A SIMPLE ELECTROPHORETIC PROCEDURE FOR SEPARATION OF RNA ON MIXED AGAROSE–ACRYLAMIDE GEL COLUMNS

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SUMMARY

A simple procedure is described for the preparation of mixed agarose–acrylamide gels for the separation of high molecular weight species of RNA by a modification of the method of PEACOCK AND DINGMAN[1]. A method for the measurement of radioactivity in fractionated gels is described which permits tritium determination with efficiencies as high as 40 %.

INTRODUCTION

The most recently exploited method of analytical resolution of RNA species is gel electrophoresis. Polyacrylamide[2,3], agar[4], agarose[5,6], and mixed polyacrylamide-agarose[1] gels have been successfully employed in electrophoretic separation of different molecular weight classes of RNA. Resolution of closely sedimenting RNA species has been achieved with RNA from numerous organisms[3,7-11]. Microelectrophoretic procedures using agarose[12] and polyacrylamide[13] gels for RNA separation have been published.

Molecular weight estimates[1,14] and relative sedimentation coefficients[7,10] can be obtained quite reproducibly for all RNA species resolved by electrophoresis. Gel electrophoresis of high molecular weight plant ribosomal RNA has permitted detailed studies of cytoplasmic rRNA and chloroplast rRNA in total RNA preparations from whole leaves[7,15] and RNA from isolated chloroplasts[7]. The resolution obtained is far greater than that realized with sucrose gradient centrifugation[16] and MAK column separations[17].

The simultaneous analysis of distribution of ultraviolet absorbing material and radioactive label after electrophoresis is often desired in studies of RNA synthesis. Such studies have frequently been performed using acrylamide gels of low concentration (2–3 %) which are very difficult to handle since these gels swell upon removal from the electrophoresis tube necessitating correction of migration distances to a standard length if molecular weight estimates are to be made. These problems are minimized using agarose–acrylamide slab gels[1].

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We wish to report a procedure modified from the methods of PEACOCK AND DINGMAN[1] which permits highly reproducible resolution by gel electrophoresis of RNA on gel columns of agarose–acrylamide. Procedures for optical and radioactive analysis of resolved RNA species are described.

EXPERIMENTAL

Materials

Acrylamide and N,N'-methylenebisacrylamide were obtained from Eastman Organic Chemicals, Rochester, N.Y., and were recrystallized twice from chloroform and acetone respectively as described by LOENING[3]. N,N,N',N'- tetramethylenediamine (TEMED) was purchased from Eastman Organic Chemicals. Agarose was purchased from Bausch and Lomb as "Seakem". All other chemicals used were reagent grade. Electrophoresis was carried out at 7 °C in a Canalco Model 6 electrophoresis apparatus.

Acrylamide stock solutions were 20 % (w/v) total acrylamide consisting of 95 % (19 g) acrylamide and 5 % bisacrylamide (1 g) in 80 ml distilled water. Other stock solutions were: 0.8 % (w/v) ammonium persulfate; 3.2 % (v/v) TEMED; buffer[1] (10 ×) consisting of 0.01 M EDTA, 0.3 M NaH$_2$PO$_4$, and 0.36 M Tris, pH 7.8. All solutions were stored in the cold. It was necessary to prepare fresh TEMED and ammonium persulfate at least once a week. Table I indicates the proportions of these

TABLE I

AMOUNTS OF STOCK SOLUTIONS NEEDED TO PREPARE 0.5 % AGAROSE–ACRYLAMIDE GELS OF VARIOUS FINAL ACRYLAMIDE CONCENTRATIONS

	% Acrylamide concentration						
	2.00	*2.25*	*2.50*	*2.75*	*3.00*	*3.25*	*3.50*
	Volume reagents for 15 gels in ml						
Agarose	0.16 g	0.16 g	0.16 g	0.16 g	0.16 g	0.16 g	0.16 g
H$_2$O	22.6	22.2	21.8	21.4	21.0	20.6	20.2
Buffer (10 ×)	3.2	3.2	3.2	3.2	3.2	3.2	3.2
TEMED (3.2 %)	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Acrylamide (20 %)	3.2	3.6	4.0	4.4	4.8	5.2	5.6
Ammonium persulfate (0.8 %)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Total volume (ml)	32.0	32.0	32.0	32.0	32.0	32.0	32.0

