Preparative Polyacrylamide Gel Electrophoresis of Ribonucleic Acid. Identification of Multiple Molecular Species of Bacteriophage T7 Lysozyme Messenger Ribonucleic Acid†

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ABSTRACT: The preparative fractionation of RNA on polyacrylamide gels using an apparatus which continuously elutes the RNA from the bottom of the gel is described. Several parameters which affect the ability to resolve RNA species of different mobility have been studied, including gel length, amperage, acrylamide concentration, amount of RNA, and elution rate. The values of these parameters which provide optimal resolution are presented and discussed. The rate of elution from the preparative gel decreases with increasing molecular weight but not in a way that can be described by a simple function. The recovery of up to 1 mg of RNA in the eluted fractions is essentially complete as measured by recovery of absorbance, radioactivity, and mRNA template activity. High molecular weight rRNA is recovered intact and there is no detectable contamination of 23S *Escherichia coli* rRNA by 16S rRNA, or *vice versa*. Analysis of the size of bacteriophage T7 lysozyme mRNA activity by preparative polyacrylamide gel electrophoresis reveals that the mRNA migrates as several discrete species with unique mobilities, suggesting among other possibilities that this mRNA may be processed prior to translation in the infected cell.

Polyacrylamide gel electrophoresis is a powerful technique for fractionating proteins and nucleic acids. Preparative techniques have been developed and used successfully in the isolation of proteins but more difficulty has been encountered in adapting this technique to the preparative fractionation of RNA. The literature contains relatively few descriptions of attempts to fractionate RNA by preparative polyacrylamide gel electrophoresis (Lanyon *et al.*, 1968; Malacinski, 1970; Popescu *et al.*, 1971, 1972; Landos and Bresnick, 1972; Shimada *et al.*, 1973) and in these reports the resolution obtained did not approach that characteristic of analytical polyacrylamide gel electrophoresis.

An exception to this statement is the use of large slab gels for the separation of oligonucleotides produced by RNase digestion of various mRNA preparations for sequence purposes (Adams *et al.*, 1969). In this case the fractions of interest are usually identified by autoradiography and then cut out of the polyacrylamide slab and extracted from the gel by homogenization and diffusion. Although time consuming, the physical extraction method is reasonably efficient for small molecular weight RNA. However, a more convenient method would be to elute the RNA continuously from the bottom of the gel even though this would decrease the resolution.

This paper includes a description and characterization of an apparatus which is capable of fractionating high molecular weight RNA on a preparative scale and which provides resolution similar to that obtained using analytical polyacrylamide gels for RNA identification. Numerous parameters affecting the preparative fractionation of RNA have been investigated. These include the effect of amperage, length of gel, elution rate, gel porosity, and size of load on RNA fractionation. RNA molecules are eluted according to their molecular weight and nearly 100% recovery of optical density, radioactivity in the form of labeled RNA, and mRNA activity in the form of T7 lysozyme mRNA activity is obtained. In addition, a demonstration that the T7 lysozyme message exists as several discrete species is described and discussed.

Materials and Methods

a. RNA Extraction. RNA was prepared from *Escherichia coli* B (Studier, 1969) and T7L infected *E. coli* B by a phenol-extraction method described by Bolle *et al.* (1968) and further described by Hagen and Young (1973).

Samples of sea urchin and mouse RNA were generously supplied by Byron Gallis. The sea urchin (*Strongylocentrotus purpuratus*) RNA was prepared by phenol extraction of sea urchin embryos in the morula (100 cell stage) developmental stage according to the method described by Aviv and Leder (1972). The mouse RNA was prepared by the same technique from GPC-11 mouse tumor cells (Riblet and Herzenberg, 1970). Mouse rRNA sediments at 18 S and 28 S and sea urchin rRNA sediments at 18 S and 26 S. The molecular weights associated with these species are taken from Loening (1968).

b. Cell-Free Protein Synthesis. The S-30 extract was prepared from *E. coli* MRE 600 as described by Capecchi (1966) and used for protein synthesis as originally described by Salser *et al.* (1967) and later outlined by Hagen and Young (1973).

c. Assay for Lysozyme Activity. The amount of lysozyme activity produced by *in vitro* protein synthesis was determined by measuring the rate of release of radioactive cell-wall components from radioactive filter discs prepared as devised by W. Leutgeb and V. Schwarz and described by Schweiger and Gold (1969) (Hagen and Young, 1973).

d. RNA Preparative Polyacrylamide Gel Electrophoresis. The preparative polyacrylamide gel electrophoresis apparatus used for RNA fractionation is shown in Plate 1 and Figure 1. The apparatus was originally designed by Dr. Gordon Hager, redesigned by Dr. Mike Gardner and Dr. Robert Cleland, and

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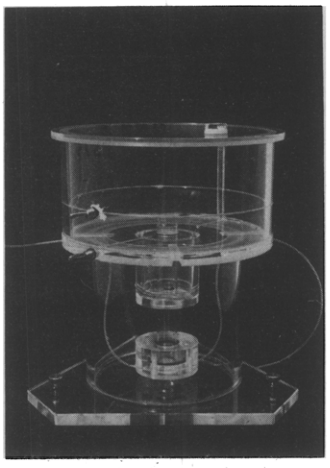

PLATE I: Preparative gel apparatus. The apparatus is shown assembled as for electrophoresis but without a gel in the gel tube or electrophoresis buffer in the reservoir chambers.

