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Molecular Weight Estimation and Separation of Ribonucleic Acid by Electrophoresis in Agarose–Acrylamide Composite Gels*

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ABSTRACT: An electrophoretic method has been developed for the analysis of ribonucleic acids (RNAs) ranging in size from 10^4 to 10^8 daltons. The method depends on the use of acrylamide gels strengthened with agarose for analysis of the larger RNAs. The resolving power of the method permitted individual characterization of RNAs in mixtures containing multiple species of RNA, without prior purification of each species; RNA molecules which differed in molecular weight by only a few per cent could be clearly distinguished, and the molecular weight of each estimated. This unusual application of electrophoretic methods for the determination of molecular weight is based on the observation that, for RNAs, smaller molecules migrate more rapidly than larger ones. The mobility and the logarithm of the molecular weight are inversely related and this relationship is approximately linear. The molecular weights estimated by this technique, although numerically dependent on values assigned to known RNA standards, are highly reproducible in gels of various composition, and are at present the best means of identification of species resolved by gel electrophoresis. By this means, liver 18S RNA is identified as a doublet of RNAs of 0.66 and 0.62 $\times$ 10^6 daltons and the analogous 16 S of *Escherichia coli* as a doublet of 0.58 and 0.54 $\times$ 10^6 daltons, while liver 5S RNA has a molecular weight of 38,000 daltons.

Several recent publications have described the usefulness of acrylamide gels in studying the electrophoretic behavior of ribonucleic acids of mol wt 10^4–10^6 daltons (Loening, 1967; Loening and Ingle, 1967; Mills *et al.*, 1967; Bishop *et al.*, 1967a,b; Peacock and Dingman, 1967). The resolution of RNA species in this molecular weight range appears much superior to that afforded by other techniques, such as centrifugation in density gradients or chromatography on methylated albumin kieselguhr.

We have been able, by incorporation of agarose in the gel mixtures, to prepare acrylamide gels of very low concentration, which still retain sufficient strength to permit easy handling. These new porous gels provide a medium suitable for the high-resolution analysis of RNAs up to 10^8 daltons, and are thus useful for the study of nuclear RNA (nRNA) (Dingman and Peacock, 1968). The addition of agarose also improves the handling characteristics of the 3.5% gels which we previously described as being useful for the study of cRNA (Peacock and Dingman, 1967).

Uriel (1966) and Uriel and Berges (1966) have described the preparation of composite agarose–acrylamide gels for protein electrophoresis, but they could

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not prepare gels with less than 3.0% acrylamide. Agar (Bachvaroff and Tongur, 1966; Tsanev, 1965) and agarose (McIndoe and Monroe, 1967) have been used for RNA electrophoresis in 2–5% gels. We have used just enough agarose to provide mechanical support for an acrylamide gel otherwise too weak to retain its shape. The size separation depends principally on the acrylamide component of the gel.

A very important property of these composite gels is that the mobility of each species is inversely related to its molecular weight. Thus it is possible to obtain on a single electrophoretogram (1) qualitative information as to type and distribution of RNA species present, (2) quantitative data on isotope incorporation, and (3) an estimate of the molecular weight of each species.

Experimental Section

Materials. The electrophoretic cell of Raymond (1962) was purchased from E-C Apparatus Co., Philadelphia, Pa. Acrylamide (catalog no. X-5521, for electrophoresis), Bis,[1] and DMAPN were obtained from Eastman Kodak, Distillation Product Industries, Rochester, N. Y. Tris was purchased from Calbiochem, Los Angeles, Calif. Agarose used in most of the experiments was "Seakem," distributed by Bausch and Lomb. It provided clear solutions at 1.0% concentrations, which gelled at about 32–33°. Agarose from Behringwerke was also satisfactory, but two lots of agarose from Mann Research Laboratories were not satisfactory for the present purpose.

Liver cRNA and a similar preparation from *Escherichia coli* were prepared as described earlier (Peacock and Dingman, 1967). The 30S, 18S, 5S, and 4S components were identified on the gels by electrophoretic analysis of fractions separated by centrifugation, and has been fully described previously (Peacock and Dingman, 1967).