components used to prepare composite gels of various final acrylamide concentrations in the presence of 0.5 % agarose. Preliminary experiments indicated that the polymerization accelerator dimethylaminopropionitrile (DMAPN) used successfully for slab gels by PEACOCK AND DINGMAN[1] was unsatisfactory for column gels due to the limited shelf life of this reagent. Use of DMAPN yielded gels which polymerized unevenly, had extremely high background absorbance (greater than 1.5 O.D. at 260 nm), had irregular absorbance along the length of the gel, and RNA samples tended to run unevenly in gels made with this reagent. Gels polymerized with TEMED had much lower background absorbance which stabilized by 4 h after initiation of polymerization.

Gels were polymerized in glass or plastic tubes 8 cm long and 5 mm I.D. The tubes were soaked for 15 min in a 1 % solution of Column Coat (Canalco, Rockville, Md.) and dried thoroughly before use to allow the gel solution to flow rapidly and evenly down the walls of the tube. Acrylic plastic tubes were preferred over glass tubes since the agarose–acrylamide gels were easily removed from the tube after electrophoresis by air pressure from a rubber bulb; gels polymerized in glass tubes tended to adhere to the walls of the tube and removal by rimming usually resulted in marring the gel which introduced optical irregularities into the gel surface.

Preparation of composite gels

Agarose stock solutions were refluxed for 15 min, filtered twice through charcoal to remove light scattering impurities, and stored indefinitely at 45°C (agarose to water proportions as in Table I). Acrylamide, buffer, and TEMED were mixed and incubated at 40°C along with a 5 or 10 ml syringe fitted with a 20-gauge needle. Parafilm was wrapped flush across one end of the gel tube so that it was watertight and a rubber collar (a serum stopper with the nipple cut off) was fitted on the open end of the tube. When the agarose solution had cooled to 40–42°C, the acrylamide mixture and the ammonium persulfate were added to it, mixed, and injected rapidly into the gel tubes with the prewarmed syringe, taking care not to introduce any bubbles into the gel column. The gels were allowed to polymerize for 45–60 min before use. Then, the rubber collar was slid down onto the tube, the excess gel trimmed neatly with a razor blade, and the bottom of the tube covered with a dialysis membrane held on with a rubber band cut from a piece of rubber tubing. The parafilm was removed and the gel tubes mounted in the electrophoresis apparatus. Buffer (1 ×) was added to the upper (cathode) and lower compartments and the gels prerun at 5 mA/gel for 45–60 min before sample application.

RNA samples were dissolved in the electrophoresis buffer (1 ×) containing 10 % sucrose and a small amount of Bromophenol Blue tracking dye. Sample solutions were prepared just before use to contain 10–30 μg RNA in up to 100 μl buffer. The sample was carefully applied to the top of the gel, and electrophoresis carried out for 2 h at 5 mA/gel.

Optical and radioactivity analysis. After electrophoresis, the gels were removed from the apparatus and scanned spectrophotometrically, stained with Methylene Blue[8], or stained with "Stains-all"[18] to reveal the presence of RNA and other macromolecular components. The gel scanner attachment (Model 2410 Linear Transport) for the Gilford Model 2000 spectrophotometer was used to scan the gels at 260 nm. The background absorbance of the gels (0.8–1.0) was subtracted with the digital absorbance control of the Gilford and the baseline adjusted with the recorder controls. The observed absorbance patterns of RNA distribution were found to correspond accurately to the patterns observed in stained gels.

For radioactivity determinations, gels were frozen on dry ice and sectioned with a slicer constructed of 90 stainless steel razor blades mounted on three brass threaded rods spaced 1 mm apart with brass washers. The thickness of the gel slice could be varied using different sized washers as spacers. Acrylic plastic plates were mounted on each end of the device for convenient handling. Gel slices were placed in scintillation vials to which 0.25 ml NCS reagent (Amersham Searle, Inc., Des Plaines, Ill.) was added. The vials were tightly capped, inclined at about a 30° angle, and in-

