Preparative Agarose Gel Electrophoresis of Ribonucleic Acid†

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ABSTRACT: A procedure for rapid, preparative purification of RNA was developed using preparative electrophoresis on 2% agarose gels. This procedure is simple, rapid, and reproducible and gives excellent separation into the three main classes of cytoplasmic RNA: (1) low molecular weight RNA containing 4–7S RNA, (2) 16S and 18S rRNA, and (3) 23S and 28S rRNA with high recovery. This method is superior to other methods such as sucrose gradients and agarose–acrylamide composite gels. Nucleic acid preparations can be electrophoresed directly after phenol deproteinization and a single ethanol precipitation without removing residual phenol.

Gel electrophoresis of RNA has been used extensively to separate RNA samples into component parts, both on an analytical (Hampel *et al.*, 1971; Loening, 1967; Peacock and Dingman, 1967) and a preparative (Zapisek *et al.*, 1969; Moriyama *et al.*, 1969; Hodnett and Busch, 1968) scale. Acrylamide and composite agarose–acrylamide gels have been the supportive medium of choice by most investigators. However, there are certain disadvantages to the acrylamide gel system: (1) the long length of time required for polymerization and preelectrophoresis of the gel, (2) potential hazard of handling the acrylamide monomer, (3) the near fluid consistency of low concentration acrylamide gels, and (4) variable polymerization as a function of pH. These major impediments are overcome by the use of pure agarose gels as the supportive medium for electrophoretic migration.

Purified agarose undergoes a simple noncovalent gelling process to form a gel which does not generate inhibitory products. A short (15-min) preelectrophoresis period is suggested to remove buffer discontinuities. This is advantageous over gels containing acrylamide. Acrylamide gels undergo a free radical polymerization which produce unwanted oxidation products. These products often produce artifacts in macromolecular electrophoresis (Loening, 1967; Fantes and Furminger, 1967).

The physical properties of a low percentage agarose gel make it amenable to preparative electrophoresis. Low concentration acrylamide and agarose–acrylamide composite gels, having similar macromolecular resolution properties on an analytical scale as low concentration agarose gels, were not suitable in our hands for preparative electrophoresis. The extreme fluidity of these gels precluded their usefulness on a preparative scale.

Agarose gels have been used for analytical separation of RNA (McIndoe and Munro, 1967) and for electroelution of RNA from analytical agarose gels (Popescu *et al.*, 1971, 1972). However these studies used small analytical size RNA samples which had considerable degradation. Using the Canalco preparative electrophoresis apparatus and 2.0% agarose gels, samples of up to 4 mg of RNA are routinely separated into major size classes in 100 min. This agarose gel electrophoresis system is more convenient and time saving than any other conventional method for separation of RNA.

Materials and Methods

Materials. Electrophoretic grade agarose powder was obtained from Bio-Rad Laboratories (Richmond, Calif.); all other chemicals used were reagent grade.

The preparative electrophoresis apparatus was from Canal Industrial Corp. (Rockville, Md.); the Model 300 power supply and PD2/320 upper column were used for all preparative experiments. The gel effluent was run through a 0.2-cm flow cell in a double-beam spectrophotometer. Fractions of 2 ml were collected using an automated fraction collector, and RNA elution was recorded by a standard chart recorder. The buffer flow was provided by a peristaltic pump. The analytical gel electrophoresis apparatus was that described by Raymond (1962) supplied by E-C Apparatus Co. (St. Petersburg, Fla.). The power supply for the analytical runs was a constant current power source. Both the preparative and the analytical gels were temperature regulated using a cooling circulator.

The buffer used throughout was Tris–EDTA–borate (pH 8.3) (0.089 M Tris–0.089 M boric acid–2.5 mM Na_2EDTA) as described by Peacock and Dingman (1968).

Agarose gels were prepared for either the analytical or preparative gels as follows: to 9 volumes of H_2O was added the desired amount of agarose. This was heated in an erlenmeyer flask with rapid stirring until the suspension began to boil. It was then refluxed for 5 min and removed from the heat. One volume of ten-times-concentrated buffer was added with stirring to give the final buffer concentration. This suspension was immediately cooled to 50° with warm water and the gel was poured. No deaeration of the suspension is needed. In the preparative column the bottom 3 cm of the inside glass surfaces had been previously sandblasted to roughen up the sides and thus effect a tighter binding of the gel to the column surface. This was found to be necessary or the gel moved in the column and sample was lost between the gel and the column sides. A piece of nylon stocking was stretched tightly over the end of the column and secured with a rubber band; this also helped secure the gel more firmly in place. Over the nylon netting was placed parafilm held in place with a rubber "O" ring. The warm gel solution was added with the aid of a syringe and plastic tubing directly into the gel column to the desired height. No layering of water or other material on top of the gel was necessary. The suspension was allowed to gel for 15

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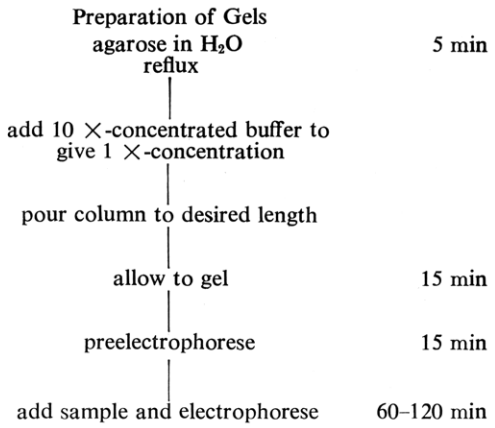

FIGURE 1: Flow sheet for both analytical and preparative agarose gels outlining the steps of preparation and the approximate amount of time required for each step.

