

Sieving by Agarose Gels and its Use During Pulsed-Field Electrophoresis

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Introduction

The use of gel electrophoresis to fractionate double-stranded DNA by length is progressively thwarted by a loss of resolution that occurs as the DNA length approaches 0.05–0.1 megabase pairs (Mb). This phenomenon is caused by elongation of the DNA random coil and end-first reptation of the elongated coil ('reptation' refers to the snake-like motion of an extended molecule through a gel mesh; reviewed by Stellwagen, 1987; Cantor, Smith and Mathew, 1988; Olson, 1989a; Serwer, 1989). Gel electrophoresis by use of an electrical field that varies in either direction or magnitude (pulsed-field gel, or PFG, electrophoresis) overcomes this limitation and can be used to improve separation by length for linear, double-stranded DNA as long as 3–6 megabase pairs. PFG electrophoresis of 0.1–6 Mb double-stranded DNA has been used to:

1. Fractionate whole chromosomal DNAs of the lower eukaryotes (Carle and Olson, 1985; Gemmill *et al.*, 1987; Smith and Cantor, 1987; Dawkins, 1989; Olson, 1989a);
2. Assist in directed cloning during the mapping of chromosomal DNAs (Collins, 1986; Michiels, Burmeister and Lehrach, 1987); and
3. Construct low-resolution restriction endonuclease maps (Shaw, 1986; Smith *et al.*, 1987; Weissman, 1987; Anand, Villasante and Tyler-Smith, 1989; Gardiner and Patterson, 1989; Gessler and Bruns, 1989).

In addition, PFG electrophoresis is useful for fractionation of shorter (0.001–0.05 Mb) double-stranded DNAs (Birren, Simon and Lai, 1990), single-stranded DNA (Lai, Davi and Hood, 1989), and even micron-sized spheres (Griess and Serwer, 1990).

Abbreviations: E , electrical field; Mb, megabase pairs; μ , electrophoretic mobility; μ_0 , μ in the absence of a gel; μ'_0 , μ extrapolated to an agarose percentage of zero; P_E , radius of the effective pore of a gel; PFG, pulsed-field gel; ψ , angle between two directions of the electrical field during PFG electrophoresis; R , radius of a sphere.

Although the effectiveness of pulsed fields has been observed during use of gels other than agarose, the majority of applications of PFG electrophoresis has been for agarose gel electrophoresis. Thus, the first topic of this review will be the structure of agarose gels and its relation to the sieving of comparatively simple particles (spheres, rods). Subsequently, the topics of discussion will be: modes of PFG electrophoresis, proposed mechanism for the effects observed, DNA standards for length and conformation and the apparatus used.

The structure of agarose gels

Agarose is the least electrically charged component of agar, a mixture of related polysaccharides obtained from several genera of red seaweed. Although derivatized to a variable extent with sulfate, pyruvate, glucuronate and methoxyl groups, the polysaccharide backbone of agarose is a linear alternating copolymer of 3,6-anhydro- α -L-galactose and β -D-galactose (reviewed by Araki, 1959; Percival and McDowell, 1967; Serwer, 1983; FMC Corporation, 1988). The molecular weight of an average, single polysaccharide chain has been found to be 120 000 (Hickson and Polson, 1968). Studies of the dependence of molecular weight on the source and purification procedure have, apparently, not been made.

Gelation of agarose requires aggregation of polysaccharide chains and is accompanied by a change in optical rotation (Dea, McKinnon and Rees, 1972; Norton *et al.*, 1986). When the agarose chain is cleaved at the position of 3,6 anhydrides that have been opened by derivatization, the changes in optical rotation occur without gelation. The following findings for this cleaved agarose indicate that an agarose dimer initially forms: (1) second-order kinetics for the change in optical rotation; and (2) light scattering consistent with a doubling in mass. Subsequently, a slower formation of higher multimers was observed (Norton *et al.*, 1986). Galactomannans can be used to gel these aggregates (Dea, McKinnon and Rees, 1972). That the dimer is a double helix of agarose is consistent with X-ray diffraction patterns obtained from dried agarose films (Arnott *et al.*, 1974). Other X-ray patterns of dried agarose films are consistent with stacked single helices (Foord and Atkins, 1989). Whatever the details at this level of resolution, the results of light scattering (Öbrink, 1968; Norton *et al.*, 1986) indicate that multiple agarose chains aggregate to form a thicker fiber (suprafiber) during gelation. Confirmation of this conclusion has been obtained from observation of the thickness of fibers in electron micrographs of thin sections of agarose gels (Amsterdam, Er-el and Shaltiel, 1975; Attwood, Nelmes and Sellen, 1988; Griess and Serwer, 1989). Fortunately, change in size during preparation for thin sectioning does not occur for either 6% agarose beads used for molecular sieve chromatography (Amsterdam, Er-el and Shaltiel, 1975) or 0.4–2.5% agarose gels used for electrophoresis (P. Serwer, M.M. Miller and G.A. Griess, unpublished observations). At higher resolution, *Figure 1* reveals the substructure of a negatively stained agarose suprafiber that was unintentionally broken from an agarose gel during elution of bacteriophage P22 empty

capsids (a P22 capsid, 31 nm in radius, is indicated by arrow 1 in *Figure 1*). This fiber consists of roughly parallel aggregates of thinner fibers; the thinnest fibers have become separated at a frayed, presumably broken, end (arrow 2 in *Figure 1*). The fibers at this frayed end have diameters of 5–15 nm, consistent with that of either a single or a double agarose chain (Arnott *et al.*, 1974). The suprafiber of *Figure 1* consists of two bundles of thinner fibers joined to form a branch (arrow 3). Presumably, such branches are necessary for gelation.

