Purification by Benzoylated Cellulose Chromatography of Translatable Messenger Ribonucleic Acid Lacking Polyadenylate*

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A population of mRNA that does not bind to oligo(dT)-cellulose (operationally defined as poly(A)- mRNA) has been purified 50-fold by benzoylated cellulose chromatography. The nominal poly(A)- mRNA is authentic mRNA on the basis of its labeling in the absence of rRNA synthesis, its sedimentation pattern, its release from polysomes by EDTA, and its activity as template in a heterologous cell-free translation system. Only 40 to 50% of rapidly labeled RNA originally retained on benzoylated cellulose is polyadenylated as judged by its subsequent retention on oligo(dT)-cellulose. Similarly, the amount of nominal poly(A)- mRNA and poly(A)+ mRNA appears to be equivalent on the basis of labeling studies and translation in the rabbit reticulocyte system. Chromatography of renal polysomal RNA labeled under conditions that suppress cytoplasmic accumulation of newly synthesized rRNA showed approximately 30 to 35% of labeled mRNA did not bind to either benzoylated cellulose or oligo(dT)-cellulose. Quantitation of rRNA-specific, methyl-labeled dinucleotides in purified nominal poly(A)- mRNA showed 98% of rRNA is removed by these chromatographic procedures. Purification of biologically active poly(A)- mRNA by benzoylated cellulose chromatography should be useful in further delineating the regulatory role of poly(A)- mRNA and in investigating the metabolism of mRNA in the cytoplasm of mammalian cells and organs.

Although the studies of mammalian mRNA have relied primarily on analysis of mRNA isolated using its 3'-OH terminal polyadenylate as a marker (1, 2), a substantial fraction (30-35%) of rapidly labeled polysomal mRNA in exponentially growing cultured mouse L-cells and HeLa cells lacks poly(A)1, judged by its inability to bind to oligo(dT)-cellulose (3-5). Translatable mRNAs for several proteins, including actin and protamine, are present in both the polyadenylated and the nonadenylated state in eukaryotic cells (6-10). Thus, analysis of only polyadenylated mRNA (poly(A)+ mRNA) may not provide a sufficiently thorough description of mRNA metabolism. For example, because the possible deadenylation of poly(A)+ mRNA would result in biologically active mRNAs that no longer contain poly(A), the existence of poly(A)-lacking mRNA (poly(A)- mRNA) raises doubts of estimates of mRNA stability assayed as disappearance of radioactivity from poly(A)+ mRNA.

Direct analysis of poly(A)- mRNA requires its purification from rRNA contaminants. We have prepared nominal poly(A)- mRNA by a combination of benzoylated cellulose and oligo(dT)-cellulose chromatography. Previous methods based on purification of messenger ribonucleoprotein from ribosomes (3, 4) are not applicable to the kidney because high endogenous ribonuclease levels extensively degrade renal mRNA. Benzoylated cellulose was employed because it binds nonadenylated polynucleotides and appears to favor the binding of molecules with little secondary structure (11), perhaps by planar base stacking interactions between the purines and pyrimidines of RNA and benzoyl residues. For example, formaldehyde-denatured 28 S rRNA binds to the column but native 28 S rRNA does not (11).

Reasoning that poly(A)+ mRNA might have less secondary structure than rRNA in aqueous solution, we investigated benzoylated cellulose as a possible method for purification of poly(A)+ mRNAs. The experiments described below show that benzoylated cellulose is effective in preparing nominal poly(A)- mRNA from polysomes of renal tubular cells and in purifying it 50-fold from rRNA contaminants.

EXPERIMENTAL PROCEDURES

Animals and Cells—Male mice (45 days old, 30 to 35 g) were purchased from Charles River Breeding Laboratories, Wilmington, MA. Radiochemicals and drugs were administered by dorsal subcutaneous injection. Mouse L-cells were donated by Dr. J. R. Greenberg (Worcester Foundation for Experimental Biology, Shrewsbury, MA) and were grown at 37°C in Dulbecco's Modified Eagle's Medium (3). Details of the conditions for labeling L-cells are given in the appropriate legends.