Methods. PRELIMINARY EXPERIMENTS. A warm solution of agarose which also contains all the reagents required for an acrylamide gel (acrylamide cross-linking reagent, accelerator, and catalyst) may be handled in either of two ways. (1) It may be kept above 35° to prevent the agarose from gelling until acrylamide polymerizes, and then subsequently cooled (method of Uriel and Berges, 1966), or (2) the solution may be cooled to 20° to permit the agarose to gel first with the acrylamide gelling last. We tried both of these approaches. At acrylamide concentrations above 3.0%, where a definite acrylamide gel formed, it made no difference which method was used. At lower acrylamide concentrations, however, where the polymerized acrylamide product was still fluid, the prior gelation of the agarose was important.

PREPARATION OF COMPOSITE AGAROSE–ACRYLAMIDE GELS. Four solutions were used in the preparation of the gels: (1) 20% acrylamide monomer (19 g of acrylamide and 1 g of Bis in 100 ml of water; (2) DMAPN, 6.4% in water; (3) ammonium persulfate, 1.6% in water; and (4) buffer consisting of Tris (108 g), disodium EDTA (9.3 g), and boric acid (55 g), in 1 l. (pH 8.3).

Gel solution (160 ml) was prepared. Water was added to 0.8 g of agarose in an amount which varied according to the amount of the acrylamide solution used. For example, for 2.0% acrylamide content, 0.8 g of agarose was placed in an erlenmeyer flask with 113 ml of water at room temperature. The mixture of agarose and water was stirred vigorously by magnetic stirring, connected to a condenser, and refluxed at 100° for 15 min. The agarose solution was cooled to 40° with running tap water set at approximately 30° (somewhat warm, to avoid local undercooling and premature gelation). Buffer (16 ml), DMAPN (10 ml), and acrylamide (16 ml) were mixed and warmed to 35°. The agarose and acrylamide solutions were mixed, the temperature was adjusted to 35°, and 5 ml of 1.6% ammonium persulfate was added. To ensure the gelation of the agarose prior to the acrylamide, we have decreased the concentration of persulfate compared to our earlier report (Peacock and Dingman, 1967). The completed gel solution was mixed well and poured rapidly into an electrophoretic cell previously equilibrated at 20°. The slot former (precooled in ice water) was inserted in place and a chilled glass rod was placed in the front part of the cell to improve cooling of this area. The agarose gelled rapidly, and after 1 hr, the acrylamide had poly-

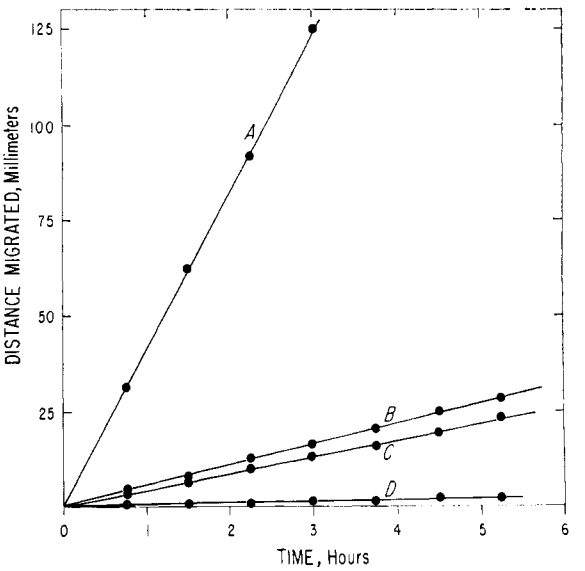

FIGURE 1: Migration distance as a function of time in 3.5% acrylamide gel. A similar result was found in a composite gel with 3.0% acrylamide and 0.5% agarose. The RNA species (from liver cytoplasm) are: A, 4 S; B, 18 S, faster component; C, 18 S, slower component; and D, 30 S.

[1] Abbreviations used: Bis, *N,N'*-methylenebisacrylamide; DMAPN, dimethylaminopropionitrile.