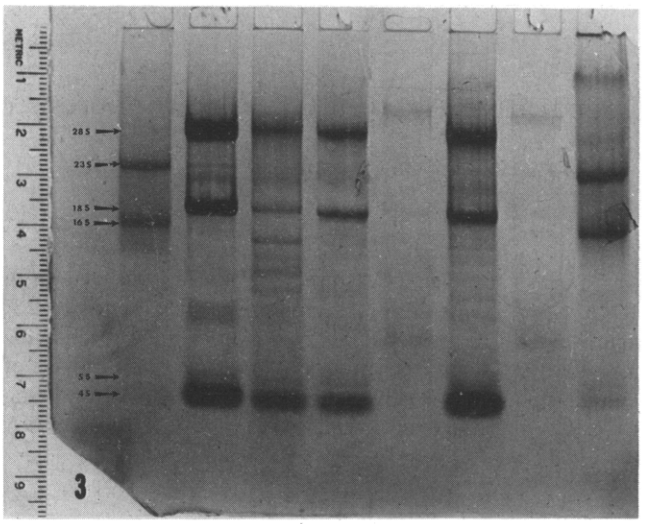

FIGURE 2: A typical 2% agarose vertical slab gel electrophoresed at 15°. Direction of migration is from top to bottom. The gel was run at a constant current of 43.6 mA for 90 min. Samples from left to right are: slots 1 and 8, *E. coli* total RNA, slots 2, 3, 4, and 6 contain rat liver cytoplasmic RNA prepared as in Materials and Methods. Slots 5 and 7 contain poly(adenylic acid)-rich RNA from rat liver prepared according to A. Weil and A. Hampel (in preparation). Sample size ranged from 10 to 20 μg of RNA. The arrows show the bands corresponding to the marker RNA species. The bands in slots 1 and 8 above the 23S RNA are due to sodium dodecyl sulfate contaminating the *E. coli* preparation. These sodium dodecyl sulfate bands stain yellow.

min, and then preelectrophoresed for an additional 15 min at 30-mA constant current (9.1 mA cm^{-2}). Samples were applied *via* a disposable syringe and plastic tubing and electrophoresis commenced at 30-mA constant current. The time required for electrophoresis varied with gel length, ranging from 90 min for 2-cm gels to 120 min for 4-cm gels. The flow rate of the elution buffer was 1.0 ml/min and fractions were collected at two minute intervals. Figure 1 summarizes the steps involved in gel preparation and shows the very short times involved in this type of RNA separation.

Analytical gels were prepared in essentially the same way, except that after the warm gel suspension had been poured into the vertical slab cell already at running temperature, the cooled slot former was introduced and a corked centrifuge tube full of ice placed behind that. The agarose was allowed to gel for 15 min. The slot former was then removed and buffer added to the electrophoresis cell. After preelectrophoresis for 15 min at 9.1 mA/cm^2 (43.6 mA), samples were applied and the gel was run at the same current. 43.6-mA constant current gave the same current density used during preparative gel runs. After electrophoresis gels were stained by the method of Dahlberg *et al.* (1969), destained under cold running tap water, and photographed.

Sucrose Density Gradient Centrifugation. RNA was run on 10–30% linear sucrose gradients in 10 mM KCl–10 mM Tris–1 mM $MgCl_2$–0.1 mM dithiothreitol (pH 7.5). Gradients were prepared using an Instruments Specialties Co. linear gradient former. RNA samples of 1 mg in 0.1 ml of gradient buffer were layered on top of 36-ml gradients. The tubes were centrifuged at 27,000 rpm in a Spinco 27.1 rotor at 4° for 18 hr. The gradients were monitored for uv absorption at 254 nm using an Instrument Specialties Co. automatic fractionator. Sedimentation values corrected to water at 20° ($s_{20,w}$) were calculated using the computer program of Dingman (1972).