Subcellular Fractionation and Preparation of RNA—Decapsulated mouse kidneys were disrupted by Dounce homogenization in ice-cold buffer consisting of 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 0.15 M NaCl, 3 mM MgCl2 (four kidneys per 3.0 ml of buffer). To prepare polysomes, postmitochondrial supernatants prepared by centrifugation of homogenates at 9,000 rpm for 10 min in the Sorvall SS-34 rotor were sedimented in 7 to 47% (w/w) sucrose density gradients in 10 mM Tris-HCl (pH 7.4), 0.50 M NaCl, 50 mM MgCl2 for 3 h at 4°C in the Beckman SW 27 rotor at 26,500 rpm (12). Ethanol-purified polysomes solubilized in 10 mM Tris-HCl (pH 9.0), 100 mM NaCl, 10 mM EDTA, 0.2 M sodium dodecyl sulfate were deproteinized by phenol/chloroform/isoamyl alcohol extraction (13). RNA also was prepared from polysomes dissociated by sedimentation in 7 to 47% (w/w) sucrose density gradients in 10 mM Tris-HCl (pH 7.4), 0.25 M NaCl, 10 mM EDTA. The conditions for sedimentation are described above.

RNA isolated for translation was extracted with guanidine HCl 3 times before deproteinization with phenol/chloroform/isoamyl alcohol (14, 15). Ribonucleoproteins ethanol precipitated from sucrose density gradients were dissolved in 5 ml of 6 M guanidine HCl, 25 mM EDTA (pH 7.4), acidified by adjustment to 100 mM potassium acetate (pH 5.0), and precipitated 60 to 90 min with 0.5 volume of 95% ethanol. Precipitated RNA was deposited at 9,000 rpm for 10 min at
0°C in the Sorvall SS-34 rotor, and the guanidine extraction repeated twice. Samples were stored gently with 5 ml of 95% ethanol, dissolved in a final volume of 10 ml of buffer containing 10 mM Tris-HCl (pH 9.0), 100 mM NaCl, 10 mM EDTA, 0.2% sodium dodecyl sulfate, and extracted sequentially with phenol, chloroform, isomyl alcohol (25/24/1) and chloroform/isomyl alcohol (24/1) until the interphase was clear (13). RNA was stored in 2 volumes of 95% ethanol.

Cytoplasmic RNA was prepared by extraction of unfractonated postmitochondrial supernatants with guanidine HCl (14, 15).

Chromatography of RNA on Benzoylated Cellulose—Benzoylated cellulose prepared by the procedure of Gillam et al. (11, 16) was used. Benzoylated cellulose bound twice as much labeled RNA-RNA retained on benzoylated cellulose, and retained RNA was eluted with 12 to 15 ml of 50% ethanol (v/v), 0.05 mM EDTA (pH 7.4) (11). Radioactivity in bound and nonbound fractions was measured by counting aliquots in clear solutions of 1.0 ml of sample and 10 ml of scintillation fluid consisting of 2 volumes of xylene, 1 volume of light mineral oil X-100 (Rohn & Haas Co., Philadelphia, PA), and 8 g of Omnifluor (New England Nuclear, Boston, MA) per liter. RNA was precipitated at -20°C by adjustment of column fractions to 0.15 M sodium acetate and 0.5 volume of isopropanol.

Oligo(T)-Cellulose Chromatography—Poly(A)± mRNA was purified using two different oligo(dT)-cellulose columns. RNA was applied to 10 ml of Tris-HCl (pH 7.4), 0.1% Sarkosyl, and adjusted to 0.45 M NaCl to the column. Polyadenylated RNA bound to the column was eluted with 10 ml of Tris-HCl (pH 7.4, 0.1% Sarkosyl. RNA was stored at -20°C in 0.2 M NaCl and 2 volumes of ethanol.

Sedimentation Velocity Analysis of RNA—RNA samples dissolved in 0.1 M of buffer containing 10 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 10 mM EDTA, and 0.2% sodium dodecyl sulfate were centrifuged in linear 34-1111 15 to 30% (w/w) sucrose density gradients in the same buffer (18). Gradients were centrifuged at 25,000 rpm for 17 h at 23°C in the SW 27 rotor. Total gradient fractions or fraction aliquot samples were counted in a gel of sample, plus 3 to 4 ml of H2O and 10 ml of xylene-based scintillation fluid.