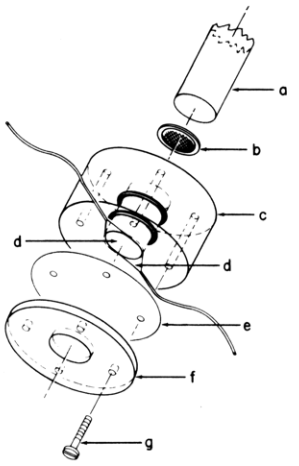

FIGURE 1: Elution cell. This figure is an extended artist's drawing of the elution cell of the preparative electrophoresis apparatus. The elements of the flow cell are indicated with letters: (a) gel tube, (b) nitex retaining ring, (c) body of elution cell with O-rings and elution tubing, (d) elution chamber, (e) dialysis mehbrane, (f) acrylic washer, and (g) nylon screw. Outside diameter of gel tube is 17 mm.

subsequently modified for RNA preparative electrophoresis by one of the authors (F. S. H.).

The reagents and concentrations used in preparing electrophoresis buffer and polyacrylamide gels are those of Loening (1969) with the exception that sodium dodecyl sulfate is deleted from the preparations. The acrylamide is electrophoresis purity grade acrylamide from Bio-Rad Laboratories and the bisacrylamide is recrystallized *N,N'*-methylenebisacrylamide from Eastman Organic Chemicals. The 1 × RNA electrophoresis buffer (REB) is 40 mM Tris (pH 7.8)–20 mM sodium acetate–2 mM EDTA; 10 ml of acrylamide gel solution is prepared by mixing 2 ml of 5 × REB, 5 μl of freshly prepared 10% (w/v) ammonium persulfate, an appropriate amount of stock acrylamide solution, 15% (w/v) acrylamide and 0.75% (w/v) bisacrylamide, and enough water to make the solution up to 10 ml.

An acid-washed, sterile gel tube 100 mm × 15 mm i.d. is closed off at one end with parafilm. The acrylamide solution is prepared, swirled to mix, and quickly pipetted into the gel tube to the desired height, usually 2–5 cm. About 1 cm of water is layered on the top of the gel solution by means of a peristaltic pump. A smooth sharp boundary is formed between the water and gel solution allowing the gel to polymerize with a flat top surface. Both top and bottom surfaces of the gel must be flat, smooth, and orthogonal to the long axis of the gel tube for optimal resolution.

After allowing the gel to polymerize, the water overlaying the gel is removed. After removing the parafilm from the end of the gel tube, the gel tube is inserted into the elution cell (Figure 1c) while both the elution cell and the bottom of the gel tube are submerged in electrophoresis buffer. This facilitates insertion of the tube and lessens the possibility of trapping air bubbles between the gel and the nitex retainer. The bottom of the gel comes to rest against the nitex retaining ring (Figure 1b) which prevents the gel from sliding out of the gel tube during electrophoresis. The insertion of the gel tube into the elution cell forms a flow chamber (Figure 1d) between the end of the gel and the dialysis membrane (Figure 1c) stretched tightly across the bottom of the elution cell, secured by means of a large acrylic washer (Figure 1f) and four screws. Care should be taken to avoid trapping air bubbles between the nitex retaining ring and the bottom of the gel since one small bubble will reduce resolution significantly.

The gel tube is inserted into the top buffer reservoir chamber and is secured by an O-ring in the floor of the chamber. The assemblage is set into the lower buffer reservoir three-fourths full of electrophoresis buffer. The assemblage is tipped to allow bubbles trapped against the dialysis membrane by the acrylic washer to escape. A peristaltic pump pumps 1 × REB through the elution chamber. The electrophoresis buffer is pumped out of the elution chamber rather than into the elution chamber because the action of the peristaltic pump creates air bubbles which can become trapped in the elution chamber if the pump is connected to the inlet tubing of the flow cell. By again tipping the top reservoir assemblage, the buffer carries out air bubbles caught in the elution chamber. Once the bubbles are removed the pump is set to a flow rate of 0.15 ml/min. The top reservoir buffer chamber is filled with 1 × electrophoresis buffer. The RNA sample is layered onto the top of the gel in a sucrose solution by means of a Carlsberg micropipet. The volume of the sample should be large enough to uniformly cover the top of the gel. Samples as large as 0.8 ml have been used without undue loss of resolution. A typical sample preparation would be: 20 μl of 50% sucrose, 4 μl of 25 × REB, and 75 μl of RNA solution; 200 μg of RNA gives optimal resolution but amounts of RNA up to 1 mg can be run with satisfactory resolution. The electrodes of the electrophoresis apparatus are connected to a power supply and a constant current is applied. The electrophoresis is carried out at room temperature. The effluent absorbance is monitored by an LKB ultraviolet absorptiometer detector unit with chart recorder prior to collection.

Detailed specifications of the apparatus are available from the authors. It is also available commercially from Savant Instruments.

Results

a. The Effect of Amperage on RNA Fractionation. The first parameter to be investigated in determining optimal conditions for RNA preparative electrophoresis was the amount of current.

In order to distinguish the effect of amperage on band shape during migration through the gel from its effect on elution from the gel, the amperage which produced flat, sharp bands was determined by electrophoresing *E. coli* and sea urchin RNA at various amperages, 15, 20, 25, 30, and 35 mA, and then staining the RNA *in situ*. The results indicated that 15 mA provided optimal resolution according to these criteria.