Preparation of RNA. Cytoplasmic RNA from rat liver was prepared as follows. Male albino rats were sacrificed *via* cervical dislocation, and livers were removed immediately, weighed, and placed in ice-cold 0.25 M sucrose. The livers were then run through a tissue press into 5 volumes of ice-cold 0.25 M sucrose. The resulting slurry was homogenized in a Potter-Elvehjem homogenizer, rotating at 1000 rpm, with four passes of the Teflon pestle (clearance 0.006–0.009 in.). This homogenate was then centrifuged at 8000*g* for 10 min. The supernatant was taken to 10 volumes with 0.05 M NaCl–1 mM Na_2EDTA (pH 7.0). Sodium dodecyl sulfate was then added to 0.5%, an equal volume of 88% phenol was added, and the mixture was shaken at room temperature for 30 min. The phases were split by centrifugation at 3400*g* for 10 min, and the aqueous phase was drawn off, restored to volume with NaCl–EDTA buffer, and rephenoled twice for 15 min. The final aqueous phase was not restored to volume. The RNA solution in the final aqueous phase was precipitated by the addition of 2 volumes of cold absolute ethanol and left overnight at −20°. The RNA precipitate was recovered by low-speed centrifugation, and the RNA was lyophilized to dryness, redissolved in a minimum volume of 0.05 M NaCl–1 mM Na_2EDTA (pH 7.0), and made 10% in sucrose. Bromophenol Blue was added and the sample was ready for electrophoresis.

Radioactive RNA preparations were prepared following the same procedure except 18 hr before sacrificing, rats were given an intraperitoneal injection of 50 μCi of orotic-6-^{14}C acid in 0.5 ml of sterile aqueous solution (specific activity 25.5 Ci/mmol, ICN). After electrophoresis of the labeled RNA an aliquot of each radioactive fraction was added to 5 ml of Aquasol (Nuclear-Chicago Corp.) and counted in a Mark II Nuclear-Chicago scintillation counter. The reported counts are those corrected for background.

Mobility Determination. From the analytical gels, mobilities were determined after staining by measuring the distance of migration of a given species and then calculating mobility (μ) as follows: $\mu = dk/it$, where d is distance migrated in centimeters; k is specific conductance of the buffer; t is time in seconds; and i is current density in amps/cm^2 (Fischer and Dingman, 1971).

A separation factor (SF) was defined and used as a quantitative measure of RNA resolution on the preparative agarose gels. The equation used to calculate the separation factor for any two RNA species, modified from Singhal and Cohn (1972), is as follows: $(D_B - D_A)/(W_A + W_B)$, where D_A is distance of peak A from the origin, D_B is distance of peak B from the