cubated at 60–70°C for 4 h; care must be taken to assure that each slice is submerged in the NCS reagent˜and that the vial caps are airtight during the incubation period. Otherwise, incomplete solubilization of the sample will occur which is recognized as opaqueness in the gel slices in contrast to the swollen, fully transparent appearance when solubilization is complete. Ten milliliters of a scintillation mixture (5 g PPO, 0.3 g dimethyl POPOP per liter toluene) was added, and the samples were counted with an efficiency of 40 % for tritium and 90 % for ^{14}C as determined by the external standard method. Fig. 1 indicates that 0.25 ml is the optimum volume of NCS for use with 2.5 % gel slices (avg. wt./slice = 20 mg) containing equal amounts of either labeled RNA or uracil. The water content in these gel slices exceeds the solubility capacity of 0.1 ml NCS reagent, and a water–toluene emulsion results which quenches strongly. Larger gel slices (such as those of DINGMAN AND PEACOCK[19], with higher water content) require more NCS reagent for similar results.

RESULTS

Molecular weight estimation

Since it has been shown that the molecular weight of high polymeric RNA is inversely proportional to its electrophoretic mobility, molecular weights could be calculated directly from measurements of the mobility of different RNA species. As given by PEACOCK AND DINGMAN[1], eqn. 1 defines the empirical relationship between RNA molecular weight and mobility,

$$\log M = \log M_0 + m \cdot \mu \tag{1}$$

where M is the molecular weight of the RNA species in question, M_0 is the intercept molecular weight, m is the slope and μ is the electrophoretic mobility of the RNA species expressed in $cm^2/V/sec$. It is apparent that M_0 and m vary as a function of
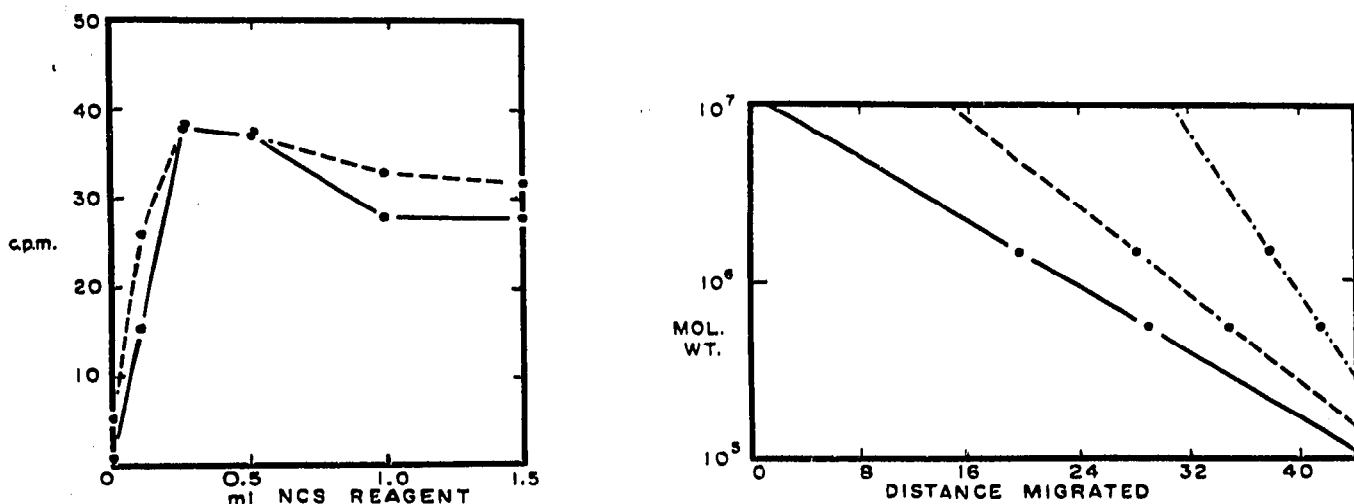

Fig. 1. Determination of optimum NCS reagent concentration for counting tritium in gel slices. ^{3}H-labeled *E. coli* RNA (– – –) c.p.m. × 10^{-3}; [5-^{3}H]uracil (———).