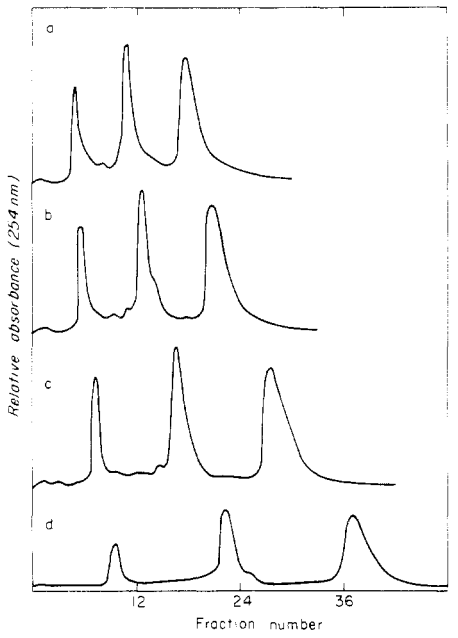

FIGURE 2: Optical density elution profiles of *E. coli* RNA preparatively electrophoresed by different amounts of amperage; 200 μg of *E. coli* RNA was electrophoresed on a 3-cm, 2.5% acrylamide gel with a constant current indicated below, an elution flow rate of 0.1 ml/min, and fraction time of 10 min. The optical density of the effluent was continuously monitored by an ultraviolet absorptiometer detector unit and recorded by a chart recorder. The optical density profiles on the chart were traced directly for this figure. The absorbance plotted on the ordinate is a relative absorbance. The fraction numbers on the abscissa are real: (a) 30 mA, (b) 25 mA, (c) 20 mA, (d) 15 mA.

Higher current caused curvature of the bands indicating differential heating of the gel.

In the next experiment *E. coli* RNA was preparatively electrophoresed at amperages of 15, 20, 25, and 30 mA under conditions of complete electrophoresis and elution. The elution profiles obtained are shown in Figure 2. The lowest amperage, 15 mA, gave the greatest separation between the optical density peaks of the stable RNA with the degree of separation decreasing with increasing amounts of current. As the current and consequently the rate of migration decreased, the peaks were broadened, but this effect did not negate the advantage of the greater peak separation at low current.

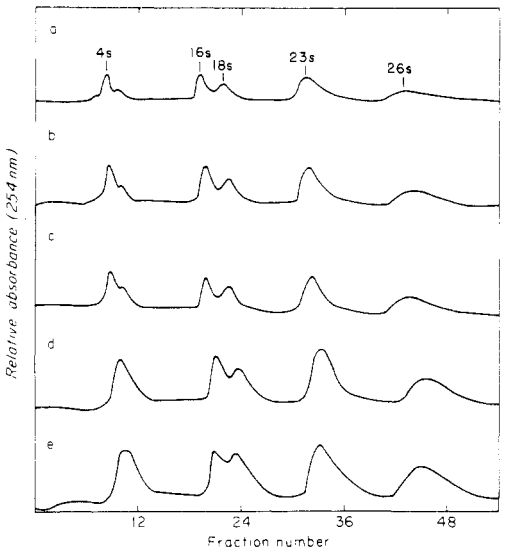

FIGURE 3: The effect of elution rate on the fractionation of *E. coli* RNA and sea urchin RNA by preparative polyacrylamide gel electrophoresis; 90 μg of *E. coli* and 115 μg of sea urchin RNA were electrophoresed on a 2.8-cm, 2.5% acrylamide gel with a constant current of 15 mA, fraction time of 10 min, and an elution flow rate as indicated below. The optical density of the effluent was continuously monitored by an ultraviolet absorptiometer detector unit and recorded by a chart recorder. The optical density profiles on the chart were traced directly for this figure. The absorbance plotted on the ordinate is a relative absorbance. The fraction numbers on the abscissa are real: (a) 0.3 ml/min, (b) 0.2 ml/min, (c) 0.15 ml/min, (d) 0.1 ml/min, (e) 0.05 ml/min.

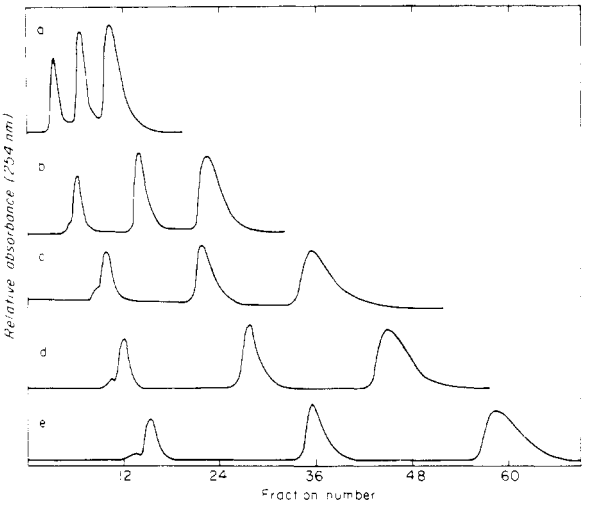

FIGURE 4: Optical density profiles of *E. coli* RNA preparatively electrophoresed on polyacrylamide gels of different lengths; 205 μg of *E. coli* RNA was electrophoresed on a 2.5% acrylamide gel, with a constant current of 15 mA, an elution flow rate of 0.15 ml/min, a fraction time of 10 min, and length of gel as indicated below. The optical density of the effluent was continuously monitored by an ultraviolet absorptiometer detector unit and recorded by a chart recorder. The optical density profiles on the charts were traced directly for this figure. The absorbance plotted on the ordinate is a relative absorbance. The fraction numbers on the abscissa are real: (a) 1-cm gel, (b) 2-cm gel, (c) 3-cm gel, (d) 4-cm gel, (e) 5-cm gel.