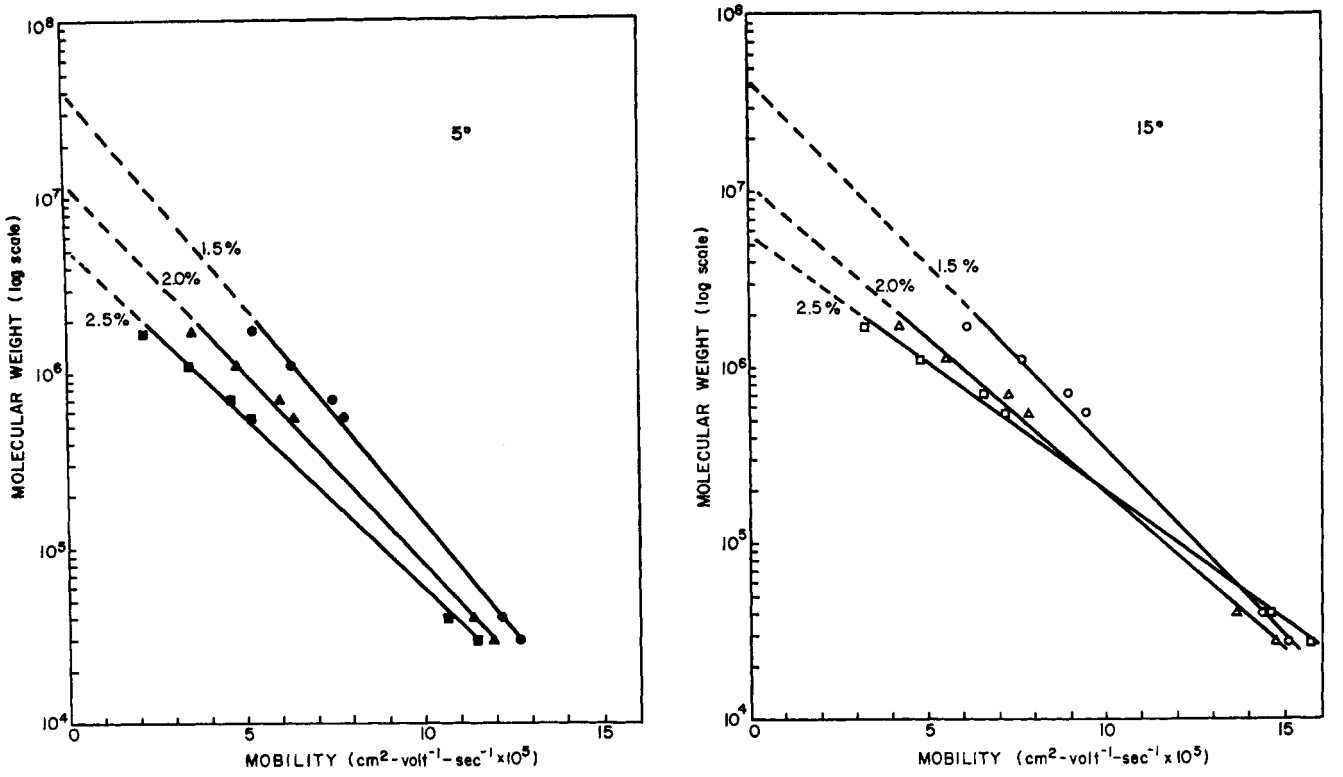

FIGURE 3: The log molecular weight of RNA is plotted as a function of mobility as determined by electrophoresis on agarose gels of various concentrations at two different temperatures: (A, left) 1.5% gels at 5° (●), 2.0% gels at 5° (▲), 2.5% at 5° (■); (B, right) 1.5% gels at 15° (○), 2.0% gels at 15° (△), 2.5% gels at 15° (□); curves were drawn using least-squares analysis.

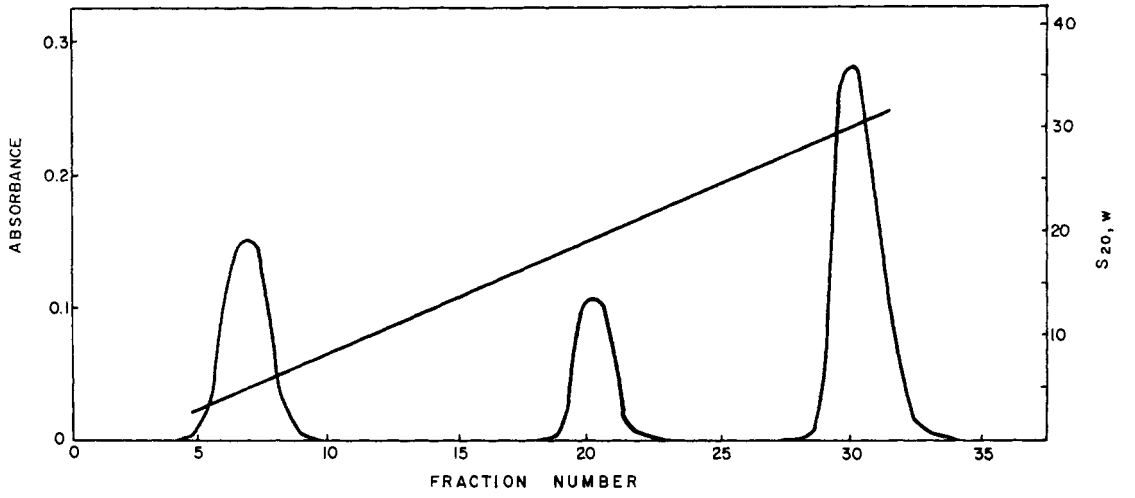

FIGURE 4: A 10–30% linear sucrose gradient of rat liver cytoplasmic RNA. A 1-mg sample of RNA in 0.1 ml was applied to the sucrose gradient and centrifuged as described in the text. The line represents $s_{20,w}$ calculated for each fraction.

origin, W_A is the width of peak A measured at the base line and W_B is the width of peak B at the base line. From the equation it can be seen that an SF of 0.5 or greater is required for complete separation of any two species.

Results and Discussion

Properties of Agarose Gels. Agarose, a natural linear polymer of galactose and 3,6-anhydrogalactose, forms a highly porous yet rigid gel at concentrations of 2.0%. These gels are easily handled and can be used in both preparative and analytical applications. As long as the stained analytical gels are kept moist they can be stored in a refrigerator for months and not lose their desirable physical properties.

Analytical Gels. Analytical agarose gels (Figure 2) were used for convenience to survey separations and to determine mobilities under conditions identical with those encountered in preparative agarose gels. When mobility is plotted against the log of the molecular weight a linear graph results (Figure 3). Each point represents the average of three electropherograms run under identical conditions. This linear function is defined by the following equation: $\log M = \log M_0 + mu$, where $\log M$ is the log of the molecular weight, $\log M_0$ is the intercept molecular weight obtained by extrapolation, m is the slope, and u is observed mobility (Peacock and Dingman, 1968). The slope and intercept molecular weights in Table I show that very good separation can be accomplished using these agarose gels. The slope is not so large as to group

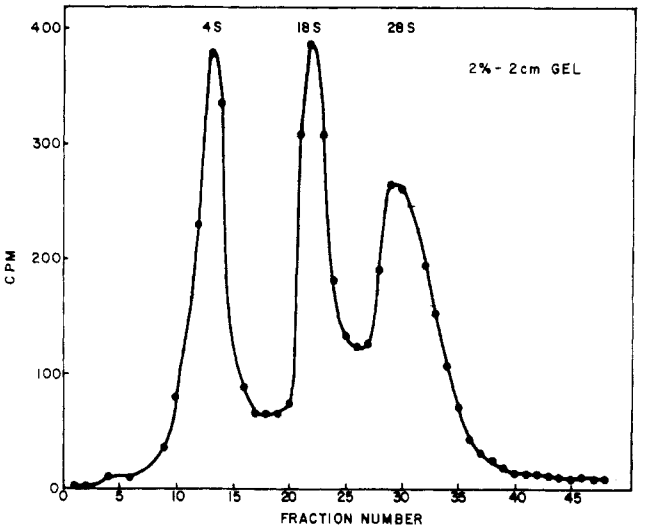

FIGURE 5: Radioactivity profile of a ^{14}C-labeled rat liver cytoplasmic RNA preparation fractionated on a 2%, 2.0-cm preparative agarose gel. A 2.5-mg sample in a 0.25-ml volume was applied to the gel and electrophoresed for 90 min.

