# Role of Molecular Conformation in Determining the Electrophoretic Properties of Polynucleotides in Agarose–Acrylamide Composite Gels*

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ABSTRACT: Electrophoresis of a variety of polynucleotides in agarose–acrylamide gels under different conditions of temperature, voltage gradient, and gel concentration revealed that, for molecules whose molecular weights exceeded 0.3–0.4 × $10^6$, single-stranded species behaved differently from double-stranded species. Specifically, such double-stranded species, unlike single-stranded species, showed positive relationships between mobility and temperature, and mobility and voltage gradient, as well as a low retardation coefficient relative to single-stranded species. Some of the limitations of polyacrylamide gel electrophoresis as a means for determining the molecular weight of unknown polynucleotides are illustrated and discussed.

We suggest that measurements of electrophoretic mobilities in these gels reflect relative molecular radii under different conditions rather than molecular weights.

In recent years, polyacrylamide gels and composite agarose–acrylamide gels have been used extensively in fractionating and analyzing RNA isolated from various cells (*e.g.*, Weinberg *et al.*, 1967; Dingman and Peacock, 1968; Loening *et al.*, 1969; Dingman *et al.*, 1969). More recently, interest has been shown in using this technique to define some of the physical properties of the molecules under investigation (*e.g.*, Bishop *et al.*, 1967; Peacock and Dingman, 1968; Loening, 1969).

We believed that a more complete investigation of the role of secondary structure in determining the electrophoretic properties of polynucleotides in these gels would prove useful. To be able to define the chemical nature (whether RNA or DNA), size, and secondary structure (whether single stranded or double stranded, linear or circular) of polynucleotides isolated from biological sources by application of a single technique, when the amount of material available is very small, should prove valuable. This paper describes the results of our investigations to date which are aimed at achieving this goal.

## Methods and Materials

*Gels.* Composite agarose–acrylamide gels containing 0.5% agarose and having a ratio of Bis:[1]total monomer of 0.05 (Peacock and Dingman, 1968) were used in all cases. TEMED (final concentration 0.031%) was used as the catalyst throughout these experiments and all gels were allowed to gel for at least 1 hr at room temperature. A prerun at 200 V (approximately 8.8 V/cm) while the apparatus was being brought to the desired temperature was also routinely performed. The buffer used (Tris–EDTA–borate, pH 8.3) was that described by Peacock and Dingman (1968), and the electrophoretic cell used was that described by Raymond (1962).

*Electrophoresis.* The power supply used was that supplied with the electrophoretic cell by E-C Apparatus Co., Philadelphia, Pa., and its voltage and current output were monitored using a Hewlett–Packard 410C voltmeter. Specific conductances were measured using a Type CDM2e conductivity meter (Radiometer, Copenhagen). Constant temperature in the electrophoretic cell was maintained by use of a thermostatically regulated external circulator. Temperatures within the gels, under conditions simulating those used to analyze polynucleotides, were measured by differential thermometry using thermistor probes connected to a Tele-Thermometer (Yellow Springs Instrument Co., Inc.). The reference probe was placed in the upper buffer reservoir, the temperature of which was measured with a standard thermometer. Measurements of gel temperature at 2.2 and 8.8 V per cm indicated that, within this range, there was no significant effect of voltage gradient on the temperature of the electrophoretic run.

*Measurements of Mobilities.* The electrophoretic mobility, $M$, was defined as $M = (d)(k)/(i)(t)$, where $d$ is the distance migrated in centimeters, $k$ is the specific conductance of the buffer at the temperature of the gel, $i$ is the current density in A/cm$^2$, and $t$ is the time in seconds. The electrophoretic runs were performed at constant voltage. In most cases the current varied by less than 10% during the run and the time-averaged value was used to calculate $M$. Replicate measurements of mobilities (done in different gels of the same composition and run at the same voltage and temperature) gave values that differed by less than 5% in the great majority of cases. The Tris–EDTA–borate buffer used had a specific conductance equivalent to 0.0085 M KCl at 25° and its specific conductance, $k$, varied with temperature as follows: $k = 2.725 \times 10^{-5}(C) + 5.35 \times 10^{-4}$, where $C$ is the temperature in degrees centigrade. Because the actual temperature in the gel was higher than that of the circulating coolant during runs below room temperature, the specific conductance value, $k$, used to calculate the mobility was that measured at the temperature of the gel. It was assumed that $k$ was not altered by the presence of the gel for the dilute gels used in this study. The distance migrated, $d$, was measured on gels stained as described by Dahlberg *et al.* (1969). Gels containing 0.5% agarose swell very little in water (Peacock and Dingman, 1968) and measurements of $d$ were not considered to be significantly affected by changes in

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[1] Abbreviations used are: Bis, *N,N'*-methylenebisacrylamide; TEMED, *N,N,N',N'*-tetramethylenediamine; SDS, sodium dodecyl sulfate.