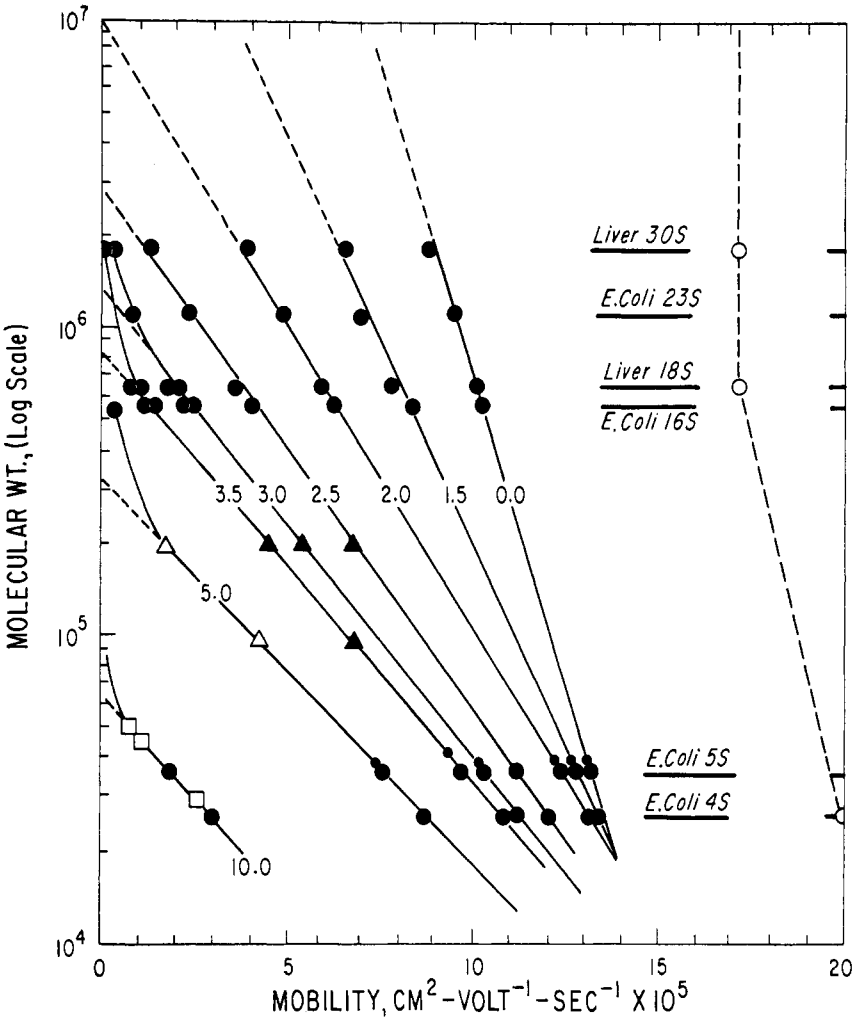

FIGURE 2: Relation between the molecular weight of RNA species in 0.5% agarose gels containing various acrylamide concentrations and their mobilities. The molecular weights used for the *E. coli* RNAs were cited by Bishop *et al.* (1967a); values for liver 30S and 18S RNA were calculated by the formula of Kurland (1960). The acrylamide concentrations are shown on each line. The dotted line represents the mobilities in free solution (Olivera *et al.*, 1964). The significance of △, ▲, □, and small circles is described in the text.

merized and the excess gel was removed from the cell.

ELECTROPHORESIS. The cell was placed vertically, buffer (diluted one-tenth) was added to the top and bottom buffer reservoirs, and the slot former was removed. The temperature of the circulating water was then reduced gradually over the next 0.75 hr to approximately 5°. During this time, 200 v was applied to the cell, constituting the prerun. Samples were applied at the end of this time, and the electrophoresis was commenced at 200 v. The time of the run was varied depending upon the circumstances, but in general was 1.5–2 hr for the 2% gels and up to 4 hr for the 10% gels. When the run was over, the gels were stained in 0.2% methylene blue as described before (Peacock and Dingman, 1967). Destaining was most conveniently done by pouring off the excess stain and covering the gel overnight with distilled water. During the day, the gel was rinsed by continuous flow of fresh water at approximately 25°. The rate at which gels of various concentrations destain was variable and frequent inspection was required to determine when destaining had been satisfactorily completed.