all of the RNA species into a narrow range of mobilities, nor is the slope so small that only a small range of RNAs (molecular weight range) can be separated on these gels. It is seen that an increase in temperature will cause an increase in mobility. The intercepts for a given gel concentration at different temperatures are identical. It is also apparent that a given separation is more complete at 5° than at 15° for a given period of electrophoresis. From Figure 3 it is concluded that gel concentration is inversely proportional to mobility.

Preparative Agarose Gels. RNA samples used for all unlabeled preparative runs came from the same rat liver cytoplasmic preparation. A sucrose gradient separation of this RNA preparation in Figure 4 shows that the major cytoplasmic RNA species 4, 18, and 28 S are present in the sample with negligible degraded RNA. Figure 5 represents a separation of labeled RNA effected by 2% agarose gel preparative electrophoresis. This electrophoretic method gives complete separation of the major cytoplasmic RNA species with a considerable savings in time. This electropherogram required only 90-min electrophoresis with a minimal preparation time (see Figure 1). The corresponding sucrose gradient separation required about 22 hr. Thus one can visualize the substantial saving in time using the preparative agarose gels. Recovery of the RNA sample from the preparative gels described was in excess of 85%.

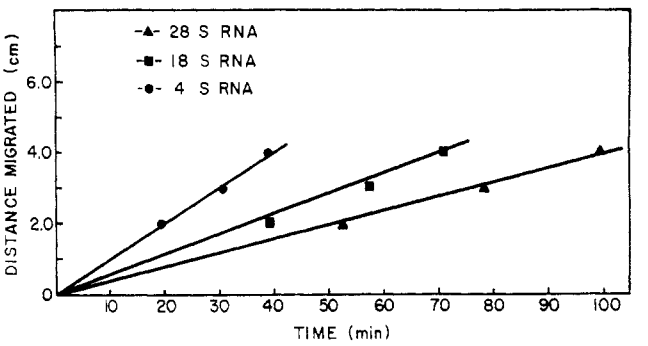

FIGURE 6: The distance an RNA species migrates as a function of time. Time required for RNA to move the length of the gel column. Electrophoresis was at 15° on 2% agarose gels with 2 mg of cytoplasmic rat liver RNA in 0.1 ml.

Agarose gel electrophoresis gives reproducibly linear separations of the RNA species. The linearity of this relationship makes predictions of elution volume of an RNA species of known size possible by simply preparing a graph as shown in Figure 6. This shows the time required for a given RNA species to migrate the length of a given preparative gel (2, 3, and 4 cm) and be eluted. The investigator is able to then preselect the time and volume of elution for any RNA species of choice, or alternatively the sedimentation value of an unknown RNA species can be found by simply determining its time of elution from a gel run under a prescribed set of conditions.

Figure 7 shows the electropherograms of actual RNA preparative runs using 2% agarose gels of various lengths. The effect of increasing gel length from 2.0 to 4.0 cm (Figure 7A–C) on separation for a given sample size (2 mg) can readily be observed. There is an increase in resolution between RNA species with increasing preparative gel length. Figure 7D shows an electropherogram using a 4-mg sample on a gel of 3-cm length. A significant loss of resolution can be corrected for by increasing gel length, as was shown in Figure 6 (Paus, 1972).

Temperature has an effect upon separation. Aggregation of the rRNA species (Figure 8) occurs and multiple peaks appear mainly in the 28S region of the electropherogram at lower temperature (Paus, 1972). This gel was run with a 4-mg sample at 4° using a 2%, 2.0-cm agarose gel. The aggregation can be seen in the form of multiple peaks in fractions 25–40, while the 28S RNA species generally has a maximum in fraction 28. As temperature is lowered and sample size increases,

TABLE I: Parameters of Agarose Gel Electrophoresis.[a]

Temp (°C)	% Gel	Slope × 10^{-4}	Log Intercept Mol Wt
5	1.5	−2.483	7.6285
	2.0	−2.203	7.0996
	2.5	−1.976	6.7216
15	1.5	−2.107	7.6521
	2.0	−1.764	7.0554
	2.5	−1.480	6.7701

[a] Slopes and intercept molecular weights were calculated using least-squares analysis. All values represent the averages of three replicate vertical slab agarose gel runs.