Chromatography of Hydrolyzed RNA on DEAE- Sephadex—RNA samples from L-cell labeled with [3H]methyl-[3H]methyl (New England Nuclear, Boston, MA) were hydrolyzed for 16 h at 37°C in 4.0 ml of 0.3 M NaOH. Hydrolysates diluted with 20 ml of H2O were adjusted to pH 7.0 with 0.3 M HCl, combined with 1 mg of renal RNA that had been digested exhaustively with pancreatic RNAse A, and applied to columns (0.8 x 17 cm) of DEAE-Sephadex (A-25, medium, Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 0.1 M NaCl, 0.1 M Tris-HCl (pH 7.4), 0.10 M NaCl, 7% glycerol, and oligonucleotides were eluted with a 200-ml linear gradient of 0.10 to 0.50 M NaCl in 10 ml Tris-HCl, 7 m urea. Fractions were individually measured for absorption of light at 260 nm, and total column fractions were counted in gels of sample, plus 3 ml of H2O, and 10 ml of xylene-based scintillation fluid.

RNA Translation in a Heterologous Cell-free System—The ability of poly(A)± mRNA and poly(A)± mRNAs to function as template was measured in an mRNA-dependent reticulocyte lysate (21). Reticulocyte lysates were purchased from New England Nuclear as the 1-[3H]leucine Translation Kit. [3H]Leucine incorporated into polypeptides was assayed as alcalase-stable, acid-insoluble radioactivity in a 1-ml aliquot sample of the 25 ml reaction (21).

Radiochemicals and 5-Fluorouracil Acid—[5-3H]Acetic acid (10 to 20 Ci/mole) in sterile aqueous solution and l-[3H]leucine ([3H]leucine (190 to 230 Ci/m mole) were purchased from New England Nuclear. Benzoylated cellulose was obtained in lyophilized form, was solubilized and completely used on the day of the experiment.

5-Fluorouracil acid (NSG-3172) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute.

RESULTS

Benzoylated Cellulose and Oligo(dT)-Cellulose Chromatography of Renal Polysomal RNA—The ability of benzoylated cellulose to bind renal mRNA was assessed by comparing the relative amounts of labeled cytoplasmic and polysomal RNA retained on benzoylated cellulose and oligo(dT)-cellulose. Benzoylated cellulose bound twice as much labeled RNA as oligo(dT)-cellulose (Table I). Because Roberts demonstrated benzoylated cellulose binds poly(A)± RNA quantitatively (11) and previous comparisons in our laboratory had shown that oligo(dT)-cellulose was as effective as poly(U) Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) in binding poly(A)± mRNA, these chromatographic results suggest benzoylated cellulose has an apparent affinity for nonadenylated RNA. As shown previously (18) and in Table I, oligo(dT)-cellulose binds approximately 20% of labeled renal polysomal RNA when mice are labeled 2 h with [3H]orotic acid; since 40% of the same labeled RNA sample binds to benzoylated cellulose, it appears benzoylated cellulose retains an amount of labeled RNA equivalent to radioactivity in poly(A)± mRNA. Therefore, characterization of the additional RNA retained on benzoylated cellulose was essential to assess the properties of the RNA and to evaluate the column in mRNA purification.

The two approaches taken to analyze RNA binding to benzoylated cellulose were to: (a) chromatograph polysomal RNA labeled under conditions that selectively inhibited the appearance of newly synthesized rRNA, and (b) chromatograph labeled polysomal RNA sequentially on oligo(dT)-cellulose and benzoylated cellulose according to the schemes shown in Fig. 1. The first series of experiments provided an initial indication of the mRNA-like properties of the poly(A)± RNA retained on benzoylated cellulose, and the second set of procedures were used to purify poly(A)± mRNA for subsequent analytical studies.