DETERMINATION OF MOBILITIES. Mobilities were calculated by first measuring the migration distance of the species in question. The voltage used in calculating the voltage gradient was the voltage indicated across the electrode (200 v usually), and the distance used was the 17-cm length of the gels (assuming the voltage gradient in the buffer was negligible). The temperature of the coolant was 5° entering the cell, and 7° leaving the cell. Mobilities are thus those observed at approximately 6° and are expressed as $cm^2\ v^{-1}\ sec^{-1}$.

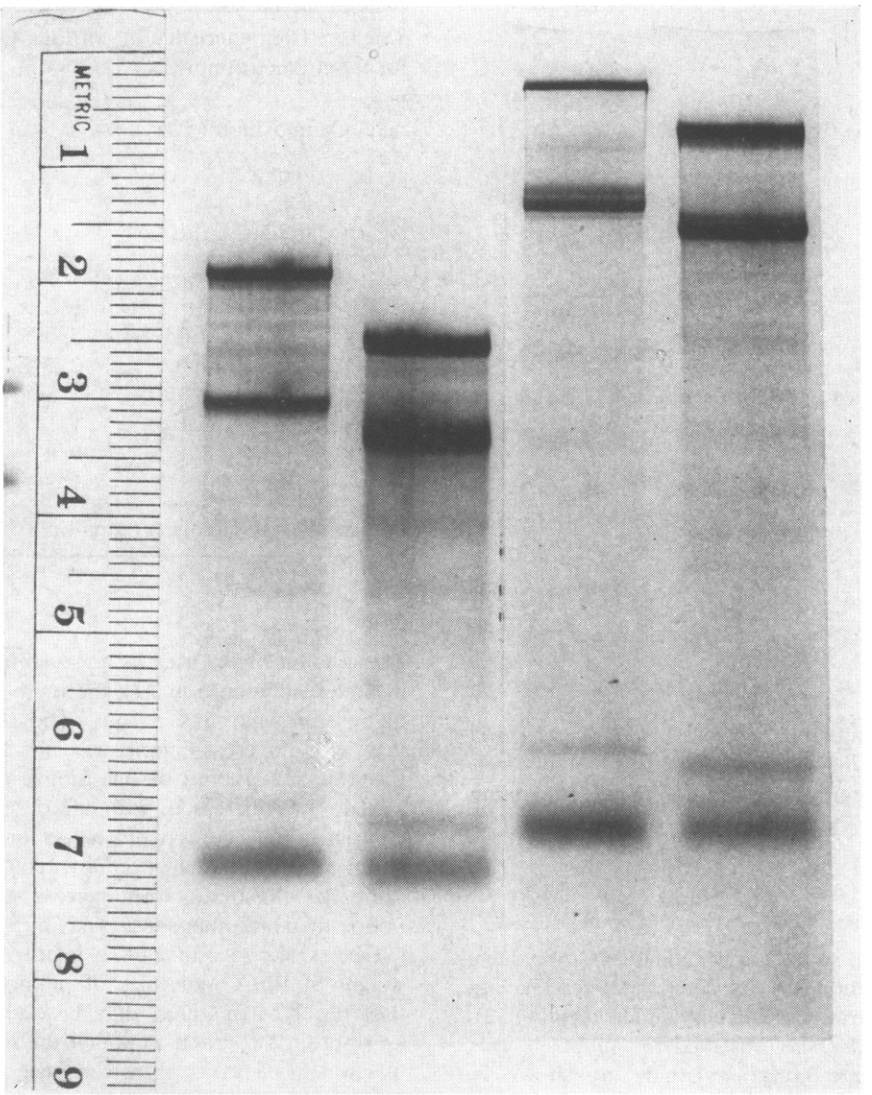

FIGURE 3: Photograph of a group of four different electrophoretograms. The origin is at 0.0 mm on the ruler. From left to right: slot 1, liver RNA in 2.0% acrylamide plus 0.5% agarose; slot 2, *E. coli* RNA in a similar gel; slot 3, liver RNA in 3.0% acrylamide plus 0.5% agarose; and slot 4, *E. coli* RNA in a similar gel. Run 90 min at 200 v; temperature approximately 2°. Major RNA classes are identified as follows. Slot 1 (liver): 19 mm, 30 S; 31 mm, 18 S; 64 mm, 5 S; and 70 mm, 4 S. Slot 2 (*E. coli*): 26 mm, 23 S; 34 mm, 16 S; 67 mm, 5 S; and 70 mm, 4 S. Slot 3 (liver): 3 mm, 30 S; 13, 14 mm, 18S doublet; 60 mm, 5 S; and 67 mm, 4 S. Slot 4 (*E. coli*): 7 mm, 23 S; 15, 16 mm, 16S doublet; 62 mm, 5 S; and 67 mm, 4 S.

Results

Physical Properties of Composite Agarose–Acrylamide Gels. Acrylamide gels of all concentrations containing 0.5% agarose are firmer and tougher than gels not containing agarose, and swell in water not nearly as much as do simple acrylamide gels. Most important, composite gels formed from 1.5 to 2.5% acrylamide were as easy to handle as the more concentrated gels, although simple acrylamide gels are fluid at these concentrations.

Gels containing 2% acrylamide and 0.5% agarose were easily cut with a multiple slicer described by Dingman and Peacock (1968). In the range 3–5% acrylamide with 0.5% agarose, the gels were more easily sectioned by removing individual sections sequentially with a microtome. All sections retained their shape and were easily transferred from the cutting device to vials for liquid scintillation counting.