TABLE II: Separation Characteristics of Preparative Agarose Gel Electrophoresis.[a]

Gel Length (cm)	Sample Size (mg)	SF 4S-18S	SF 18S-28S
2.0	1.0	1.058	0.779
2.0	2.0	0.853	0.418
3.0	2.0	1.094	0.577
3.0	4.0	0.833	0.372
4.0	2.0	1.212	0.665

[a] Separation factors were calculated for a series of agarose gel electropherograms. Distances migrated for the various species were taken from the absorption profiles. All gels were run at 15°.

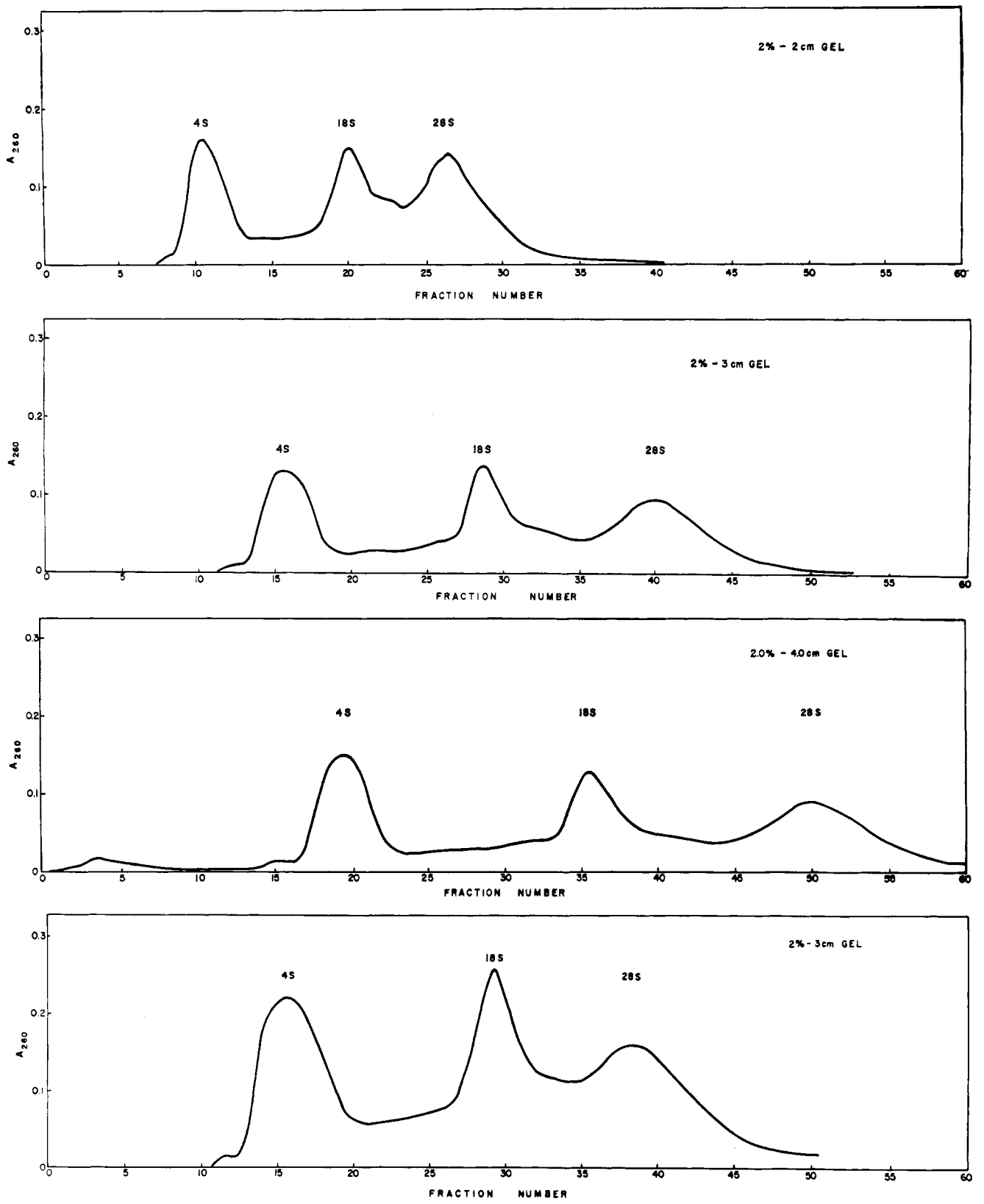

FIGURE 7: Absorption profiles of a series of 2.0% agarose preparative gels. Column lengths were (A) 2.0 cm, (B) 3.0 cm, and (C) 4.0 cm. Sample size was constant at 2.0 mg of RNA in 0.1 ml for the gels in A, B, and C. D is a 3-cm agarose gel with a sample of 4.0 mg of RNA in 0.2 ml. All gels were run at 15°.

RNA component separation correspondingly decreases. We have also observed a large loss of resolution using large sample volumes. It is for these reasons that all preparative runs are carried out at 15° with the most concentrated sample possible. This helps to sharpen separations by reducing aggregation and peak broadening due to low temperatures and large sample sizes.

