Electrophoresis of Duplex Deoxyribonucleic Acid in Multiple-Concentration Agarose Gels: Fractionation of Molecules with Molecular Weights between 2×10^6 and 110×10^6†

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ABSTRACT: By use of multiple-concentration agarose gels, the distance migrated by linear, duplex DNA during agarose gel electrophoresis has been measured as a function of agarose concentration (A) and molecular weight of the DNA (M_r) for molecules with an M_r between 1.88×10^6 and 110×10^6. Conditions for obtaining a linear plot of log M_r as a function of distance migrated (D) have been found for DNA molecules with an M_r between 5.47×10^6 and 110×10^6; improved conditions for fractionating such molecules are described. The ratio of the D of a DNA with an M_r of 26.5×10^6 to the D of a DNA with an M_r of 77×10^6 was plotted as a function of A and electrical field (E). By use of A values between 0.075 and 0.70% and a constant E, this plot had a single maximum. The maximal value of the above ratio was a decreasing function of E, and the A at this maximal value was also a decreasing function of E.

Agarose gel electrophoresis is used for the fractionation of linear, duplex DNA by molecular weight (M_r). For DNA molecules with M_r values below 10×10^6 linear plots of log M_r as a function of distance migrated (D) are obtained in gels with an agarose concentration (A) of ≥0.4%; however, as M_r increases above 10×10^6 these plots become progressively nonlinear and the distances separating different DNAs decrease (Helling et al., 1974; McDonnell et al., 1977). Improved separations of duplex DNA molecules with M_r values above 10×10^6 have been obtained by lowering the A used to 0.1–0.3% and by reducing the electrical field (E) to 0.02–0.5 V/cm (Hayward, 1974; Kaplan & Wilcox, 1977; McDonnell et al., 1977; Fangman, 1978). However, a systematic study of all of the parameters affecting the fractionation of duplex DNA molecules with M_r values above 10×10^6 has not been made; previously reported procedures appear to be inadequate for fractionating DNAs with an M_r of $>10 \times 10^6$ during studies of viral assembly and during studies of eukaryotic chromosomes. In the present communication additional data concerning the dependence of such fractionations on E, A, and the concentration of sample are presented. Improved conditions for obtaining separations of DNA molecules with M_r greater than 10×10^6 are described. For reduction of errors from differences in E and temperature among gels of different A values, determinations of D as a function of M_r and A were made in multiple-concentration agarose gels (Serwer, 1980); the values of A used were between 0.075 and 0.70%.

Materials and Methods

Bacteriophage and Bacterial Strains. The source, growth, and purification of bacteriophages T7, T5 and T4 and their bacterial hosts have been previously described (Serwer et al., 1978).

Buffers and Reagents. Tris–EDTA buffer and Tris–Mg buffer have previously been described (Serwer et al., 1978). The electrophoresis buffer was 0.05 M sodium phosphate, pH 7.4, and 0.001 M EDTA. Ethidium bromide was purchased from Calbiochem (B grade) and was filtered through a Millipore filter (0.45 μm). Restriction enzyme *Mbo*I was purchased from New England Biolabs, Beverly, MA.

Release of DNA from Bacteriophages. Dilutions of intact bacteriophages were made in Tris–Mg buffer. For electrophoresis of mature bacteriophage DNA, the DNA was released from appropriately diluted bacteriophages as previously described (Serwer et al., 1978). T7 DNA for digestion with *Mbo*I was isolated by phenol extraction as previously described (Serwer, 1974).

Digestion of T7 DNA with MboI. DNA obtained by phenol extraction from bacteriophage T7 was digested to completion by *Mbo*I in a buffer containing 2.0 mM NaCl, 4.8 mM KCl, 8.0 mM Tris-HCl, pH 7.4, 8.0 mM $MgCl_2$, 0.80 mM dithiothreitol, 0.20 mM EDTA, and 160 μg/cm³ bovine serum albumin. Five of the seven *Mbo*I fragments were visible on the gels used here: (A) M_r 14.2×10^6; (B) M_r 5.47×10^6; (C) M_r 2.53×10^6; (D) M_r 2.06×10^6; (E) M_r 1.88×10^6 (McDonnell et al., 1977).