b. The Effect of Elution Rate on RNA Fractionation. *E. coli* and sea urchin RNA were preparatively electrophoresed at elution rates of 0.05, 0.1, 0.15, 0.2, and 0.3 ml/min while all other conditions were held constant. The pattern of fractionation was followed by monitoring the optical density of the effluent flowing from the electrophoresis elution chamber (Figure 3). At low elution rates the optical density peaks are somewhat broader and have a tendency to merge into one another as shown by the 16S and 18S peaks eluted at 0.05 ml/min (Figure 3a). At high elution rates the RNA is considerably diluted in the elution chamber. The resolution at intermediate elution rates seems optimal since the peaks eluted at these elution rates are the most symmetrical and the RNA is not diluted unnecessarily.

c. The Effect of Gel Length on RNA Fractionation. In order to study the effect of gel length on the resolution of RNA eluted from the gels, *E. coli* RNA was preparatively electrophoresed on gels 1, 2, 3, 4, and 5 cm in length (Figure 4). Increase in gel length is accompanied by a substantial increase in distance between the peaks. It seems clear that the longer gels produce better resolution of the RNA species but the use of longer gels is also accompanied by a decrease in peak height and a broadening of the optical density peaks due to the added diffusion during the longer electrophoresis runs.

d. The Effect of Acrylamide Concentration on RNA Fractionation. Mixtures of *E. coli,* sea urchin, and mouse tumor RNA were preparatively electrophoresed on gels containing 2.0, 2.2, 2.35, and 2.5% acrylamide. Mouse rRNA was included to provide a molecular weight marker larger than the 26S rRNA component of sea urchin. The fractionation of the RNA was monitored by recording the absorbance of the elution buff-

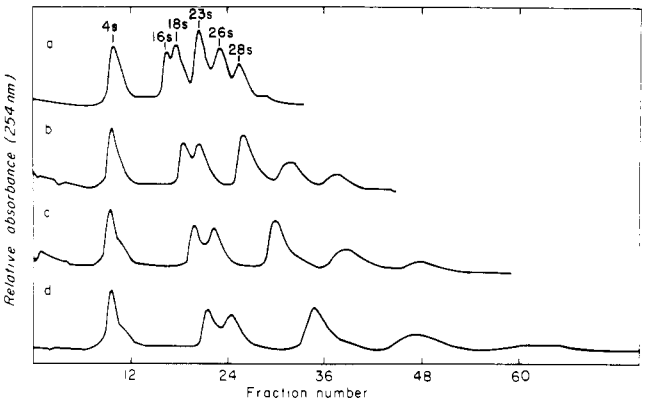

FIGURE 5: Optical density profiles of *E. coli*, sea urchin, and mouse RNA preparatively electrophoresed on polyacrylamide gels of different % acrylamide. Mixtures of 100 μg of *E. coli* RNA, 90 μg of sea urchin RNA, and 78 μg of mouse RNA were electrophoresed on a 3-cm gel, with a constant current of 15 mA, fraction time of 10 min, elution flow rate of 0.15 ml/min, and % acrylamide as indicated below. The optical density of the effluent was continuously monitored by an ultraviolet absorptiometer detector unit and recorded by a chart recorder. The optical density profiles obtained on the charts were traced directly for this figure. The absorbance plotted on the ordinate is a relative absorbance. The fraction numbers on the abscissa are real: (a) 2.0% gel, (b) 2.2% gel, (c) 2.35% gel, (d) 2.5% gel.

er flowing from the elution chamber during electrophoresis (Figure 5). Analyzing these data one observes that the fractionation obtained on the 2.0% gel resolved the rRNAs into distinguishable but overlapping peaks. With increasing acrylamide concentration the peaks become better resolved, particularly the peaks representing the high molecular weight RNA. The greater peak separation produced by the higher per cent gels is also, however, accompanied by a decrease in peak height and an increase in peak width such that in the 2.5% gels some of the peaks of higher molecular weight RNA almost disappear. The decreased peak height of the larger RNA molecules is probably due to their experiencing more difficulty leaving the gel matrix and entering the elution chamber. Because of this reduced exit rate from the gel, the larger RNAs become greatly diluted by the elution buffer and cause the peak profiles to be lowered and broadened. This problem will be further discussed in the next section.

e. Are RNAs Eluted According to Their Molecular Weight? It has been shown by Loening (1969) and others that there is a linear relationship between the mobility of RNA in polyacrylamide gels and the logarithm of its molecular weight. When initial fractionations of RNA by preparative polyacrylamide gel electrophoresis were tested for this property it seemed that the same relationship did not hold for the separation of RNAs by this method. It was therefore of interest to determine whether this deviation from the expected results developed during the migration of the RNA through the gel material or whether the discrepancy was produced during elution of the RNAs from the gel surface.