Benzoylated Cellulose Chromatography of Selectively Labeled Renal mRNA—Benzoylated cellulose chromatography of polysomal RNA labeled in the presence of 5-fluorouracil acid to suppress labeling of rRNA (22, 23) gave results similar to those observed when RNA labeling proceeded normally (Table I). Benzoylated cellulose bound twice as much selectively labeled polysomal mRNA as oligo(dT)-cellulose, but approximately 35% of labeled mRNA did not bind to either benzoylated cellulose or oligo(dT)-cellulose (Table I, Experiment 2). The increased retention of labeled RNA on benzoylated cellulose was not due to binding of labeled RNA because 5-fluorouracil completely inhibits cytoplasmic accumulation of newly synthesized rRNA for several hours after injection (22, 23). Cytoplasmic RNA synthesized in the presence of the

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**Table I**

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<th>Experiment</th>
<th>Oligo(dT)-cellulose</th>
<th>Benzoylated cellulose</th>
</tr>
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<td>5-Flurouracil</td>
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Purification of Poly(A)-lacking mRNA from Mouse Kidney

**Fig. 1. Purification of poly(A)-mRNA.** In Scheme A, RNA from renal polysomes was chromatographed on benzoylated cellulose (see under "Experimental Procedures"), and RNA retained on benzoylated cellulose was chromatographed subsequently by two passages on oligo(dT)-cellulose. RNA that bound to oligo(dT)-cellulose was designated poly(A)+ mRNA, and RNA which did not bind to oligo(dT)-cellulose was termed poly(A)- mRNA. In Scheme B, polysomal RNA was chromatographed on oligo(dT)-cellulose; the bound fraction was designated poly(A)+ mRNA and the nonbound poly(A)- mRNA was chromatographed on benzoylated cellulose. Poly(A)- mRNA retained on benzoylated cellulose was designated poly(A)-mRNA.

**Fig. 2. Sedimentation properties of poly(A)-containing and poly(A)-lacking messenger RNA.** Mice (4) were labeled for 3 h with 250 μCi of [3H]orotic acid 10 min after injection of 1 μmol of 5-fluoroorotic acid. The postmitochondrial supernatant was sedimented in a 7 to 47% sucrose density gradient containing 10 mM Tris-HCl (pH 7.4), 0.25 M NaCl, 10 mM EDTA for 3 h in the SW 27 rotor (see under "Experimental Procedures"). RNA prepared from ribonucleoproteins in the 30 to 100 S region was sedimented in 15 to 30% sodium dodecyl sulfate-containing sucrose density gradients after fractionation on oligo(dT)-cellulose (see under "Experimental Procedures"). Radioactivity was measured by counting 1.4-ml gradient fractions in gel phase xylene-based scintillation fluid. A, RNA not retained on oligo(dT)-cellulose; B, RNA bound to oligo(dT)-cellulose.

**Fig. 3. Sedimentation properties of selectively labeled mRNA purified by benzoylated cellulose chromatography.** Mice (4) were labeled 4 h with 100 μCi of [3H]orotate 10 min after injection with 1 μmol of 5-fluoroorotate. The renal postmitochondrial supernatant was sedimented in an EDTA-containing sucrose density gradient (Fig. 2). RNA prepared from the 30 to 100 S region (Fig. 2) was chromatographed on benzoylated cellulose and oligo(dT)-cellulose as shown in Fig. 1, Scheme A. A, RNA retained on benzoylated cellulose but not on oligo(dT)-cellulose; B, RNA retained on benzoylated cellulose and oligo(dT)-cellulose. Arrows indicate positions of nominal 28 S and 18 S rRNA markers.

Drug was evaluated by sedimentation velocity analysis of nominal poly(A)+ RNA and poly(A)- mRNA prepared by oligo(dT)-cellulose chromatography (Fig. 2). The data showed labeled poly(A)- RNA was mRNA-like in its sedimentation properties, thus demonstrating the effectiveness of 5-fluoroorotate in suppressing labeling of renal rRNA and in identifying in kidney a class of mRNA-like RNA that does not bind to oligo(dT)-cellulose. Therefore, benzoylated cellulose has an affinity for some nonadenylated mRNAs which do not bind to oligo(dT)-cellulose.