Agarose Gel Electrophoresis. Electrophoresis in gels of several different agarose concentrations was performed by embedding several agarose gels (running gels; Miles HGT[P] agarose was used) within a 1.5% agarose frame (Seakem ME agarose) and layering identical samples at the origin of each running gel prior to electrophoresis in an apparatus for horizontal slab gels. This procedure has previously been described in more detail (Serwer, 1980). The gel consisting of both the running gel and the surrounding frame is referred to as a multigel. Electrophoresis in agarose slab gels of a single agarose concentration (Miles HGT[P] agarose) was performed as previously described (Serwer, 1980). All electrophoresis was performed in electrophoresis buffer at room temperature (25 ± 3 °C) by using the E values indicated. Fluctuations in E were ±8%.

For preparation of samples for electrophoresis, a mixture of bacteriophage DNAs in Tris–EDTA buffer was added to 2.6 volumes of a buffer containing 2% sucrose, 0.005 M sodium phosphate, pH 7.4, 0.001 M EDTA, and 400 μg of bromophenol blue per cm³. Slow pipeting with a 50-μL micropipet (1 mm in diameter) was used to avoid shear-induced breakage of the DNA.

In preliminary experiments the concentration of phosphate used in the electrophoresis buffer was varied. Use of 0.03 M phosphate or less in the electrophoresis buffer resulted in zones of DNA that were more variable in sharpness and less sharp than the zones obtained with the higher phosphate concen-

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tration used here. Although attempts to use 0.05% agarose in running gels were sometimes successful during preliminary studies (intact viral particles were the samples; P. Serwer, unpublished data), samples leaked into the running gel during roughly 50% of the attempts to use 0.05% gels. The use of gels with an A below 0.075% was not attempted in the present study.

Photography of Gels. After electrophoresis gels were stained by slowly drawing off the electrophoresis buffer (a turkey baster was usually used) and filling the electrophoresis apparatus with 1 $\mu g/cm^3$ ethidium bromide in 0.001 M sodium EDTA, pH 7.4. After 3 h the solution of ethidium bromide was carefully removed and the multigel was illuminated from the top with a short wavelength ultraviolet transilluminator (Ultraviolet Products, Inc.); photographs of the orange ethidium bromide fluorescence were taken as previously described (Serwer et al., 1978). Because the background fluorescence decreases with A, dodging was used during the printing of multigels to keep the backgrounds in the running gels roughly the same.

Theoretical Considerations. During electrophoresis in the absence of a solid support it has been shown that the electrophoretic mobilities of linear, duplex DNA molecules with M_r values between 0.2×10^6 and 110×10^6 are independent of M_r (Olivera et al., 1964). Therefore, for DNA molecules with M_r values in this range it is expected that a plot of D as a function of A extrapolated to an A of 0 (D_o) will produce a D_o which is independent of M_r. Semilogarithmic plots of this type are linear for proteins in polyacrylamide gels (Ferguson, 1964; Rodbard & Chrambach, 1971) and for DNA with an M_r below 10×10^6 in agarose gels (Johnson & Grossman, 1977). By use of agarose multigels, it was shown (1) that a semilogarithmic plot of D as a function of A is linear at the lower values of A for molecules with M_r below a critical value (M_c) that depends on E and (2) that D_o is independent of M_r for $M_r \leq M_c$ (see Results). Therefore, for measurement of the effects of A on D, D/D_o was plotted semilogarithmically as a function of A; D_o was determined by prior extrapolation using DNA molecules with an $M_r \leq M_c$.

For quantitation of the separations of DNAs, M_r is plotted semilogarithmically as a function of D/D_o [see McDonnell et al. (1977)]. The negative of the slope of this plot, K_m, is a measure of the distance between two DNA molecules after electrophoresis for a fixed time; the lower the K_m, the further apart will be the two DNA molecules. An advantage of using D/D_o rather than D on the abscissa is that K_m values from gels run for different times can be compared.

Mature T7 DNA and larger DNA molecules do not diffuse enough to perceptibly broaden DNA bands during staining in ethidium bromide for times up to 6 days (data not shown). Thus, for times of electrophoresis within this range diffusion-induced broadening of DNA bands does not affect resolution for molecules with $M_r \geq 26.5 \times 10^6$. Therefore, resolution is determined by (a) factors, excluding diffusion, that affect the width of bands of DNA (narrowing of bands during entrance of DNA into the gel and electrophoresis-induced broadening of bands) and (b) the ratio of distances migrated by two DNA molecules differing in M_r. Raising the ratio of the D of the smaller DNA to the D of the larger DNA optimizes the resolution obtainable if the time of electrophoresis is not restricted (lowering K_m optimizes resolution if the time is held constant).