A summary of several preparative electropherograms is given in Table II. The separation factors were calculated from measurements made to the middle of the appropriate ab-

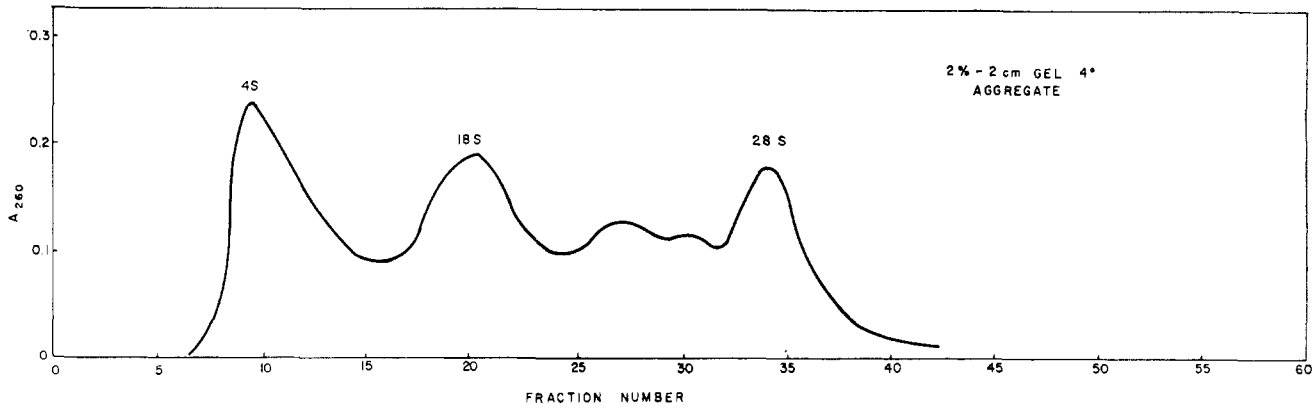

FIGURE 8: Electrophoresis of a large RNA sample at low temperatures on a 2.0%, 2.0-cm agarose gel. Gel was run at 4° with a sample of 4 mg of RNA in 0.2 ml.

sorbancy peaks from the electropherograms. These are given for 4S–18S RNA separation and 18S–28S RNA separation. It can be seen that most separation factors are greater than the 0.5 SF value required for complete separation. Several of these electropherograms are shown in Figure 7.

Figure 9 shows that SF is a linear function of column length for a given sample size at a given temperature. From this, the general reproducibility of this method is reiterated. By using this graph one can determine the length a given percentage agarose column must be in order to resolve the component parts of a given RNA sample. For example, in order to completely separate all three of the major species of rat liver cytoplasmic RNA in the minimum amount of time, it would be necessary to have a 2% agarose preparative gel 2.6 cm in length giving an SF of 0.5 or greater.

The purity of the individual peak fractions from preparative agarose electropherograms with separation factors greater than 0.5 is complete. Figure 10A shows a preparative electropherogram on 2 mg of RNA in a very small sample volume (0.15 ml). Peak tubes of the various RNA species from the electropherogram were then pooled, precipitated, and reseparated on 10–30% linear sucrose gradients with $s_{20,w}$ calculated for each peak. Figure 10B shows the size of peak I from the electropherogram and identifies it as 4 S with no contamination by larger or smaller RNA species. Figure 10C shows peak II to be 18 S in size with no contamination and Figure 10D shows peak III to be 28 S in size with no contamination by other RNA species.

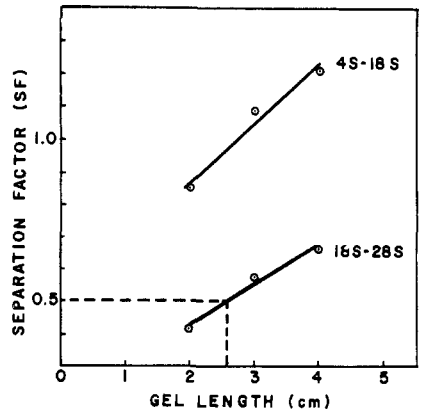

FIGURE 9: Separation factor calculated for a series of 2% agarose gels of varying lengths. Identical sample sizes of 2 mg of RNA in 0.1 ml were used. Separation factor is calculated for the 4S–18S and the 18–28S RNA peak separations.