The size of poly(A)- mRNA, selectively labeled in the presence of 5-fluoroorotate, and then purified by benzoylated cellulose and oligo(dT)-cellulose chromatography (see Fig. 1 and Table II) was examined in sucrose density gradients (Fig. 3). Purified nominal poly(A)+ mRNA had sedimentation properties similar to those of both poly(A)+ mRNA and nominal poly(A)- RNA prepared by oligo(dT)-cellulose chromatography of RNA labeled in the presence of 5-fluoroorotate. These similarities are apparent by comparison of Fig. 3A with Fig. 3B and with Fig. 2A. RNA that was selectively labeled in the presence of 5-fluoroorotate and did not bind to benzoylated cellulose or oligo(dT)-cellulose was also similar to poly(A)- mRNA with respect to its sedimentation profile (data not shown). These results suggest the existence of additional mRNA-like RNAs that cannot be purified by benzoylated cellulose chromatography. In summary, nominal poly(A)- mRNA purified by oligo(dT)-cellulose chromatography of polysomal RNA retained on benzoylated cellulose is comparable in size to authentic poly(A)+ mRNA.
RNA, i.e. RNA not bound to oligo(dT)-cellulose (Fig. 1, Scheme B), was chromatographed on benzoylated cellulose, a quantity of labeled RNA equivalent to the amount of radioactivity in poly(A)' mRNA was retained (Table II). Furthermore, an apparent specificity of the column for mRNA was suggested by its retention of 19% of pulse-labeled [3H]poly(A)-more, an apparent specificity of the column for mRNA was clearly labeled polysomal RNA that binds to oligo(dT)-cellulose (Table II and Ref. 18) and thus are consistent with a selectivity of benzoylated cellulose for renal mRNA. The unique binding capabilities of benzoylated cellulose were further illustrated by the fact that less than 0.5% of the same pulse-labeled poly(A)' RNA was retained on oligo(dT)-cellulose when chromatographed a second time. The similarity of the [3H]/[14C] ratio of poly(A)' mRNA and of poly(A)' mRNA suggested the mRNA-like properties of poly(A)' RNA retained on benzoylated cellulose. Both classes of mRNA had approximately 10-fold higher [3H]/[14C] ratios than the RNA that failed to bind to either column.

Oligo(dT)-Cellulose Chromatography of RNA Retained on Benzoylated Cellulose—When RNA previously bound to benzoylated cellulose was chromatographed subsequently on oligo(dT)-cellulose only 40% of the labeled RNA was retained (Table II, Experiments 3 and 4). This 40% value corresponds well with the finding that only 40% renal polysomal mRNA selected for labeling in the presence of 5-fluoroorotate contains poly(A) as judged by its binding to oligo(dT)-cellulose (Table I, Experiment 2), thus providing further evidence that the selected RNA was authentic poly(A)' mRNA nominally lacking in poly(A). We observed that only 40% of labeled mRNA was polyadenylated for RNA from polysomes (Table II, Experiment 3), from unfractionated cytoplasm (Table II, Experiment 4), and from RNA from 30 to 80 S particles released from polysomes by dissociation with EDTA (Table I, Experiment 3 and Table II, Experiment 2). In conclusion, the data in Table I and II show that nearly 60% of newly synthesized mRNA in renal proximal tubular cells lacks poly(A) according to its inability to bind to oligo(dT)-cellulose.

### Table II

**Purification of poly(A)-lacking mRNA**

RNA samples prepared as described below were chromatographed on benzoylated cellulose or oligo(dT)-cellulose (see under "Experimental Procedures"). Experiment 1A: polysomal RNA prepared from mice labeled 24 h with 10 µCi of [14C]orotate and 60 min with 100 µCi of [3H]orotate was chromatographed on oligo(dT)-cellulose. Experiment 1B: benzoylated cellulose chromatography of poly(A)-lacking RNA prepared in 1A. Experiment 2: poly(A)-lacking RNA prepared by oligo(dT)-cellulose chromatography of polysomal RNA from four mice labeled 4 h with 100 µCi each of [14C]orotic acid was chromatographed on benzoylated cellulose. Experiment 3: polysomal RNA retained on benzoylated cellulose (see Table I, Experiment 1) was chromatographed on oligo(dT)-cellulose. Experiment 4: control, cytoplasmic RNA from four mice labeled with 100 µCi each of [14C]orotic acid was chromatographed on oligo(dT)-cellulose (see Table I, Experiment 2). RNA retained on benzoylated cellulose was chromatographed on oligo(dT)-cellulose. 5-Fluoroorotate, as in control, except mice were labeled 10 min after injection of 1 µmol of 5-fluoroorotic acid.