Fig. 2. Molecular weight and mobility of *E. coli* rRNA on agarose–acrylamide gels of different concentrations. 2 % acrylamide (–·–·–); 2.5 % acrylamide (– – – –); 3 % acrylamide (———). Electrophoresis carried out at 5 mA/gel for 2 h.

the concentration (*i.e.*, pore size) of the acrylamide in the gel[1]. The variation of m with gel concentration is illustrated in Fig. 2 using phenol-extracted *Escherichia coli* ribosomal RNA. Mobility was determined from the relationship

$$\mu = k(X_i - X_i{}^0)/(dv/dx)\cdot t \qquad (2)$$
$$= (kX_i)/(dv/dt)\cdot t$$

where X_i is the distance migrated from the origin, $X_i{}^0$, by any molecule i measured as Gilford chart divisions, dv/dt is the electromotive force gradient in V/cm, t is the time of electrophoresis in seconds, and k is a constant converting migration distance from chart divisions to centimeters actual migration in the gel. At 5 mA/gel, dv/dx was measured to be 8 V/cm for a 2.5 % acrylamide–0.5 % agarose gel.

To determine molecular weights of unknown RNA species, *E. coli* 23s (1.1×10^6 daltons) and 16s (0.56×10^6 daltons) rRNA were used as standards to determine M_0 and m. From the measured mobilities of these standards, a straight line plot of log M vs. mobility (μ) was constructed. The molecular weights of various high molecular weight species of jack bean leaf RNA were determined on 2.5 % acrylamide–0.5 % agarose gels on five different occasions using a total of ten different RNA preparations; *E. coli* rRNA was added to one gel of each phenol-extracted leaf RNA preparation to provide an internal standard. As shown in Fig. 3, M_0 varied somewhat from day to day with different gel preparations, but the slope (m) did not vary significantly. Reproducible values of M were obtained in each case, but it was imperative to run internal standards with each set of gels because of the slightly variable displacement
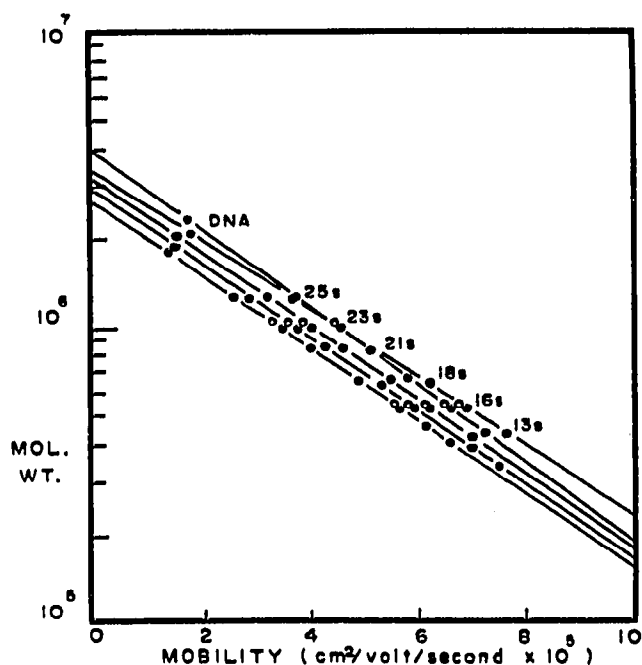

Fig. 3. Log molecular weight *vs.* electrophoretic mobility of jack bean RNA. *E. coli* rRNA standards, open circles; jack bean RNA, closed circles. *E. coli* data points are average of two determinations; jack bean 25s and 18s rRNA values are average of six determinations; 23s and 16s jack bean rRNA values are average of four determinations relative to 25s and 18s rRNA. Each line represents data from six gels polymerized and run at the same time.

of M_0. The major species of high molecular weight RNA observed in the leaf preparations had molecular weights of 1.29 and 0.67 × 10^6 daltons representing cytoplasmic rRNA and 1.06 and 0.55 × 10^6 daltons representing chloroplast rRNA[7]. These values are in close agreement with those obtained by LOENING[20] with pure acrylamide gels. Some minor species of RNA were also observed.