Results

(A) Distance Migrated as a Function of Time and the Concentration of DNA. Before measuring the dependence of D on A and E, it is necessary to (1) use DNA concentrations low enough so that D is not a function of the concentration of DNA and (2) determine whether D is a linear function of time. For measurement of the dependence of D on the concentration of DNA, electrophoretic profiles of mixtures of the mature DNAs from bacteriophages T7 (M_r of DNA = 26.5×10^6; McDonnell et al., 1977), T5 (M_r of DNA = 77×10^6; Shaw et al., 1979), and T4 (M_r of DNA = 110×10^6; Freifelder, 1970) were obtained by using 1200, 600, 375, 150, 76, and 38 ng/cm^2 of each DNA (Figure 1a–f, respectively; the cross-sectional area of the sample was 0.16 cm^2). It was found that D values obtained with 150 ng/(cm^2 band) (Figure 1, slot d) did not differ measurably from D values measured at lower concentrations of DNA (Figure 1, slots e and f). As the concentration of DNA increased above 150 ng/(cm^2 band) the bands of DNA broadened and decreased in mobility. All experiments presented below using mature DNAs from bacteriophages T7, T5, and T4 were performed with concentrations of DNA below 150 ng/(cm^2 band) to avoid dependence of measurements on the concentration of DNA.

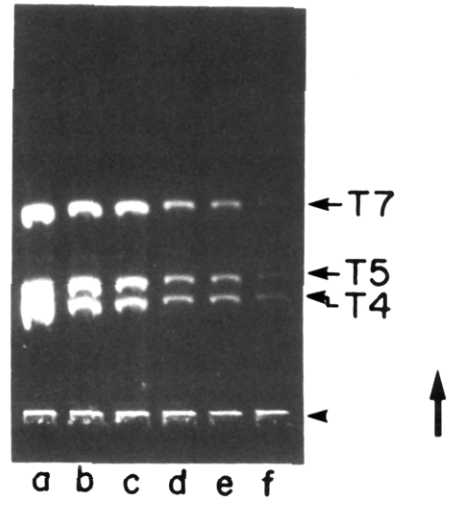

FIGURE 1: Electrophoresis as a function of the concentration of DNA. Mixtures of DNA extracted from bacteriophages T7, T5, and T4 that had (a) 192, (b) 96, (c) 60, (d) 24, (e) 12, and (f) 6.1 ng of each DNA in 40 μL were made and subjected to electrophoresis at 0.34 V/cm for 20.1 h in a 0.15% agarose slab gel as described under Materials and Methods [the concentrations of DNA in ng/(cm^2 band) were 1200, 600, 375, 150, 76, and 38, respectively]. The arrowhead indicates the origin of electrophoresis; the arrow indicates the direction of electrophoresis.

For determination of the distance migrated as a function of time, the sample used for Figure 1e was subjected to electrophoresis in a 0.15% agarose slab gel; additional, identical samples were placed in adjacent wells of the gel at 6 times after the start of electrophoresis at 0.34 V/cm. At the conclusion of the electrophoresis the D of each DNA was determined as a function of time. This relationship was found to be linear for all DNAs (the shortest time was 8.3 h; the longest time was 40.0 h).

(B) D as a Function of A and M_r. For determination of D as a function of A and M_r, an *Mbo*I digest of mature T7 DNA was mixed with the mature DNAs from bacteriophages T7, T5, and T4; this mixture was subjected to electrophoresis at 0.34 V/cm in an agarose multigel with running gels of 0.075, 0.10, 0.15, 0.20, 0.30, 0.50, and 0.70% agarose for 20.0 h (Figure 2). In Figure 3a D/D_o is plotted semilogarithmically as a function of A for duplex DNA molecules with molecular weights of 110×10^6, 77×10^6, 26.5×10^6, 14.5×10^6, 5.47×10^6, and 1.88×10^6. This plot was linear for the DNA with an M_r of 1.88×10^6 and became progressively more concave