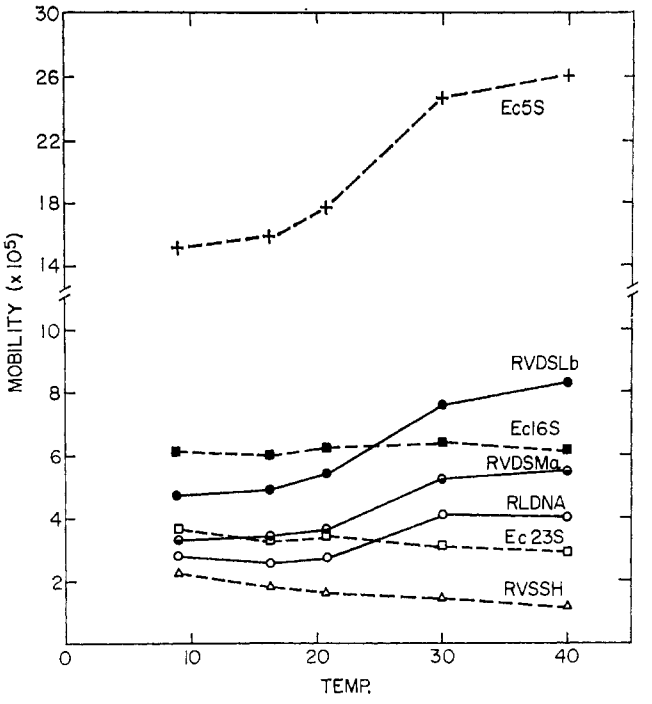


FIGURE 1: Effect of temperature on the electrophoretic mobility of some polynucleotides in agarose–acrylamide gels. In each case the gels were 2.2% with respect to acrylamide and were run at 8.8 V/cm. Dashed lines refer to single-stranded species, solid lines to double-stranded species.

the dimensions of the gel during staining and destaining. Finally, in most cases all of the polynucleotides studied here were simultaneously analyzed side by side in the same gel, so that comparison of their mobilities under identical conditions was facilitated.

*Materials.* Rat liver cytoplasmic RNAs were isolated as described previously (Dingman *et al.*, 1970) and *Escherichia coli* rRNAs were a gift from Dr. A. E. Dahlberg. Rat liver DNA was isolated from rat liver nuclei using preparative isopycnic centrifugation in CsCl (Dingman and Sporn, 1967). Reovirus (a gift from Dr. A. J. Shatkin) was treated briefly at room temperature with 0.5% SDS, then shaken very gently with one volume water-saturated phenol. Following centrifugation at 5° the aqueous layer was retreated with 3.5 volumes of phenol to concentrate the viral RNA (Dingman and Peacock, 1971) and the aqueous phase stored at −80°. T4 phage and λ phage (gifts of Dr. M. Gellert) were treated similarly except that the first aqueous phase was dialyzed for 24 hr against 2 mM $Na_3EDTA$ and the resulting DNA solutions stored at 2°. A wide-bore capillary pipet was used to apply samples of phage DNA to the gels. Single-stranded reovirus RNAs were produced by heating the double-stranded forms to 80° for 3 min in the presence of 50% $Me_2SO$, followed by quenching in ice. Because some RNAs are composed of a number of subspecies separable by gel electrophoresis, to simplify matters the abbreviations shown in Table I are used throughout this paper. Table I also lists the molecular weights that have been assumed for each of the polynucleotides used in this study. Presumed single-stranded species are shown in graphs connected by a dashed line and presumed double-stranded species by a solid line.