Mixtures of *E. coli*, sea urchin, and mouse tumor RNA were preparatively electrophoresed on 5-cm gels of 2.0, 2.2, 2.35, and 2.5% acrylamide. The period of electrophoresis was controlled such that the bands of rRNAs remained on the gels in comparable positions. The gels were removed from the gel tubes, stained for RNA and destained. The distance the RNAs had migrated was measured and plotted against the logarithm of the molecular weight of the RNA as shown in Figure 6. This study reveals that the RNAs migrate through the gel material at a rate which is inversely linearly proportional to the logarithm of their molecular weights at all of the acrylamide concentrations tested, as is routinely observed in the smaller diam-

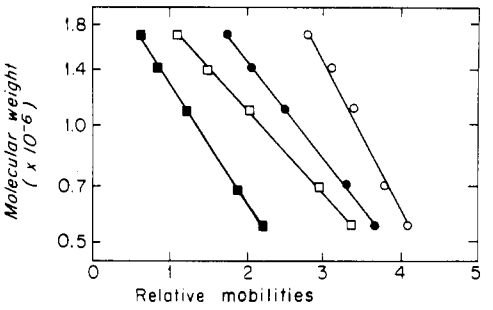

FIGURE 6: Relationship between molecular weight and electrophoretic mobility of RNA electrophoresed on different % polyacrylamide gels in the preparative electrophoresis apparatus. Mixtures of 75 μg of *E. coli* RNA, 150 μg of sea urchin RNA, and 150 μg of mouse tumor RNA were electrophoresed on 5-cm polyacrylamide gels with a constant current of 15 mA and a time period of electrophoresis necessary to electrophorese the rRNAs to comparable positions in the gels. The gels were removed from the gel tubes and stained for 1 hr with a solution of 0.2% Methylene Blue, 0.2 M sodium acetate, and 0.2 M acetic acid. The gels were destained overnight in a large tub of water agitated with a stirring bar. The migration distances of the RNAs (16 S, 18 S, 23 S, 26 S, and 28 S) were measured by eye with a ruler. Since the RNA bands had been electrophoresed to similar positions in the gels for better resolution and comparison, plotting the distance migrated against the log of the molecular weight of the RNAs would have produced overlapping lines. Therefore, the relative mobilities were plotted against the log of the molecular weight of the RNAs in order to generate each curve. The position of each curve along the abscissa is arbitrary. The molecular weight values of *E. coli* rRNAs are those of Stanley and Bock (1965) and for sea urchin and mouse rRNA are those of Loening (1968). The per cent gel used is noted in order from left to right: (□) 2.5%; (■) 2.35%; (●) 2.2%; and (○) 2.0%.

eter analytical gels. This finding suggested that the problem was one of elution of the RNAs rather than the migration of the RNAs in the gel material.

To substantiate the foregoing conclusion the elution profiles of the experiment described in the preceding section and shown in Figure 5 were analyzed to obtain the elution positions of RNAs fractionated by gels of 2.0, 2.2, 2.35, and 2.5% acrylamide. The elution positions of the RNAs were denoted by the fraction in which the RNAs were collected. The fraction numbers thus obtained were plotted against the logarithm of the molecular weight of the RNAs as shown in Figure 7. It is clear that the points plotted in Figure 7 do not fall on straight lines. It appears, therefore, that the deviation from the straight lines shown in Figure 6 develops during elution of the RNA. This deviation from linearity is less pronounced as the per cent acrylamide in the gel decreases. Even though RNA is not fractionated strictly according to the logarithm of its molecular weight by this preparative method, estimations of molecular weight of the RNA separated by this method can still be made if appropriate molecular weight standards are used.

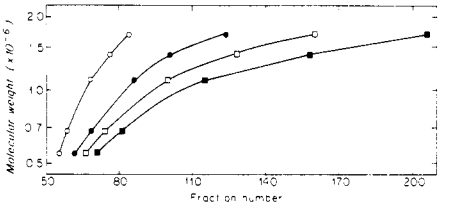

FIGURE 7: Relationship between molecular weight and elution position of RNA preparatively electrophoresed on different % polyacrylamide gels. Elution positions of 16S, 18S, 23S, 26S, and 28S RNA (*E. coli*, sea urchin, and mouse tumor RNA) were determined from elution profiles of Figure 5. The elution positions were denoted by the fraction number in which the RNA was collected. The fraction number was plotted against the log of the molecular weight of the RNA. The per cent gel used is noted in order from left to right: (○) 2.0%; (●) 2.2%; (□) 2.35%; and (■) 2.5%.

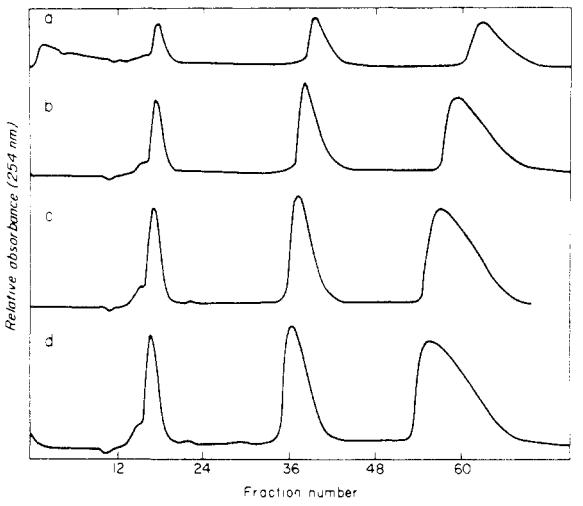

FIGURE 8: The load capacity of the preparative electrophoresis apparatus. Different amounts of *E. coli* RNA as indicated below were electrophoresed on a 5-cm, 2.5% polyacrylamide gel with a constant current of 15 mA, an elution rate of 0.15 ml/min, and a fraction time of 10 min. The same gel was used for all of the fractionations. The optical density of the effluent was continuously monitored by an ultraviolet absorptiometer detector unit and recorded by a chart recorder. The absorbance profiles on the chart were traced directly for this figure. The absorbance plotted on the ordinate is a relative absorbance. The fraction numbers on the abscissa are real: (a) 246 μg, (b) 492 μg, (c) 738 μg, (d) 984 μg.