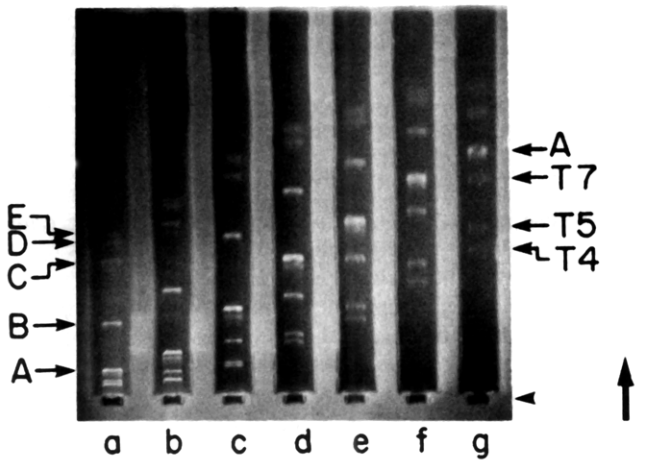

FIGURE 2: An agarose multigel of a mixture of DNAs. A mixture containing the following duplex DNA molecules in 50 μL was layered at the origin of each running gel of a multigel and was subsequently subjected to electrophoresis at 0.34 V/cm for 20.0 h as described under Materials and Methods: mature DNA of bacteriophage T4, 9.0 ng; mature DNA of bacteriophage T5, 9.0 ng; mature DNA of bacteriophage T7, 9.0 ng; an *Mbo*I completion digest of mature T7 DNA, 80 ng (five fragments visible, A–E; McDonnell et al., 1977). The concentrations of agarose in the running gels are (a) 0.70, (b) 0.50, (c) 0.30, (d) 0.20, (e) 0.15, (f) 0.10, and (g) 0.075%. The arrowhead indicates the origin of electrophoresis; the arrow indicates the direction of electrophoresis.

as M_r increased. However, as A approached 0 these plots became linear for values of $M_r \leq 26.5 \times 10^6$. The value of D_o for M_r values $\leq 26.5 \times 10^6$ (M_c) was independent of M_r, in agreement with the data of Olivera et al. (1964). Presumably, the plots for M_r values of $> 26.5 \times 10^6$ in Figure 3a would become linear at lower A values than 0.075%.

Values of M_r are plotted semilogarithmically as a function of D/D_o in Figure 3b. A linear plot was obtained with A = 0.10% for values of M_r between 5.47×10^6 and 110×10^6; K_m in the linear region of the 0.10% gel was 3.0. As A increased above or decreased below 0.10%, the plots became progressively more nonlinear. Values of K_m for molecules with $M_r \geq 26.5 \times 10^6$ progressively increased with $A \geq 0.20\%$. For the 0.10% running gel the difference in the D values of molecules with M_r 5.47×10^6 and 110×10^6 was 4.40 cm.

(C) Effect of E on DNAs with M_r between 26.5×10^6 and 110×10^6. For optimization of the resolving power of gels for DNA molecules with M_r between 26.5×10^6 and 110×10^6, the effects of E on the relative D values of DNA molecules from bacteriophages T4, T5, and T7 were measured in gels with A values between 0.075 and 0.70%. Electrophoresis of these three DNAs was performed in multigels using E values of 0.17, 0.34, and 0.70 V/cm. The D of T7 DNA [D(T7)] and the D of T5 DNA [D(T5)] were measured in each running gel and D(T7)/D(T5) was plotted as a function of A for each of the above E values. At each value of E a single peak was found in this plot (Figure 4). The height of this peak decreased as a function of E from 2.03 at 0.17 V/cm to 1.50 at 0.70 V/cm. The value of A at which the maximum value of D(T7)/D(T5) occurred (A_{max}) also decreased as a function of E from 0.20 to 0.30% with E = 0.17 V/cm to 0.15% with E = 0.70 V/cm. The peaks in Figure 4 for E values of 0.17 and 0.34 V/cm are skewed; the peak is steeper for A values less than A_{max} than it is for A values greater than A_{max}. At 0.17 and 0.34 V/cm the values of D(T7)/D(T5) dropped so rapidly with A less than A_{max} that values of this ratio became less at these lower voltages than they were at 0.70 V/cm.