## Results

*Effect of Temperature.* The effect of temperature on the electrophoretic mobility of polynucleotides in agarose–acrylamide gels was investigated over a temperature range of from 9 to 40°, both in 1.7 and 2.2% acrylamide gels. Figure 1 illustrates the results obtained with representative species of polynucleotides using 2.2% acrylamide gels and a voltage gradient of 8.8 V/cm. The mobilities of double-stranded molecules and low molecular weight single-stranded species increase in going from 16 to 30° while the mobilities of high molecular weight single-stranded species decline or remain constant. Similar results were obtained using 1.7% gels.

This phenomenon is further detailed in Table II where the "apparent" molecular weights of several single-stranded species of RNA (relative to *E. coli* 16S and *E. coli* 5S RNAs) have been determined at five different temperatures. The apparent molecular weight is defined here as that obtained by plotting the logarithm of the molecular weight of two reference species of RNA against their mobility and then deriving the apparent molecular weights of other species of polynucleotides by assuming a linear relationship between the log of the molecular weight and the electrophoretic mobility (Bishop *et al.*, 1967; Peacock and Dingman, 1968; Loening, 1969). The results in Table II show that the apparent molecular weight arrived at by this technique is sensitive to temperature, especially for high molecular weight species electrophoresed at elevated temperatures.

TABLE I: Abbreviations and Molecular Weights for the Polynucleotides Referred to in this Report.

| Polynucleotide | Abbrev[a] | Assumed Mol Wt ($\times 10^{-6}$) | Ref |
|---|---|---|---|
| T4 phage DNA | T4 | 130 | *b* |
| λ phage DNA | λ | 33 | *c* |
| Rat liver DNA | RLDNA | | |
| Rat liver 28S RNA | RL28S | 1.5 | *d* |
| Rat liver 18S RNA | RL18S | 0.69 | *d* |
| *E. coli* 23S RNA | Ec23S | 1.1 | *e* |
| *E. coli* 16S RNA | Ec16S | 0.56 | *e* |
| *E. coli* 5S RNA | Ec5S | 0.036 | *f* |
| *E. coli* precursor 16S RNA | Ecp16Sa | | |
| Reovirus, double-stranded, heavy RNA | RVDSHa | 2.3 | *g* |
| Reovirus, double-stranded, medium RNA | RVDSMa | 1.3 | *g* |
| Reovirus, double-stranded, light RNA | RVDSLb | 0.8 | *g* |
| Reovirus, single-stranded, heavy RNA | RVSSH | 1.15 | *h* |
| Reovirus, single-stranded, medium RNA | RVSSM | 0.65 | *h* |
| Reovirus, single-stranded, light RNA | RVSSL | 0.4 | *h* |

[a] Small letters, at the end of an abbreviation, are used to designate subspecies in the order of increasing mobility. [b] Rubenstein *et al.* (1961). [c] MacHattie and Thomas (1964). [d] This paper (see values obtained at 21°, Table II). [e] Kurland (1960). [f] Rosset *et al.* (1964). [g] Bellamy *et al.* (1967). [h] These values were assumed from the weights of the double-stranded forms of reovirus RNA.

TABLE II: Apparent Molecular Weights ($\times 10^{-6}$) of Some Single-Stranded RNAs as Determined at Different Temperatures by Agarose–Acrylamide Gel Electrophoresis.[a]

| | RNA | | | | | | |
|---|---|---|---|---|---|---|---|
| Temp (°C) | RVSSH | RVSSM[b] | RVSSL[b] | RL28S | RL18S | Ec23S | Ecp16Sa |
| 9 | 1.80 | 1.02 | 0.52 | 1.94 | 0.74 | 1.16 | 0.67 |
| 16 | 1.80 | 1.12 | 0.60 | 1.73 | 0.72 | 1.16 | 0.67 |
| 21 | 1.65 | 1.15 | 0.61 | 1.50 | 0.69 | 1.10 | 0.67 |
| 30 | 1.20 | 0.97 | 0.66 | 1.03 | 0.65 | 0.93 | 0.67 |
| 40 | 1.10 | 0.91 | 0.66 | 0.97 | 0.64 | 0.87 | 0.59 |

[a] The gels in each case were 2.2% in acrylamide and were run at 8.8 V/cm; the reference RNAs were *E. coli* 16S and *E. coli* 5 S with assumed molecular weights of $0.56 \times 10^6$ and $0.036 \times 10^6$, respectively (see Table I). [b] Because RVSSM is composed of two subspecies and RVSSL is composed of four subspecies, the mobilities of the subspecies were averaged prior to determining their apparent molecular weights.