f. Effect of Load Size on RNA Fractionation. In order to determine the amount of RNA which is fractionated with optimal resolution and also to determine the maximum amount of RNA which can be fractionated with satisfactory results, varying amounts of *E. coli* RNA were electrophoresed on a 5-cm, 2.5% polyacrylamide gel. The results are shown in Figure 8 for 0.25, 0.50, 0.75, and 1.0 mg of RNA. Optimal resolution, as judged by the symmetry and the separation of the peaks in the absorbance profile, is achieved with 0.25 mg of RNA. Lower amounts of RNA were also tested and did not significantly improve the fractionation profile. As the amount of RNA is increased above 0.25 mg there is a significant broadening and tailing of the RNA peaks, although the separation is still adequate with 1 mg of RNA. It also appears in the figure that the peak-to-peak distance decreases as increasing amounts of RNA are electrophoresed. Part, but not all, of this effect is caused by using the same gel for all of the fractionations presented in Figure 8. Repeated use of the same gel results in an unexplained increase in mobility during the subsequent run, but this effect is not large enough to account for the increase in mobility that occurs when large amounts of RNA are electrophoresed. That the effect of rerunning the gel is too small to account for the mobility increase observed in Figure 8 has been verified by electrophoresing the same amount of RNA on the same gel several times. Therefore, we attribute part of the loss of resolution at high RNA concentrations to an enhanced mobility of the RNA which might be caused by an increased local ionic concentration in the RNA band.

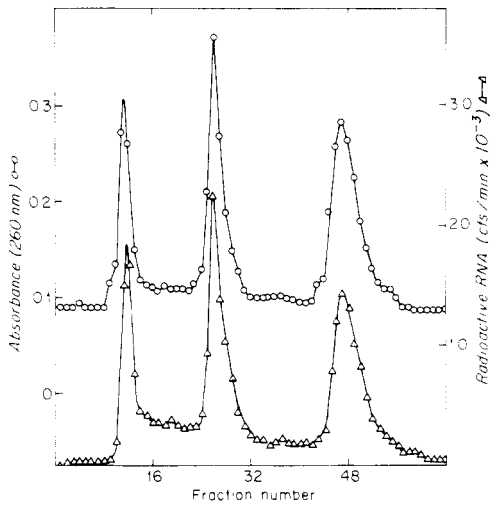

FIGURE 9: Optical density and radioactivity profiles of labeled *E. coli* RNA fractionated by preparative polyacrylamide gel electrophoresis; 100 μg of labeled *E. coli* RNA (prepared from cells grown for 0.2 generation in the presence of [^{3}H]uridine) was electrophoresed on a 2.5-cm, 2.7% polyacrylamide gel with a constant current of 15 mA, an elution flow rate of 0.1 ml/min, and a fraction time of 10 min. The absorbance at 260 nm of each fraction was determined by a Beckman spectrophotometer after collection. The background absorbance was not subtracted from plotted data; 300 μl of the 1-ml fractions were counted in Bray's solution to determine the amount of radioactivity in each fraction. The recoveries of absorbance and radioactivity are shown in Table I: (○) $A_{260\ nm}$; (△) cpm ^{3}H.

TABLE I: Recovery of Optical Density, Radioactive RNA, and Lysozyme mRNA Activity after Fractionation by Preparative Polyacrylamide Gel Electrophoresis.

	Absorbance 260 nm	Radioactive RNA (cpm)	Lysozyme mRNA Activity (cpm)
Total applied to gel	2.48	91,880	99,000
Total recovered from gel	8.10	90,400	153,700
Total background	5.70	2,460	70,000
Net recovered	2.40	88,940	83,700
% recovered	97	97	84

If more than 1 mg of RNA is applied to the gel, there is a significant loss of rRNA that cannot be recovered from the gel. In particular the 23S rRNA is recovered in poor yield when more than 1 mg of RNA is electrophoresed. We attribute this selective loss to aggregation which we assume occurs when the RNA reaches high concentrations as it stacks prior to entering the gel.

g. Recovery, Integrity, and Purity of Fractionated RNA. As discussed in a previous section (e), high molecular weight RNA is retarded in leaving the gel. In order to determine whether this effect also causes a loss of some of the RNA applied to the gel, we measured the recovery of RNA eluted from the gel as determined by three parameters: optical density, radioactivity, and mRNA template activity. Radioactive *E. coli* RNA was layered on a 2.5-cm, 2.7% polyacrylamide gel and preparative electrophoresis was carried out as described previously. The profile of radioactivity and absorbance is shown in Figure 9. As expected from the long labeling time essentially all of the radioactivity is associated with the tRNA and rRNA.

The recovery of absorbance and radioactivity in the eluted fractions was determined. These data, shown in Table I, indicate that 97% of both radioactivity and absorbance are recovered in the fractions eluted from the gel. Thus, even in a gel of high acrylamide concentration (2.7%) virtually all of the RNA is eluted, even though the 23S rRNA is retarded in leaving the gel (Figure 7).

In order to determine whether or not the size of the RNA is altered by the fractionation procedure, the fractions containing the 16S and 23S RNA species from the initial separation were separately pooled and concentrated by ethanol precipitation.