The data obtained with E = 0.17 V/cm from the experiment in Figure 4 were also plotted as in Figure 3a for T4, T5, and

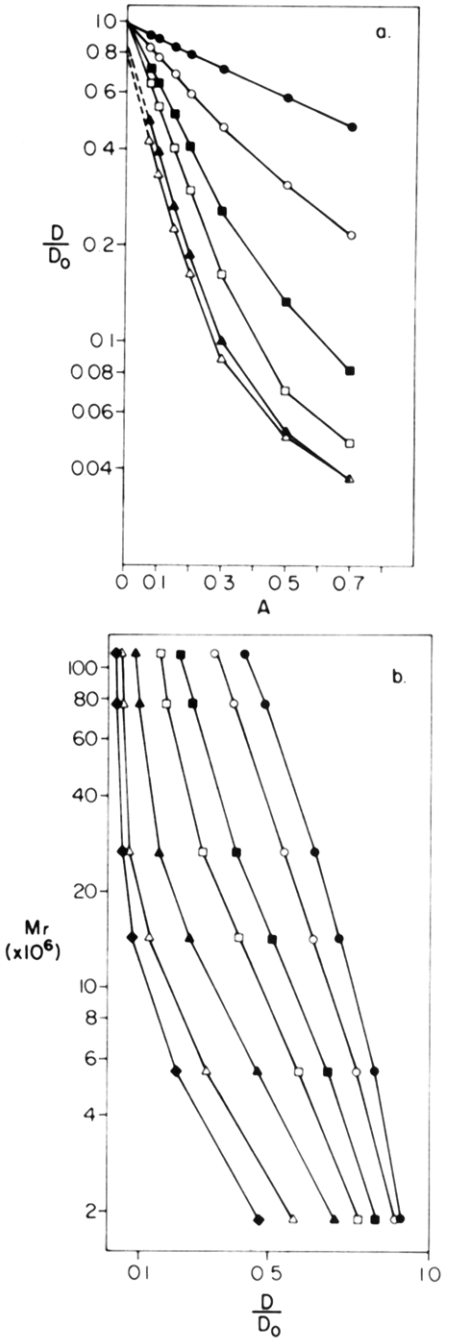

FIGURE 3: D as a function of A and M_r. (a) Values of D/D_o are plotted semilogarithmically as a function of A for different M_r values by using data obtained from the gel in Figure 2. (●) M_r 1.88×10^6; (○) M_r 5.47×10^6; (■) M_r 14.2×10^6; (□) M_r 26.5×10^6; (▲) M_r 77×10^6; (△) M_r 110×10^6. (b) Values of log M_r are plotted as a function of D/D_o for different values of A by using data obtained from the gel in Figure 2. (●) A = 0.075%; (○) A = 0.10%; (■) A = 0.15%; (□) A = 0.20%; (▲) A = 0.30%; (△) A = 0.50%; (♦) A = 0.70%.

T7 DNAs. It was found that the plots for all three DNAs were less curved than those at the higher E in Figure 3a; all three curves became linear as A approached 0, and D_o was the same for all three DNAs within experimental error. Plots of the data in Figure 4 were also made as in Figure 3b. It was found that the plots for E = 0.17 V/cm and A = 0.075, 0.10, and 0.15% were linear. Plots for increasing values of A became progressively nonlinear (data not shown).

Of the conditions thus far explored, the above data suggest that optimization of the resolving power of gels for DNA molecules with M_r values close to the M_r of T7 DNA will be obtained with E = 0.17 V/cm and A = 0.20–0.30%. T7 DNA

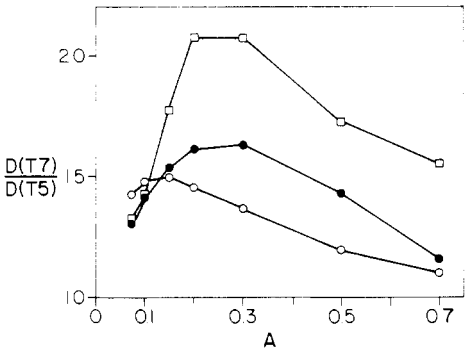

FIGURE 4: Electrophoresis as a function of E and A. A 50-μL mixture containing 9.0 ng each of the mature DNAs from bacteriophages T4, T5, and T7 was layered at the origin of each gel of each of three multigels. The multigels were subjected to electrophoresis at 0.17, 0.34, and 0.70 V/cm for 40.0, 20.0, and 10.0 h, respectively. The D(T7)/D(T5) ratio was calculated and plotted as a function of A at each value of E used. (□) E = 0.17 V/cm; (●) E = 0.34 V/cm; (○) E = 0.70 V/cm.

was subjected to electrophoresis with DNA from deletion mutants of T7 in 0.25% gels at 0.17 V/cm to test the resolving power of such gels. A mutant DNA 1.6% less massive than T7 DNA (the DNA of C63, a 1.6% deletion was used; Simon & Studier, 1973) was resolved from T7 DNA after 220 h of electrophoresis (D = 10.1 cm); this appeared to be the limit of resolution for this value of D (data not shown). Electrophoresis for longer times should give a roughly proportional improvement in resolution.