Electrophoretic Properties of RNA in Composite Acrylamide–Agarose Gels. The migration of RNA molecules in these gels was linear with time even when this mobility was slow (Figure 1). The log of the (assumed) molecular weight of the RNA was inversely related to its mobility in these composite gels (Figure 2). A similar relation has been observed by Bishop

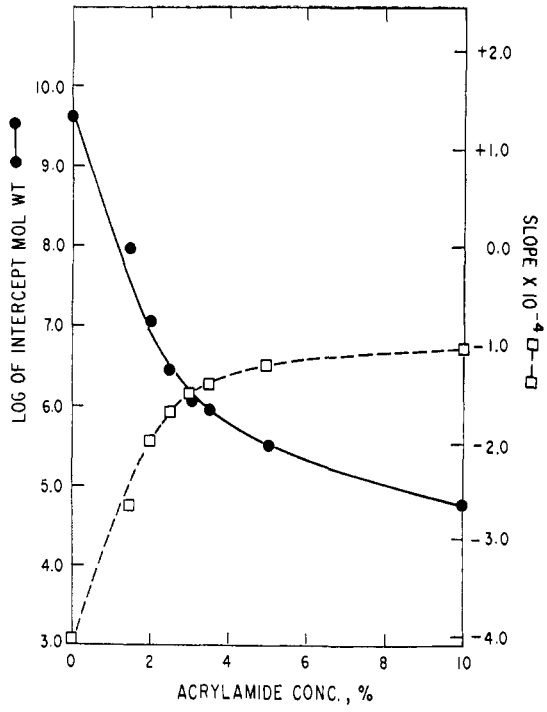

FIGURE 4: The intercept molecular weight (M_0) and slope (m) of the line relating log M and mobility as a function of acrylamide concentration in gels containing 0.5% agarose.

et al. (1967a) in simple acrylamide gels. We have studied this relationship in composite gels having different concentrations of acrylamide (Figure 2). Results typical of those observed in composite gels containing 2.0 and 3.0% acrylamide are shown in Figure 3. The lines in Figure 2 are all described by the relationship $\log M = \log M_0 + m\mu$, where M is the molecular weight of the species under study, M_0 is the intercept molecular weight obtained by extrapolation of the linear portion of the curve to the ordinate, μ is the observed mobility, and m is the slope. Both the intercept molecular weight (M_0), and the slope (m) are a function of the concentration of acrylamide (Figure 4). It is apparent that M_0 rises sharply as the concentration of acrylamide drops. The highest value of M_0 in the present experiments is determined by the properties of the agarose support. The intercept molecular weight (M_0) is a reproducible characteristic of each gel. It is not, however, the molecular weight of the largest RNA that will enter the gel; RNA species of molecular weight substantially above this limit entered the gels and had measurable migration velocities. The relationship of the slope (m) to gel concentration (Figure 4) mirrors that of M_0, becoming rapidly more negative as the acrylamide concentration decreases.