<table>
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<th>Experiment</th>
<th>Oligo(dT)-cellulose</th>
<th>Benzoylated cellulose</th>
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<tr>
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<td>Bound</td>
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<td>[3H]</td>
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<td></td>
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<tr>
<td>2. B</td>
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<tr>
<td></td>
<td>[14C]</td>
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<td>3. Control</td>
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<td>108,368</td>
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<td>4. 5-Fluoroorotate</td>
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</tbody>
</table>

### Table III

**RNA content of fractions purified by sequential benzoylated cellulose: oligo(dT)-cellulose chromatography**

RNA content of samples dissolved in H2O measured by absorption at 260 nm. RNA content was computed using 1.0 A260 unit/40 µg of RNA. RNA was extracted from renal (Experiments 1 and 2) or L-cell (Experiments 3 and 4) cytoplasmic ribonucleoproteins precipitated from postmitochondrial supernatants with 70 mM MgCl2 (24, 25). [3H]RNA not retained on benzoylated cellulose; bc', dt', RNA bound first to oligo(dT)-cellulose; bc', dt'; RNA bound first to benzoylated cellulose and also bound to oligo(dT)-cellulose; dt', bc', RNA not bound to oligo(dT)-cellulose nor to benzoylated cellulose; dt', bc', RNA which was not retained on oligo(dT)-cellulose, but did bind to benzoylated cellulose.

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</tr>
<tr>
<td>2</td>
<td>445</td>
</tr>
<tr>
<td>3</td>
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Fig. 4. Sedimentation properties of RNA fractionated by benzoylated cellulose chromatography. Mice (4) were labeled with 200 pCi each of \([\text{H}]\)orotic acid. Ribonucleoproteins precipitated from postmitochondrial supernatants with 70 mM MgCl₂ (24, 25) were deproteinized, and RNA was fractionated by chromatography on benzoylated cellulose and oligo(dT)-cellulose (see under “Experimental Procedures”). RNA samples were sedimented in 15 to 30% sodium dodecyl sulfate-containing sucrose density gradients (Fig. 2). A, RNA retained on oligo(dT)-cellulose; B, RNA not retained on benzoylated cellulose; C, RNA retained on benzoylated cellulose but not on oligo(dT)-cellulose; D, RNA retained on benzoylated cellulose and on oligo(dT)-cellulose. Arrows indicate positions of nominal 28 S and 18 S rRNA markers.

Fig. 5. DEAE-Sephadex chromatography of hydrolyzed methyl-labeled L-cell RNA fractionated on benzoylated cellulose. L-cells (500 ml, 7.8 \(\times\) 10⁶ cells/ml) were deposited by centrifugation and resuspended in medium supplemented with 20 mM formate and 20 \(\mu\)M methionine (27). Cells labeled 2 h with 3 \(\mu\)Ci/ml of L-[methyl\(\text{H}\)]methionine were deposited, washed twice with 25 ml of Earle’s solution, and in 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1.5 mM MgCl₂. RNA prepared from ribonucleoproteins precipitated from the postmitochondrial supernatant with 70 mM MgCl₂ (Fig. 4) was chromatographed successively on benzoylated cellulose and oligo(dT)-cellulose (Fig. 1). The RNA fractions shown were hydrolyzed at 37°C in 0.3 M NaOH neutralized, diluted 10-fold, and chromatographed on DEAE-Sephadex columns (18 \(\times\) 0.7 cm; see under “Experimental Procedures”). Gradients were eluted with 200 ml of 0.1 to 0.5 M NaCl gradients containing 20 mM Tris-HCl (pH 7.4) and 7 M urea. Absorbance at 260 nm was measured, and radioactivity was determined by counting the 1.85-ml fractions in 3 ml of H₃O plus 10 ml of xylene-based scintillation fluid. A, RNA not retained on benzoylated cellulose; B, RNA retained on benzoylated cellulose, but not retained on oligo(dT)-cellulose.
approximately 2.7% of methyl-labeled cytoplasmic rRNA. Similar calculations based on \(^{3}H\)methyl label in the mononucleotide (−2) and dinucleotide (−3) peaks in Fig. 5 showed that less than 17% of the \(^{3}H\)methyl label in poly(A)\(^{+}\) mRNA was due to rRNA. Since 30% of rRNA methyl activity was in mononucleotides the total methyl label in nominal poly(A) mRNA due to rRNA contamination would be equal to 1689 counts/min (poly(A)\(^{+}\) mRNA dinucleotide label) plus 2689 counts/min \(\times 0.3\) (the estimate of rRNA methyl mononucleotides in poly(A)\(^{−}\) mRNA) or a total of 3495 counts/min. Therefore, of the 21,198 total methyl counts/min in poly(A)\(^{−}\) mRNA, 3495 + 21,198, or 16.5% result from rRNA contamination. Assuming nominal poly(A)\(^{+}\) mRNA in kidney is methylated to the same extent as poly(A)\(^{+}\) mRNA (26), this 16.5% value probably represents the maximum rRNA content of nominal poly(A)\(^{+}\) mRNA purified on benzoylated cellulose.

