in the replicative cycle of an RNA virus (Stern and Friedman, 1969). The order of elution of these RNAs from the column is dependent on the proportion of ribonuclease-resistant RNA present in the molecule and is independent of relative molecular size.

The urea gradient employed in the present studies probably interrupted the secondary forces which bound polynucleotides to the column. In this case, unlike the NaCl elution patterns, elution was a function of size, as was demonstrated by the poly(I) and poly(U) series of homopolymers. Polynucleotides are also separated on the basis of size on DEAE-cellulose columns in the presence of urea (Tomlinson and Tener, 1962, 1963). Apparently, benzoylation of the hydroxyl groups of the cellulose increases the secondary binding forces between the polynucleotides and the column resin since the chain lengths of the polymers which are capable of being resolved are increased by several orders of magnitude.

The BD-cellulose column facilitates separation of partially base-paired RI's and the RF eluted by NaCl, from the single-stranded viral RNA forms eluted by the urea gradient (Stern and Friedman, 1969). In the present experiments, the homopolymers poly(I) and poly(U) were analogs of the single-stranded viral RNAs in their chromatographic patterns. However this elution pattern is not characteristic of all naturally occurring single-stranded RNAs. Predominantly single-stranded RNA molecules such as the natural messenger and viral genome of bacteriophages R17 or Q_β contain a large number of base-paired regions (Adams *et al.*, 1969; Billeter *et al.*, 1969). The proportion of double-stranded RNA may be high. Indeed, single-stranded RNA from the RNA phage MS-2, known to have an unusually high degree of structure (Strauss and Sinsheimer, 1963), is eluted from a similar column with NaCl alone (Sedat *et al.*, 1967).

The synthetic homopolymers poly(U) and poly(I), the mixed copolymer poly(A,U,C), and poly(A) following formaldehyde treatment as well as the single-stranded viral RNAs (Stern and Friedman, 1969) were eluted by the gradient of urea from the BD-cellulose column. Clearly there are major differences in secondary structure between these RNAs. The features these RNAs shared to explain their elution from the column under the same conditions are not known.

The present studies indicated there was no single basis of separation of synthetic RNAs on this column. Size, configuration, degree of secondary structure, and base composition all played roles in determining the chromatographic properties of the polymers. The important consideration, however, is that it introduces to the field of nucleic acid chromatography a separatory technique which functions on an entirely different basis from the commonly used procedures, such as sucrose density centrifugation and polyacrylamide gel electrophoresis, techniques which are unable to separate RNAs of similar relative molecular size. The demonstration of the ability to separate the RI and RF from single-stranded RNA forms of the SFV despite similar relative sedimentation values (Stern and Friedman, 1969), together with the studies presented in this communication, suggest that an approach to the separation of mRNA and hnRNA from the other RNAs of the animal cells, particularly from rRNAs and rRNA precursors, may be possible with the BD-cellulose column.

In conclusion, BD-cellulose column chromatography is a means for studying the physical properties of naturally occurring and synthetic RNA chains, and it represents an adjunct to the field of nucleic acid chromatography.

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Chemical Modification of the Fluorescent Base in Phenylalanine Transfer Ribonucleic Acid*

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ABSTRACT: The tRNA^{Phe} of wheat germ, tRNA$_1^{Phe}$, exhibits fluorescence in solution due to the presence of a fluorescent base, Y_w^1, adjacent to the 3′ end of the anticodon. When this tRNA was exposed to ammonium carbonate at pH 9, it was converted into tRNA$_2^{Phe}$ which exhibits the same fluorescence but is chromatographically distinct from tRNA$_1^{Phe}$. The conversion was due to the modification of Y_w^1 to a new fluorescent base, Y_w^2, which has a free acidic group (pK_a = about 4) not present in Y_w^1. Thus at around neutrality tRNA$_2^{Phe}$ has an acidic group with a net negative charge on the base adjacent to the 3′ end of the anticodon; in every other respect it is identical with tRNA$_1^{Phe}$. The specific modification had no effect upon the rate at which the tRNA was esterified by the Phe-tRNA synthetase, but it reduced the rate of poly(U)-directed polyphenylalanine synthesis. The free base Y_w^2 can be further degraded by alkaline hydrolysis to Y_w^3 and then to Y_w^4 without any change in the spectrum of the fluorescent chromophore.

In the conversion of Y_w^3 into Y_w^4 a blocking group is removed from an aliphatic amino group on a side chain. These results indicate that Y_w^1 is similar to the Y base of yeast in having a blocked amino acid side chain on the characteristic Y base chromophore. This paper presents evidence that Y_w^1 differs from the Y base of yeast in the structure of the distal portion of the side chain. The Y base of beef tRNA^{Phe} is indistinguishable from that of wheat germ. A simple procedure was found for purifying wheat germ and yeast tRNA^{Phe} employing two benzoylated DEAE-cellulose columns, one run in the presence of unbuffered $MgCl_2$ and one in the presence of EDTA buffered at pH 4.5.

The major phenylalanine tRNAs (tRNA^{Phe}s) isolated so far from eukaryotic organisms have all been distinguished by the presence of an unusually hydrophobic and highly fluorescent base, the Y-type base. A base of this type was first detected in the tRNA^{Phe} of yeast by RajBhandary *et al.* (1967) and was called Y. It was found to be located contiguous with the 3′ end of the anticodon. A related Y base was found in the same position in the structure of the tRNA^{Phe} of wheat germ by Dudock *et al.* (1969). A Y-type base is present, presumably in the same location, in the tRNA^{Phe}s from rat liver (Fink *et al.*, 1968), beef liver (Yoshikami *et al.*, 1968), and peas (G. A. Everett, personal communication). The Y base has not been detected in any other species of tRNA other than tRNA^{Phe} (Yoshikami *et al.*, 1968). The Y base thus appears to have a role unique to the function of the tRNA^{Phe}s of eukaryotes, yet it is not an essential feature of tRNA^{Phe} in general since it is not present in the tRNA^{Phe} of *Escherichia coli* (Barrell and Sanger, 1969).

Other tRNAs exhibit, in the same locus adjacent to the 3′ end of the anticodon, a wide variety of hypermodified residues (Schweizer *et al.*, 1969) such as 1-methylinosine (Holley *et al.*, 1965), N^6-isopentenyladenosine (Biemann *et al.*, 1966), N^6-isopentenyl-2-methylthioadenosine (Burrows *et al.*, 1968), and *N*-(purin-6-ylcarbamoyl)threonine ribonucleoside (Schweizer *et al.*, 1969). It has been found that most of the tRNAs which have an A as the 3′ base of the anticodon possess a hypermodified residue such as N^6-isopentenyladenosine or N^6-

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