Determination of relative sedimentation coefficients

    Sedimentation coefficients, relative to an internal standard, can be obtained from acrylamide gels since $S_{20,w}$ of RNA with molecular weights similar to those of *E. coli* rRNA have been shown to be inversely proportional to their electrophoretic mobility on acrylamide gels[7,10]. This linear relationship also holds true for mixed agarose–acrylamide gels as shown in Fig. 4 in which a plot of $S_{20,w}$ is superimposed
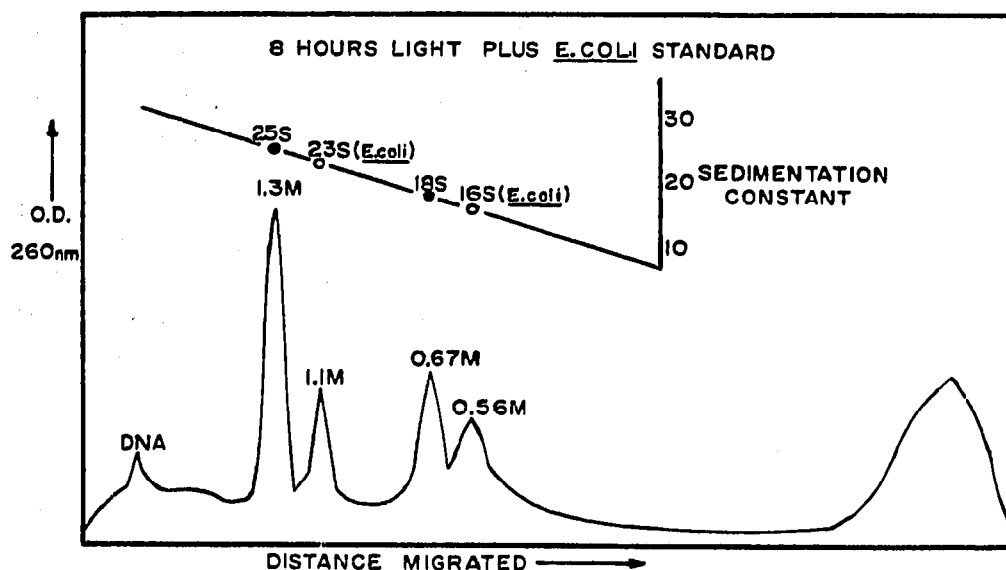

Fig. 4. Agarose–acrylamide (2.5 %) gel trace of RNA from jack bean leaves exposed to 8 h light. Linear relationship between sedimentation constant and mobility is represented by plot of S value *vs.* relative mobility (distance migrated). $M = 10^6$ mol. wt. Electrophoresis for 2 h at 5 mA/gel.

on an absorbance pattern obtained with a mixture of *E. coli* rRNA and jack bean RNA. The linearity of $S_{20,w}$ *vs.* mobility does not hold for low molecular weight 4 and 5s RNA on these gels. These values are similar to those obtained by sucrose gradient centrifugation[10,21] and acrylamide gel electrophoresis[7] for plant ribosomal RNA. It is preferable to refer to the molecular weights of resolved RNA species, since $S_{20,w}$ estimates from gels, however reproducible, do not represent actual sedimentation data.