Several possible explanations exist for the failure to detect methyl-labeled 5′-terminal “caps” (net charge −5) in nominal poly(A)\(^{+}\) mRNA (Fig. 5). First, since “caps” were not observed in poly(A)\(^{+}\) mRNA (data not shown), the labeling conditions would have been inadequate to incorporate sufficient radioactivity in “caps.” Second, the incorporation of methyl label into de novo synthesized purine rings may not have been inhibited sufficiently as illustrated by the high percentage of methyl-labeled mononucleotides in rRNA (28). Third, the mRNA may have been partially degraded, but sedimentation analysis of L-cell mRNA showed no sign of degradation. However, estimation of rRNA contamination by quantitating methylated dinucleotides remains a valid approach regardless of the absence of labeled “caps.”

**Activity of Poly(A)\(^{−}\) mRNA in a Cell-free Translation System**—Poly(A)\(^{−}\) mRNA purified by benzoylated cellulose and oligo(dT)-cellulose chromatography was highly active in stimulating protein synthesis in an mRNA-dependent rabbit reticulocyte lysate (Table IV). Addition of 1 μg of nominal poly(A)\(^{−}\) mRNA to the lysate stimulated \(^{3}H\)leucine incorporation into protein 15 times greater than background. Under the same conditions, poly(A)\(^{+}\) mRNA purified by two passages on oligo(dT)-cellulose produced 25-fold incorporation over background. However, if nominal poly(A)\(^{−}\) mRNA was purified further by a second passage on benzoylated cellulose, poly(A)\(^{−}\) mRNA and poly(A)\(^{+}\) mRNA showed comparable activities per μg of RNA added to the lysate (Table IV).

By demonstrating the translational activity of poly(A)\(^{−}\) mRNA, these data appear to establish benzoylated cellulose chromatography as a valuable method for purification of biologically active mRNAs that cannot be isolated on oligo(dT)-cellulose.

**DISCUSSION**

Benzoylated cellulose should prove useful in the study of poly(A)\(^{−}\) mRNA in mammalian organs. Until now, analysis of poly(A)\(^{−}\) mRNA primarily has relied on the use of inhibitory drugs, on studies of cultured cell lines low in ribonuclease, on developing systems that do not synthesize rRNA in the early postfertilization periods, or on anucleate mutants (3–10, 30). The study of mRNA metabolism in mammalian organs and the role of poly(A)\(^−\) mRNA in compensatory organ growth, however, cannot be investigated by employing inhibitory drugs. Benzoylated cellulose chromatography, as described by Roberts (11), is as rapid and convenient as oligo(dT)-cellulose chromatography, and effective in the partial purification of biologically active mRNA molecules that do not bind to oligo(dT)-cellulose.