Discussion

By use of concentrations of DNA below 150 ng of DNA per cm^2 band, conditions for the fractionation by agarose gel electrophoresis of linear, duplex DNA molecules with M_r between 1.88 × 10^6 and 110 × 10^6 have been explored. An overnight electrophoresis is desirable for convenience and rapid determination of results. By use of E = 0.34 V/cm and A = 0.10%, a linear semilogarithmic plot of M_r as a function of D/D_0 for M_r between 5.47 × 10^6 and 110 × 10^6 was achieved; in an overnight (20 h) electrophoresis the DNA with an M_r of 5.47 × 10^6 was separated from the DNA with an M_r of 110 × 10^6 by 4.4 cm. By use of similar conditions, concatemers attached to a rapidly sedimenting, replicating form of bacteriophage T7 DNA (Serwer, 1974) are currently being analyzed.

For the purpose of obtaining better resolution of DNA molecules with M_r between 26.5 × 10^6 and 110 × 10^6 than that obtained above, E is lowered and A is raised (Results, section C). At E = 0.17 V/cm and A = 0.20–0.30% the highest value of D(T7)/D(T5) was achieved. However, the time required to obtain comparable separations is roughly 3 times as great in 0.20% gels at 0.17 V/cm as it is in 0.10% gels at 0.34 V/cm. Because bands formed by T7 and larger DNAs do not become broadened by diffusion for times up to 6 days, such an increase in the time of an electrophoresis will not decrease resolution. Thus, the inconvenience of the longer times is the only barrier to their use.

In a previous study (Fangman, 1978) the highest ratio of the D of linear, duplex DNA from bacteriophage λ [D(λ); M_r of λ DNA = 31 × 10^6] to D(T4) was 1.74, achieved with A = 0.10% and E = 0.02 V/cm; the calculated time for a 1-cm separation was 123 h. By use of a D(λ) determined from the plot for A = 0.15% in Figure 3b, the predicted D(λ)/D(T4) for A = 0.15% and E = 0.34 V/cm is a comparable 1.69, but the time needed to achieve a 1.0-cm separation is only 12.7 h. By use of the data in Figure 4, D(λ)/D(T4) can be made as high as 2.3 (A = 0.20% and E = 0.17 V/cm; time for a 1-cm separation = 37.6 h).

The ratio of the distance sedimented by T5 DNA to the distance sedimented by T7 DNA during zone sedimentation in sucrose gradients is 1.42 (Serwer et al., 1978), a poorer separation than that obtainable by agarose gel electrophoresis. The concentration of DNA at which band spreading becomes a problem during velocity sedimentation is 100–300 ng of DNA per cm^2 band [calculated from the data in Serwer et al. (1978)], not significantly different from the concentration of DNA at which band spreading becomes a problem during agarose gel electrophroesis (Results, section A).

If DNA is treated as a sphere, a linear semilogarithmic plot of D/D_0 as a function of A is predicted theoretically (Rodbard & Chrambach, 1970); by use of $A \leq 0.9\%$, linearity of such plots has been observed for spherical capsids of bacteriophage T7 (Serwer, 1980). Therefore, the nonlinearity of plots in Figure 3a indicates that during agarose gel electrophoresis the outline of the DNA is either deformed from the spherical shape it presumably had before electrophoresis or changed in radius. The extent of the alteration apparently decreased as M_r decreased (Results, section A) and as E decreased (Results, section C). Deformation of DNA during agarose gel electrophoresis has previously been proposed (Fangman, 1978).

The inverse dependence reported here of E on the optimal A for fractionating duplex DNA may also occur during electrophoresis of DNA with higher values of M_r than used in the present study. Thus, the possibility of using electrophoresis in agarose gels to fractionate intact DNA molecules from eukaryotic chromosomes is not as remote as would otherwise be thought.

Acknowledgments

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