Optical and radioactivity analysis

    Integration of peak areas of different RNA species resolved in 260 nm absorbance traces permits determination of molar ratios of the cytoplasmic and chloroplast-associated rRNA. Combined with labeled RNA precursor studies, a great deal of useful information may be obtained conveniently using these mixed gels. Fig. 5 demonstrates a background absorbance trace of a typical 2.5 % gel along with a
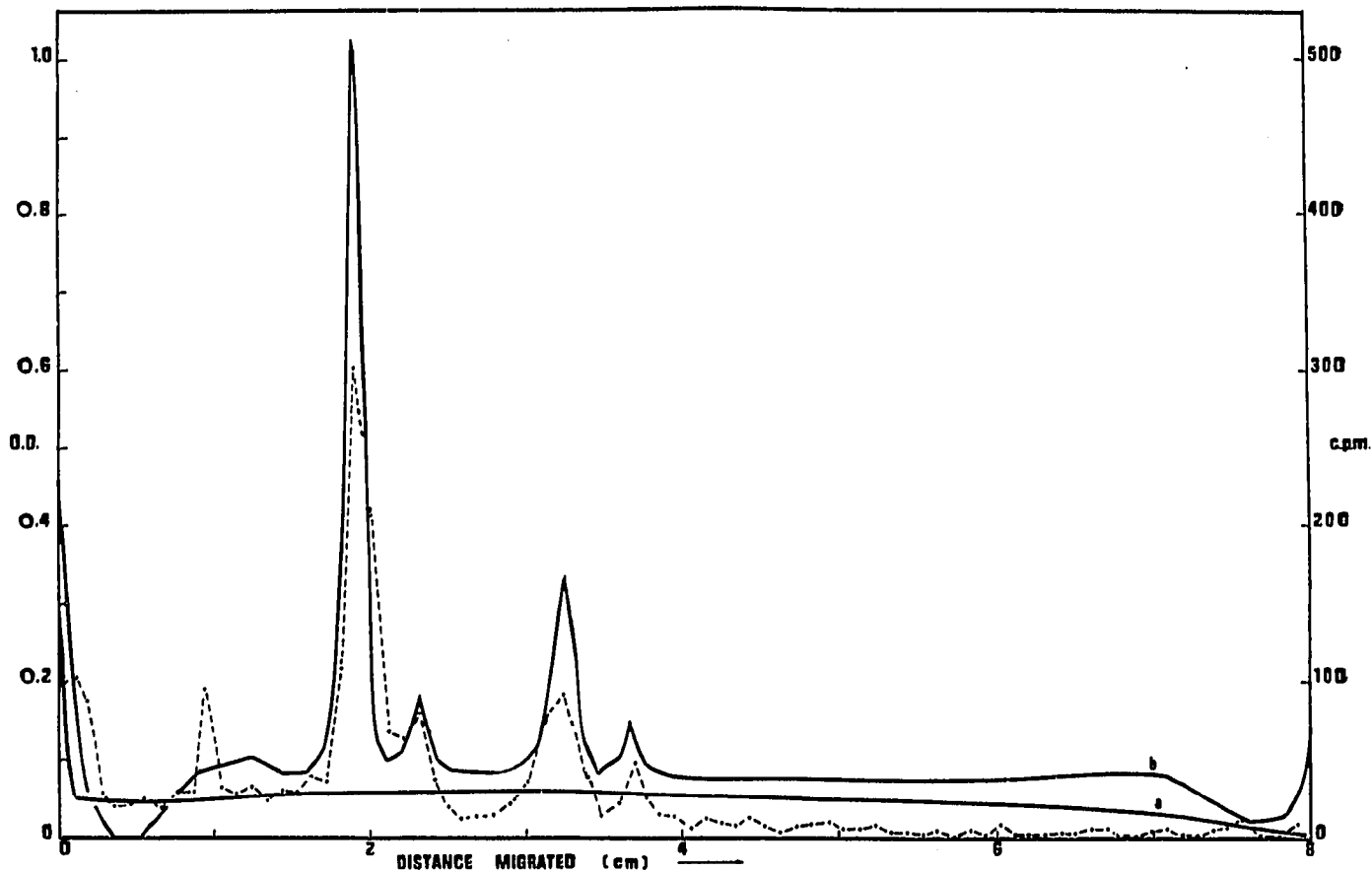
Fig. 5. Resolution of high molecular weight species of phenol-extracted[15] ribosomal RNA from greening leaves of jack bean allowed to incorporate [5-³H]uracil for 4 h. Typical background absorbance of standard blank gel (a); optical profile of RNA (b); O.D. at 260 nm (———————) ; ³H c.p.m. (– – – –).

trace obtained with leaf RNA from plants which had been allowed to incorporate [³H]uracil for 4 h in the light. The RNA in this preparation is highly intact and no degradation peaks are found in the absorbance trace. All four major species of rRNA are resolved both optically and by radioactivity distribution. Further studies have shown that differential rates of synthesis of these RNA species can be demonstrated using these techniques with similar preparations of intact RNA[15].

DISCUSSION

A simple procedure has been developed to apply the use of mixed agarose–acrylamide gels to the separation of RNA species by electrophoresis on gel columns. This method is modified from that developed by PEACOCK AND DINGMAN[1] for slab gels, but has the obvious advantages that less raw materials are required for each analysis, the column gels are more convenient to handle for large numbers of samples, and direct determination of RNA distribution on the gel by spectrophotometry reduces the time factor for analysis and provides a permanent, quantitative record of the results.

It is imperative to use TEMED rather than DMAPN in order to obtain con-

sistent results with these gels, although it does not appear to be necessary with slab gels[1,18]. Uneven, high background absorbance as well as variable mobility and smearing of RNA bands indicates the need for a new reagent supply when DMAPN is used.

The properties of these mixed gels are identical to those described by PEACOCK AND DINGMAN. Estimates of molecular weight, sedimentation coefficients, and molar ratios are obtained which are in close agreement with literature values.

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