Numerous other gels of varying composition were studied and the slope and intercept values found characteristic for these materials are shown in Table I.

TABLE I: Characteristics of Various Gel Formulations for RNA Electrophoresis.

Gel Composition (%)				
Agarose	Total Acrylamide[a]	Bis	M_0	m ($\times 10^{-4}$)
1.0			1.5×10^8	−3.4
2.0			6.5×10^6	−4.9
1.0	1.0	0.05	5.0×10^6	−2.0
0.5	3.5	0.0	2.2×10^6	−2.0
0.5	3.5	0.05	1.3×10^6	−1.5
0.5	3.5	0.10	1.1×10^6	−1.5
0.5	3.5	0.175	0.9×10^6	−1.4
	3.5	0.175	1.2×10^6	−2.3

[a] Includes weight of acrylamide and bisacrylamide.

The amount of Bis used as a cross-linking agent made a modest difference in M_0, the more cross-linked gels being somewhat less porous. The resolution of the gels with no cross-linking was not as good as with the standard amount of Bis. Simple agarose gels also sieved; M_0 increased with decreasing the agarose concentration. The slope (m) is less for a simple agarose gel of a given M_0 than it is for a composite gel of the same M_0. Resolution on agarose gels was notably poorer than on composite gels having a similar M_0.

ESTIMATION OF MOLECULAR WEIGHT. The molecular weight of RNA molecules of unknown size, even if they occur as mixtures, may be estimated by use of an appropriate graph in which the log of the molecular weight of two or three reference RNAs is plotted against the observed mobility (as in Figure 2). An illustration of the application of this method is shown in Figure 2. The open triangles are observed mobilities of two minor RNA species from liver cytoplasm (see Peacock and Dingman, 1967) plotted, where data were available, on the 3.5, 3.0, and 2.5% lines formed by the reference RNAs (*E. coli* 23, 16, 5, and 4 S). The molecular weights of these two unknown species of RNA were then estimated from the ordinate as 2 and 0.94×10^5 daltons. These same RNA species could now be used as standards on the 5.0% acrylamide gel (closed triangles, Figure 2). A similar process was used to obtain the molecular weights of nRNA species (Dingman and Peacock, 1968) shown as open squares on the 10% gel line. The small circles in Figure 2, just above points for *E. coli* 5S RNA, are data points for liver 5S RNA. They fall, with small variation, on a line corresponding to a molecular weight of 38,000 daltons, somewhat larger than *E. coli* 5S RNA.

Figure 3 and Peacock and Dingman (1967) show that there are two components in liver 18S RNA and in *E. coli* 16S RNA. We estimate from their mobilities in 3.0 and 3.5% gels (Figure 2) that the

molecular weight of the two components of liver 18 S are 0.66 and 0.62 $\times$ 10^6 daltons. The molecular weight of the two components of the *E. coli* 16 S are 0.58 and 0.54 $\times$ 10^6 daltons.

Discussion

The physical properties of dilute acrylamide gels when supported by 0.5% agarose are surprising in view of the characteristics of each of these gels separately. Thus, the agarose at 0.5% is just able to maintain itself without flaking and crumbling and the acrylamide possesses so little structure that it is very nearly fluid. As has been found with other reinforced materials such as fiberglass and plastic, these composite gels possess physical properties unexpected from the properties of each constituent separately. Agarose and acrylamide differ in the chemical structure of the basic polymeric unit. Agarose, which is polyhydroxylic, is more hydrophilic than acrylamide and it may be that an appropriate balance between these two characteristics is an important feature in producing gels suitable for electrophoresis of RNA.