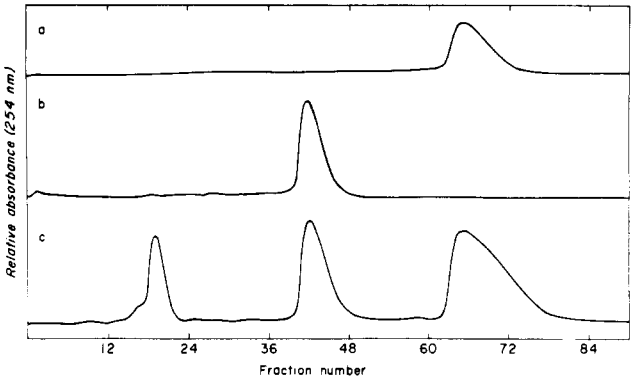

FIGURE 10: Reelectrophoresis of 16S and 23S *E. coli* rRNA; 1 mg of *E. coli* RNA was electrophoresed on a 5-cm, 2.5% polyacrylamide gel with a constant current of 15 mA, an elution rate of 0.15 ml/min, and a fraction time of 10 min. The absorbance of the effluent was continuously monitored by an ultraviolet absorptiometer detector unit and recorded by a chart recorder. The fractions containing the 16S and 23S RNA were pooled separately, made 0.2 M in NaOAc (pH 5.2) and precipitated with three volumes of 95% ethanol. The RNA was collected by centrifugation, washed with 70% EtOH, dried under vacuum at room temperature, and resuspended in 100 μl of H_2O. The total samples were separately reelectrophoresed on the same gel and under the same conditions used for the initial fractionation: (a) rerun 23S RNA, (b) rerun 16S RNA, (c) initial fractionation of *E. coli* RNA.

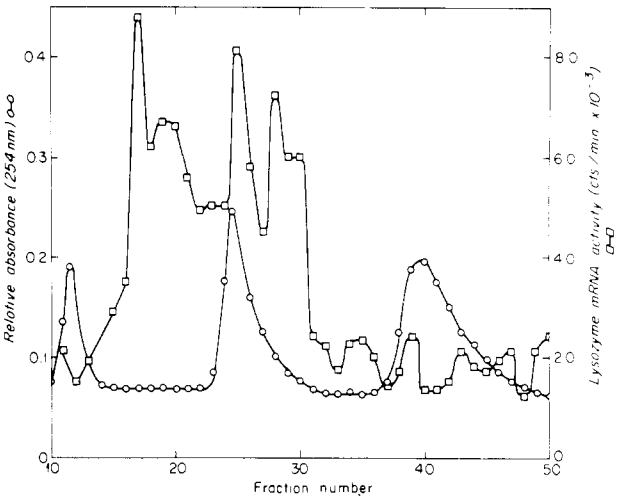

FIGURE 11: Absorbance and T7 lysozyme mRNA activity profiles of RNA fractionated by preparative polyacrylamide gel electrophoresis; 180 μg of RNA extracted from T7L infected *E. coli* B at 14 min after infection was electrophoresed on a 2.8-cm, 2.6% polyacrylamide gel with a constant current of 15 mA, an elution flow rate of 0.1 ml/min, and a fraction time of 10 min. The optical density of the effluent was continuously monitored by an ultraviolet absorptiometer detector unit and recorded by a chart recorder. The absorbance profiles on the chart were transposed to graph paper for this figure. The RNA in each fraction was ethanol precipitated, washed with 70% ethanol, collected by centrifugation, and dried under vacuum at room temperature. The RNA was resuspended in 40 μl of distilled H_2O and used directly for *in vitro* protein synthesis; 25 μl of the 100-μl reaction mix was assayed for lysozyme activity by filter assay (25 μl of reaction mix, 0.5 ml of 0.1 M NH_4OAc, and filter disc incubated together for 1 hr at 37°). The filter was removed and the solution counted in 18 ml of Bray's solution: (○) $A_{260\,nm}$; (□) lysozyme mRNA activity.

The complete samples were reapplied to the gel and again fractionated. As is shown in Figure 10, the RNA species migrate to the positions expected of intact 16S and 23S RNA. There is no evidence for breakage of the RNA.

This experiment also indicates that there is no detectable contamination of 16S RNA with 23S RNA, and *vice versa*. The amount of each species recovered during reelectrophoresis is reduced slightly from the initial fractionation due to losses through collection and precipitation.

Equally as important as total recovery is the ability to recover intact and biologically functional RNA after the preparative separation procedure. Figure 10 shows that rRNA is not detectably degraded during electrophoresis and elution. In order to determine whether biologically active mRNA could be eluted from the gel, we measured the recovery of T7 lysozyme mRNA activity. RNA extracted 14 min after T7 infection and containing lysozyme mRNA activity (Hagen and Young, 1973) was preparatively electrophoresed on a 2.8-cm, 2.6% polyacrylamide gel. The RNA in the eluted fractions was alcohol precipitated after adding tRNA as a carrier. The RNA in each fraction was tested for its ability to stimulate *in vitro* synthesis of lysozyme. The profiles of absorbance and lysozyme mRNA activity are shown in Figure 11. The optical density profile records the usual distribution of 4S, 16S, and 23S rRNA. The lysozyme mRNA activity is distributed in at least three major peaks, ranging in apparent molecular weight from about 0.7×10^6 to less than 0.5×10^6. The observation that lysozyme mRNA activity does not migrate as a single discrete component has been observed repeatedly, although the distribution of mRNA activity among the peaks shown in Figure 11 does vary in different experiments. The possible significance of this observation will be presented in the Discussion.