The utility, reproducibility, and high resolution of this agarose gel electrophoresis system can easily be recognized. Complete RNA separations can be routinely performed in one-half to one-eighth the time required by other conventional

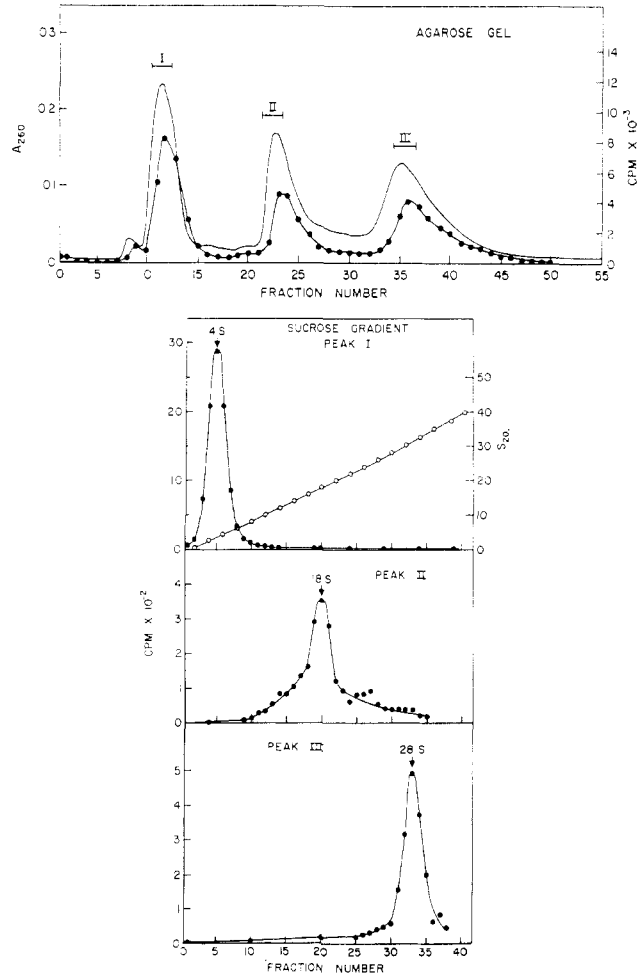

FIGURE 10: Sucrose gradient centrifugation of eluted RNA species from a preparative agarose gel. (A, upper) A 2-mg sample of ^{14}C-labeled rat liver cytoplasmic RNA (91,000 cpm) in 0.150 ml was applied to a 2.5%, 2.0-cm preparative agarose gel and electrophoresed for 110 min. Fractions I, II, and III were pooled, precipitated using CETAB (Sibitani (1970)), and centrifuged on 10–30% linear sucrose gradients (B, C, and D, lower). The samples in 0.50 ml were run for 18 hr in an SW27 rotor as described in the text. The line in B represents $s_{20,w}$ calculated for each fraction in each of the three sucrose gradients B, C, and D.

methods, the hazards imposed by the acrylamide monomer are nonexistent, and its physical properties lend themselves to large size RNA separations.

As seen from the linear plots of mobility *vs.* log molecular weight (Figure 3) the molecular weight of an unknown RNA species can be estimated from its mobility if it is analytically coelectrophoresed with appropriate standards. This agarose gel system can be used on both the preparative and analytical scale with equal ease and resolution.

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Mechanism of Inactivation of L-Glutamate Dehydrogenase by Pyridoxal and Pyridoxal Phosphate†

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ABSTRACT: We have studied the inactivation of glutamate dehydrogenase by pyridoxal and pyridoxal phosphate. Kinetic measurements on the sodium borohydride reduced adduct of pyridoxal phosphate and glutamate dehydrogenase show that the inactivation is reflected in the V_{max} parameter and not in the K_m parameter, indicating that the residual activity is due to unmodified enzyme. Ligand binding studies have established that allosteric activators form complexes with both the pyridoxal and pyridoxal phosphate modified enzymes, the stability and spectral properties of which closely resemble those of the native enzyme. In contrast, we found no evidence of complex formation of the pyridoxal phosphate modified enzyme with substrate acids or coenzymes. The pyridoxal modified enzyme did form a stable complex with NADPH which resembled that formed with the native enzyme. Formation of ternary complexes between the pyridoxal modified enzyme, NADPH, and the substrate acids was not observed, however, nor were complexes with glutamic and α-ketoglutaric acids detected with this protein. We conclude that the loss of catalytic activity which parallels the incorporation of pyridoxal (phosphate) into glutamate dehydrogenase occurs because the modified enzymes are unable to form complexes with the substrate acids.

Several group specific reagents have been applied to the modification of glutamate dehydrogenase (L-glutamic acid: NAD(P) oxidoreductase (deaminating) (EC 1.4.1.3)). Pyridoxal phosphate (Anderson *et al.*, 1966) has been shown to be one of the most site-specific reagents (Piszkiewicz *et al.*, 1970; Moon *et al.*, 1972). Piszkiewicz *et al.* (1970) have shown that a single lysine residue per polypeptide reacts with pyridoxal phosphate, later established as *lys 126* in the revised amino acid sequence (Piszkiewicz *et al.*, 1970; Moon *et al.*, 1972).

Piszkiewicz and Smith (1971a) have shown that approximately 90% of the catalytic activity of glutamate dehydrogenase is reversibly lost when reacted with pyridoxal phosphate. The inactivation was attributed to imine formation between pyridoxal phosphate and the ϵ-amino group of *lysine 126*. The kinetics and equilibria of inactivation by pyridoxal and pyridoxal phosphate have also been investigated (Piszkiewicz and Smith, 1971a,b). The inactivation by these two aldehydes was shown to be consistent with imine formation with a group on the enzyme which has a $pK_{app} \approx$

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