We do not know the mechanism by which these gels produce the size discrimination which results in the electrophoretic separation. One possibility is that there are tunnels throughout the gel through which RNA molecules pass with a tumbling motion proportional to their chain length. That is, the longer strands of RNA require a tunnel with a somewhat larger cross-section. The frictional resistance of the solution in the tunnel presumably acts as the viscous force limiting the velocity. It appears from the data in Figure 2 and Table I that agarose has a substantial effect on this viscosity, for the velocities are reduced appreciably. The acrylamide, on the other hand, has some effect on the width of the tunnel openings because increasing the acrylamide concentration results in gels which are progressively less permeable to large molecules (Figure 2). In addition, Figure 2 shows that the effect of acrylamide concentration is not as great on the smaller RNA molecules as it is on the larger ones.

The relation between the log of the molecular weight and the mobility is clearly an empirical one for which we have no satisfactory explanation. Fortunately, this relationship changes in a consistent way from one gel concentration to another, with simple identifiable changes in the parameters M_0 and m (Figures 2 and 4). Calibration of these gels may be accomplished by determining the mobilities of RNAs of known molecular weight (1) by the presence in the sample of certain suitable calibrating standards, such as, liver cytoplasmic RNA which contains 30S, 18S, and 5S RNA, or (2) by the preparation of additional samples containing such standards run in another slot on the same gel. The molecular weight of an unknown RNA species, determined from a plot of the above data, as shown in Figure 2, is a more reproducible property than its relative mobility since the latter varies with gel concentration. For this reason, the estimated molecular weight is a better identification than the relative mobility. The precision with which the true molecular weights of various RNAs fit into this relationship is not known at present. In addition to errors in the measurement of mobilities on the gel, there are uncertainties in reported values of the molecular weight of several species.

Figure 2 shows that RNA molecules larger than the intercept molecular weight limit enter the gel with definite mobilities. One interpretation of this result is that the first few millimeters of the gel possess a structure more porous than the rest of the gel. This interpretation is unlikely in view of Figure 1, which shows that the migration distance is proportional to the time, even for large species which spend a substantial portion of the time in this first part of the gel. However, the use of the first portion of the gel for the determination of molecular weights is not recommended.

It appears from Figure 2 that the maximum resolution between two species of RNA with similar molecular weights occurs when the mean molecular weight is approximately one-half of the intercept molecular weight (M_0). Thus, to determine a suitable circumstance for the resolution of the *E. coli* 16S doublet whose mean molecular weight is approximately 5.4 $\times$ 10^5, we should choose a gel whose M_0 is approximately 10^6, that is, one containing between 3 and 3.5% acrylamide. To discriminate between liver 5 S and *E. coli* 5 S in which the mean molecular weight is of the order of 3.5 $\times$ 10^4, we should choose a 10% gel in which the M_0 is approximately 7 $\times$ 10^4. On the other hand, nRNAs of the order of 4.0–5.0 $\times$ 10^6 will be best resolved on 2% gels, in which the M_0 is 10^7. The estimation of molecular weights of two species of 18S RNA found in liver and the two species of 16S RNA found in *E. coli* affords one type of distinction that may be made between species of RNA occurring in the same tissue whereas the 10% difference observed between *E. coli* 5S and rat liver 5S RNA indicate how species of RNA from different sources may be compared. The assignment of absolute values of molecular weight to various RNA is, of course, limited by the standards employed and thus the method can only be used as a secondary method for the determination of molecular weights. Nonetheless, for many of the less common species of RNA which we have described (Peacock and Dingman, 1967), there is as yet no other readily applied method for the determination of the molecular weight.

Separation of RNAs by electrophoresis in gels is different in several important ways from separation by ultracentrifugation. (1) The properties of an RNA molecule which determine its mobility in an electrical field are different from the properties which determine its sedimentation velocity in a gravitational field. (2) In centrifugation, the large molecules move faster, while in the electrophoretic analysis, the small molecules move faster. (3) The electrophoretic method has much higher resolution, and each fraction is very much less contaminated with other RNAs than is the

case for centrifugation. These differences suggest that new information concerning metabolic patterns may emerge from electrophoretic studies. Such studies should complement, and perhaps, by virtue of the higher resolution, even supplant earlier results based on less complete analysis.

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