*Effect of Voltage Gradient.* It was observed that high molecular weight double-stranded species showed a pronounced increase in mobility when the applied voltage was increased. This effect was observed at all gel concentrations tested (*i.e.*, in 1.7, 2.2, 2.7, 3.0, and 5.0% acrylamide gels). Some of these results are given in Table III. It will be noted that the effect is much less pronounced for low molecular weight double-stranded molecules such as RVDSLb. Also of interest was the tendency for high molecular weight single-stranded molecules to show a decrease in mobility with increasing voltage gradients (*e.g.*, RVSSH, Table III). The mobility of other single-stranded polynucleotides was not affected by a change in voltage gradient.

TABLE III: Effect of Voltage Gradient on the Mobilities of Some Single- and Double-Stranded Polynucleotides in Agarose–Acrylamide Gels at 21°.

| Polynucleotide | Gel Concn | Voltage Gradient (V/cm) | Mobility ($cm^2 \times V^{-1} \times sec^{-1} \times 10^5$) |
|---|---|---|---|
| T4 | 2.7 | 2.2 | 1.15 |
| | 2.7 | 8.8 | 2.50 |
| λ | 2.7 | 2.2 | 1.16 |
| | 2.7 | 8.8 | 2.68 |
| RLDNA | 1.7 | 2.2 | 1.76 |
| | 1.7 | 8.8 | 4.19 |
| | 2.2 | 2.2 | 1.33 |
| | 2.2 | 8.8 | 2.71 |
| | 2.7 | 2.2 | 1.13 |
| | 2.7 | 8.8 | 2.51 |
| RVDSHa | 1.7 | 2.2 | 3.09 |
| | 1.7 | 8.8 | 4.04 |
| | 2.2 | 2.2 | 2.24 |
| | 2.2 | 8.8 | 2.68 |
| | 2.7 | 2.2 | 2.04 |
| | 2.7 | 8.8 | 2.50 |
| RVDSLb | 1.7 | 2.2 | 7.40 |
| | 1.7 | 8.8 | 7.57 |
| RVSSH | 1.7 | 2.2 | 5.77 |
| | 1.7 | 8.8 | 4.04 |
| RVSSM | 1.7 | 2.2 | 8.29 |
| | 1.7 | 8.8 | 7.78 |
| Ec23S | 1.7 | 2.2 | 7.60 |
| | 1.7 | 8.8 | 7.32 |
| Ec16S | 1.7 | 2.2 | 10.4 |
| | 1.7 | 8.8 | 10.2 |
| Ec5S | 1.7 | 2.2 | 20.7 |
| | 1.7 | 8.8 | 20.2 |

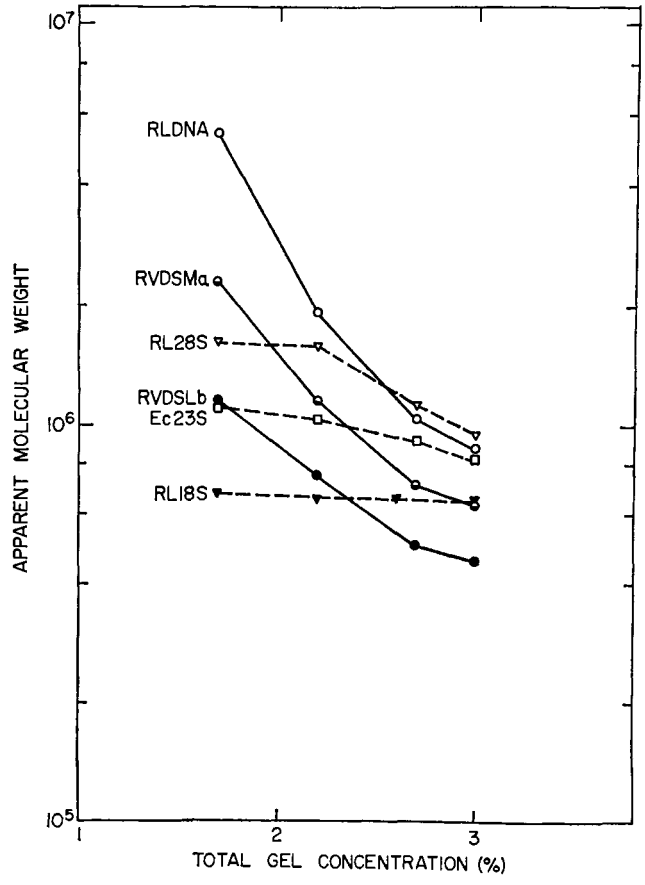


FIGURE 2: Effect of gel concentration on the apparent molecular weight of some polynucleotides in agarose–acrylamide gels (see text for definition of apparent molecular weight). These gels were all run at 21° and 2.2 V/cm. Dashed lines refer to single-stranded species, solid lines to double-stranded species.