Roberts has suggested the poly(A)\(^{+}\) mRNA association with benzoylated cellulose results from the favored binding of molecules with limited secondary structure to benzoyl residues (11). However, the mechanisms of nominal poly(A)\(^{−}\) mRNA binding to benzoylated cellulose are not understood although similar principles may be involved. Not all labeled poly(A)\(^{−}\) mRNAs in our experiments bind to benzoylated cellulose; the nonbound mRNAs may represent a population of mRNA molecules which have more secondary structure than the mRNAs which bind to benzoylated cellulose. An alternative explanation for some mRNAs not binding to benzoylated cellulose is that the binding capacity of the columns may have been exceeded, but sample overloading seems unlikely since a 2- to 4-fold difference in sample RNA content did not substantially alter the fraction of RNA retained. We have also noted that when small amounts of polysomal RNA (less than 10 μg) were applied to benzoylated cellulose, approximately 90% of the input RNA was retained thus eliminating the ability of the column to discriminate between rRNA and mRNA. We speculate, therefore, that benzoylated cellulose contains sites to which any RNA may bind and that molecules with less secondary structure may be more effective in competing for those binding sites. Presaturation of the column with several milligrams of tRNA before application of trace levels of RNA may help reduce the apparent nonspecific binding.

Our studies suggest more renal mRNA lacks poly(A) than in other systems more commonly studied. Because the kidney cells labeled in vivo are nondividing and are not synthesizing DNA, these poly(A)\(^{−}\) mRNAs are nonhistone mRNAs. Previous studies of nonhistone poly(A)\(^−\) mRNAs in mammalian cells estimated the poly(A)\(^−\) mRNA content of exponentially growing cultured HeLa and L-cells at approximately 30% (3–5). The fraction of mRNA lacking poly(A)\(^{+}\) may be higher in kidney because of its nonproliferative state.

In this report, mRNA retained on benzoylated cellulose but not on oligo(dT)-cellulose was referred to as poly(A)\(^{−}\) mRNA or nominal poly(A)\(^{−}\) mRNA. It is judged to be authentic mRNA by its association with polysomes, its continued appearance in the absence of cytoplasmic rRNA labeling, its release from polysomes by EDTA, its heterogeneous sedimentation pattern, and its activity as template in an mRNA-dependent rabbit reticulocyte lysate. The designation of these mRNAs as poly(A)\(^{−}\) lacking was less rigorous, relying solely on
the inability of this RNA to bind to oligo(dT)-cellulose. Therefore, the possibility of short poly(A), or oligoadenylate sequences, at the 3′-OH terminal of these RNAs cannot be excluded.

Although the presence of oligoadenylate on this nominal poly(A)− mRNA has not been established, this class of poly(A)− mRNA has escaped attention in studies of poly(A)− mRNA metabolism (18, 31, 32). Estimates of mRNA catabolism in kidney determined by disappearance of radioactivity from RNA binding to oligo(dT)-cellulose should be reconsidered since an estimated 60% of renal cytoplasmic mRNA lacks poly(A) assayed by ability to bind to oligo(dT)-cellulose.

The demonstration by several laboratories (6–9) that eukaryotic cells contain translatable mRNA sequences distributed between the nominal poly(A)+ and poly(A)− RNA populations raises the possibility of a kinetic relation between these classes of mRNA. Although labeling studies suggested that radioactivity accumulated in poly(A)+ mRNA and poly(A)− mRNA at the same rate in HeLa cells (5), the metabolic fate of individual poly(A)− mRNA sequences in relation to poly(A)+ mRNA is still unknown. Thus, some mRNAs may lose poly(A) (or a sufficient length of poly(A) to preclude binding to oligo(dT)-cellulose), enter the poly(A)− pool, and continue to be translated. In this case, quantitation of label in poly(A)+ mRNA sequences would be an inadequate measure of actual mRNA turnover.

A recent abstract by Van Ness et al. (33) described the use of benzoylated cellulose to prepare putative poly(A)-lacking mRNA from mouse brain polysomes. Poly(A)+ mRNA prepared by Van Ness et al. hybridizes to unique sequence DNA and has a total sequence complexity comparable to poly(A)+ mRNA. Although there were sequences common to poly(A)+ mRNA and poly(A)− mRNA, these classes of brain mRNA were comprised mostly of different sequences. Thus, the data of Van Ness et al. combined with ours suggest benzoylated cellulose chromatography selects a population of mRNA not isolated by poly(A) selection techniques. Benzoylated cellulose chromatography should provide sufficient purification of poly(A)+ mRNA for increased translational efficiency and also should permit more accurate quantitation of molecular hybridization studies of poly(A)+ mRNA sequences.

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REFERENCES
Purification by benzoylated cellulose chromatography of translatable messenger ribonucleic acid lacking polyadenylate.

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