The recovery of lysozyme mRNA activity was determined by summing the lysozyme mRNA activity measured in all of the fractions and comparing the total so obtained with the amount of mRNA activity in the RNA applied to the gel. This calculation (Table I) reveals that 84% of the mRNA activity is recovered from the gel. The per cent recovery of biologically active mRNA from the gel is more variable than the recovery of absorbance, which is always approximately 100%. The main problem, however, seems to be in precipitating the small quantity of mRNA rather than maintaining the integrity of the mRNA. Another problem in recovering mRNA activity is that some preparations of tRNA added as carrier inhibit the translational system (C. Pachl, personal communication).

Discussion

The isolation of RNA and particularly mRNA is becoming an increasingly important aspect of the study of genetic regulatory mechanisms in both procaryotes and eucaryotes. For some time now the isolation of RNA has relied primarily upon techniques involving ultracentrifugation. In this paper we describe some of the conditions necessary to achieve optimal resolution of RNA species of different molecular weight using a preparative polyacrylamide gel electrophoresis apparatus which is relatively inexpensive to build and easy to use. Because this apparatus has considerable potential for the fractionation of RNA and proteins, a detailed description of the parameters we have investigated which influence the resolution of RNA species is presented. Although not discussed in this paper, the apparatus appears to be suitable for preparative protein fractionation (M. Gardner and R. Cleland, personal communication).

RNA molecules migrate through the preparative polyacrylamide gel just as they do in cylindrical analytical RNA gels, but at the end of the gel they are electrophoresed into a small chamber which is continuously flushed with buffer. Buffer flushed from this chamber can be collected in fractions and subsequently examined for the activities of interest. In comparing the preparative gel apparatus to the more conventional separation technique for RNA molecules, zonal centrifugation in sucrose gradients, several advantages of the gel method are apparent. Perhaps the most important of these is the improved resolution afforded by preparative gel electrophoresis and the

ability to influence this resolution very dramatically by changing any one of several parameters. In particular, simply by making a longer gel in the same apparatus, a considerable increase in resolution is possible (Figure 4).

The criterion we have used to optimize the separation of RNA molecules of different molecular weights is the elution pattern of the rRNA species, usually consisting of a mixture of total *E. coli* and sea urchin (*S. purpuratus*) RNA or *E. coli*, sea urchin, and mouse RNA. This mixture of RNA comprises molecules ranging in molecular weight from 25,000 to 2×10^6. In fact, we have concentrated our attention on the resolution of the rRNA species since these species, ranging in size from 0.5 to 2×10^6, migrate at a rate which is proportional to the logarithm of their molecular weights in the gel system discussed (Figure 6). The separation between the 16S rRNA of *E. coli* and the 18S rRNA of *S. purpuratus* in particular provides a useful estimate of the resolution in the different experiments. As can be seen in Figures 3 and 5 these two RNA species can be almost completely separated preparatively on a relatively short (3 cm) gel.

Despite the fact that RNA migrates in the gel at a rate which is linearly proportional (inversely) to the logarithm of its molecular weight, its rate of elution does not obey the same simple relation. Thus several standards of known molecular weight should be included in the gel if an estimate of the molecular weight of an unknown RNA species is to be obtained.

Our main interest in developing a high-resolution preparative gel technique is to fractionate active mRNA from bacteriophage-infected cells. The data in Table I indicate that it is possible to recover nearly 100% of the mRNA template activity for a specific enzyme, bacteriophage T7 lysozyme, in the fractions eluted from the preparative gel.

The electrophoretic analysis of T7 lysozyme mRNA activity reveals that this mRNA has a wide range of mobilities, appearing as discrete peaks of activity as shown in Figure 11. We have no evidence that the separation of lysozyme template activity into discrete peaks corresponds to lysozyme mRNA molecules of different molecular weight although this seems to be the simplest explanation for the different mobilities. Other possible explanations for the multiple peaks of mRNA activity that are shown in Figure 11 are that they are due to aggregation of the RNA or that they represent different forms of the same molecule. Whatever the correct explanation for the mobility distribution is, we do not know whether the distribution observed is representative of the sizes of the mRNA being translated in the infected cell or if it is due to posttranslational degradation either *in vivo* or *in vitro*. However, Dunn and Studier (1973) have shown that the early T7 mRNAs are produced by endonucleolytic cleavage of a large precursor RNA molecule. Thus, there is a precedent for the processing of T7 bacteriophage mRNA and this might be the correct explanation for our observations. The distribution of mRNA activity that we observe could be due to the processing of one or more large mRNA precursors to produce redundant mRNA molecules, several of which contain the coding sequences for lysozyme.

It is tempting to speculate that the multiple peaks of lysozyme mRNA activity are related to our previous observation of a lag in translation of the lysozyme mRNA *in vivo* (Hagen and Young, 1973). Translation *in vivo* could require processing of a high molecular weight precursor. We are exploring these possibilities further by attempting to use denaturing conditions during preparative electrophoresis to eliminate aggregation (Staynov *et al.*, 1972) and by trying to cleave T7 lysozyme mRNA *in vitro* with *E. coli* RNase III which is thought to process the early T7 mRNA precursor. Other possible explanations for multiple lysozyme messengers include multiple initiation or termination sites for transcription.

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Added in Proof

Since this manuscript was written an article appeared describing the preparative fractionation of RNA on agarose gels using a commercially available apparatus (Weil and Hampel, 1973).

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