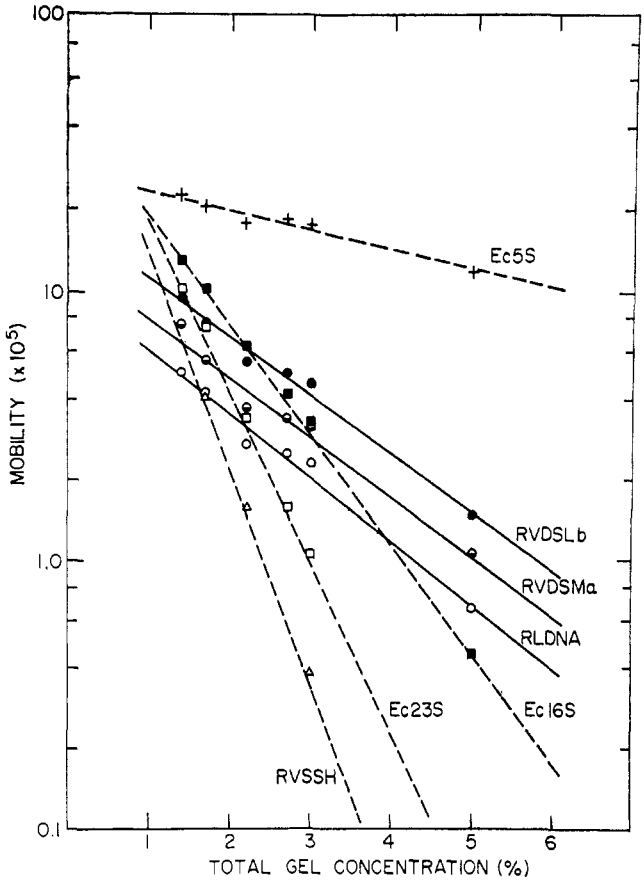


FIGURE 3: Effect of gel concentration on the electrophoretic mobility of some polynucleotides in agarose–acrylamide gels. These gels were all run at 21° and 8.8 V/cm. Dashed lines refer to single-stranded species and solid lines to double-stranded species; these lines were calculated to be the best linear fit by least-squares regression analysis.

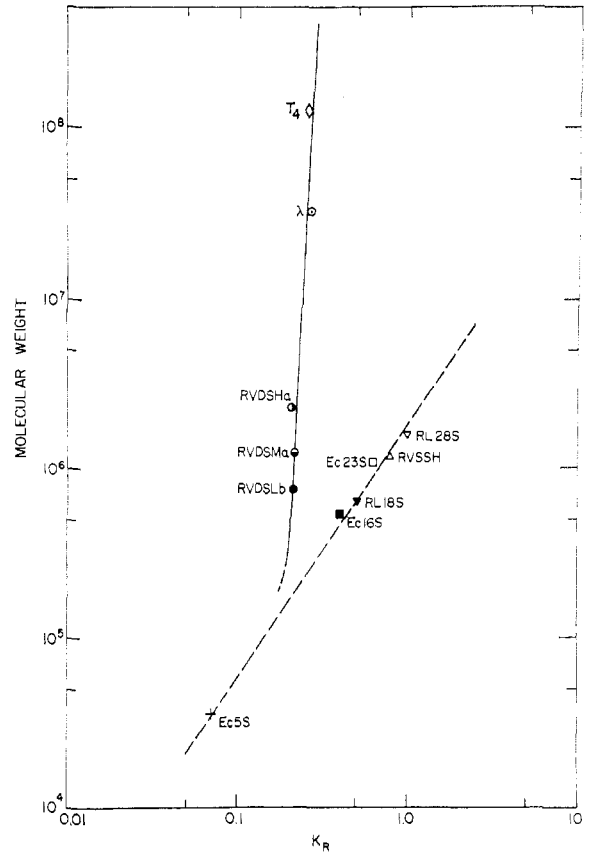


FIGURE 4: The relationship of $K_R$ (the retardation coefficient, see text) to molecular weight for some single-stranded (dashed line) and double-stranded (solid line) polynucleotides.

*Effect of Gel Concentration.* Single- and double-stranded molecules were also distinguishable by their behavior in gels of different acrylamide concentrations. This effect can be illustrated in two ways. In Figure 2 the apparent molecular weight of three species of double-stranded and three species of single-stranded molecules relative to Ec16S and Ec5S is shown at four different acrylamide concentrations. In agreement with previous reports (Peacock and Dingman, 1968; Loening, 1969), the apparent molecular weight of rRNA species was independent of gel concentration except for the higher molecular weight species at the higher gel concentrations, where there is a diminution in the apparent molecular weight because plots of log molecular weight *vs.* mobility are not strictly linear under these conditions (Peacock and Dingman, 1968). However, the most striking feature of Figure 2 is the continued, rapid increase in the apparent molecular weight of the double-stranded species with decreasing gel concentration.

Another manner in which these data can be presented is shown in Figure 3. Here the following relationship has been assumed: $\log M = \log M_0 - K_R T$ (Ferguson, 1964; Rodbard and Chrambach, 1970), where $K_R$ is the retardation coefficient and $T$ is the gel concentration (with respect to acrylamide). Because of the presence of agarose in these gels, the value for $M_0$ (the mobility at zero gel concentration) may not be particularly useful for these studies. Figure 3 illustrates the results obtained at 21° and 8.8 V/cm; very similar results were obtained at 21° and 2.2 V/cm. The important finding here is that the slope, $K_R$, appears to be a function of the molecular weight for single-stranded species, whereas $K_R$ varies very little with molecular weight for double-stranded species. The relationship between $K_R$ and molecular weight is shown graphically in Figure 4. From this figure it is apparent that one could not, by this technique, distinguish single-stranded from double-stranded polynucleotides by their behavior in agarose–acrylamide gels when their molecular weights were less than about $0.3 \times 10^6$.

## Discussion

The purpose of these investigations is to establish the electrophoretic behavior of polynucleotides of different primary and secondary structure in agarose–acrylamide gels. The experiments presented here suggest that if the molecular weight of a polynucleotide is greater than $0.3–0.4 \times 10^6$, a decision as to whether it is single- or double-stranded may be made on the basis of its electrophoretic behavior in gels. The distinguishing features of double-stranded species are (1) a positive relation between mobility and temperature, (2) a positive relation between mobility and voltage gradient, and (3) a retardation coefficient lower than single-stranded species of similar molecular size. Intuitively, these results might be explained in part by the assumption that highly double-stranded molecules migrate end-on in the gels, thus presenting the gel with a profile of small cross-sectional area. Further,

as the voltage gradient is increased, the shear stress on the molecules increases and the double-stranded molecules would be the ones capable of orienting in a manner which minimizes the frictional resistance to their motion (and thus inceases their mobility), analogous to the phenomenon of flow birefringence seen with highly asymmetric molecules. On the other hand, the single-stranded species (even with a significant amount of double strandedness secondary to intramolecular interactions) can be assumed to present rather globular profiles to the gel, the cross-sectional areas of which are not significantly altered by various orientations. The relatively low melting temperatures recorded for single-stranded species suggests that these molecules occupy larger volumes as the temperature increases. For this reason, single-stranded species might show decreasing electrophoretic mobilities with increasing temperature. However, because the viscosity of the gel medium might also be expected to decrease with increasing temperature, only relatively high molecular weight, single-stranded molecules might show a loss in mobility with increasing temperature, as was found in the experiments reported here.

With respect to the use of polyacrylamide gel electrophoresis to estimate the molecular weight of polynucleotides (Bishop *et al.*, 1967; Peacock and Dingman, 1968; Loening, 1969), the data presented here indicate that such measurements are affected by temperature, voltage gradient, and gel concentration, and, most strikingly, by molecular conformation. Thus, molecular weight values arrived at by this technique are subject to ambiguity. Perhaps it would be more accurate to view such data as reflecting the relative molecular radii of the molecules under examination (Rodbard and Chrambach, 1970).

## Acknowledgments

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## Added in Proof

We have recently found that single-stranded polydeoxyribonucleotides also show an increase in mobility with an increase in voltage gradient, but only at the higher gel concentrations.

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