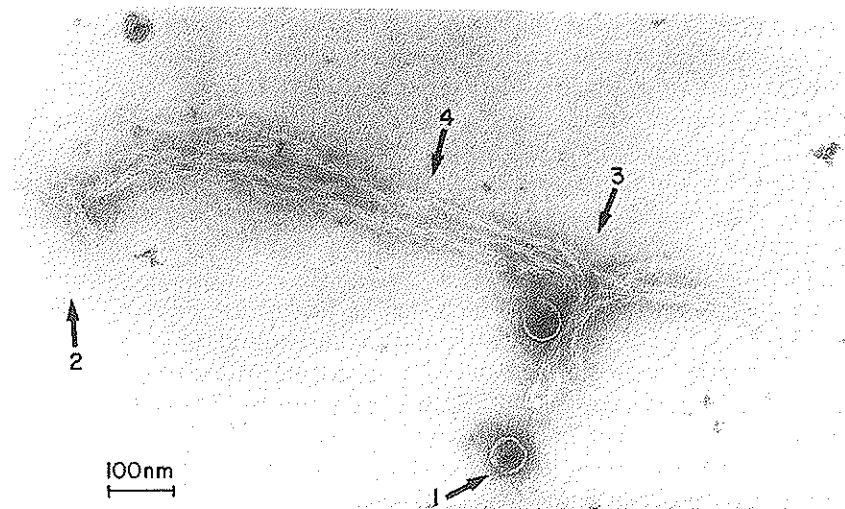


Figure 1. A branched agarose suprafiber. After fractionation of empty bacteriophage P22 capsids by use of agarose gel electrophoresis (*see Serwer, 1987a*), the capsids were eluted from the agarose gel directly to the surface of a carbon support film and prepared for electron microscopy by negative staining with uranyl acetate (Griess *et al.*, 1990). In addition to the P22 capsids, agarose fibers broken from the gel were transferred to the support film and negatively stained.

When observed in either thin sections (Attwood, Nelmes and Sellen, 1988; Griess and Serwer, 1989) or freeze-etched specimens (Waki, Harvey and Bellamy, 1982), the distribution of suprafibers is non-random. For the most dilute gel that could be thin-sectioned, 0.4%, micron-sized pores appeared to be surrounded by an agarose network of a more concentrated gel (Griess and Serwer, 1989). An explanation for this order in the arrangement of suprafibers is a pre-gelation phase separation that has been observed for agarose, both pre- and post-gelation, by use of light scattering (Pines and Prins, 1973; San Biagio *et al.*, 1986).

In contrast to conventionally cross-linked polyacrylamide gels, the fibers of agarose gels are sufficiently immobile so that no effect of their mobility on the diffusion of proteins is observed (Sellen, 1986). In addition, at any given gel concentration, the pores of agarose gels are much larger than the pores of

conventionally cross-linked polyacrylamide gels (Righetti, Brost and Snyder, 1981; Serwer, 1983). These observations are both explained by the presence of suprafibers and higher order structure in agarose, but not to the same extent (or, possibly, at all) in typical cross-linked polyacrylamide gels. Other characteristics explained by the presence of suprafibers and higher order structure are the comparatively great strength of agarose gels (gels as dilute as 0.03% can be used for electrophoresis; Serwer, Moreno and Griess, 1988) and the incomplete return to full size that occurs when a dried agarose is rehydrated. In contrast, cross-linked polyacrylamide gels do return to full size when rehydrated.

Incomplete rehydration of agarose gels helps to explain a property useful for in-gel detection procedures: the retention of DNA by an agarose gel that has been dried and rehydrated. By drying and rehydrating an agarose gel, DNA-DNA hybridization can be performed in-gel, for the detection of complementary nucleotide sequences (Mather, 1988). In-gel hybridization minimizes variability in results that can occur during pre-hybridization transfer. After agarose gel electrophoresis, pre-hybridization transfer (Southern, 1975) is usually used before hybridization-based detection of DNA. For in-gel hybridization, the following problems with the initial procedures have been solved: (1) the removal of liquid-phase water during drying of the gel, (2) curling of the gel during drying, and (3) disintegration of the gel during washing (Son, Watson and Serwer, 1990). Unusual difficulties in the transfer of DNA after performing PFG electrophoresis in the field inversion mode (this mode is described in a subsequent section) have been reported (van Deventer and von Hoff, 1990). In-gel hybridization is a useful alternative when transfer procedures cause difficulties.

Sieving during gel electrophoresis: basic observations

During electrophoresis in the absence of a gel, the electrophoretic mobility, μ ($\mu =$ the velocity of a particle, divided by the electrical field, E) is, in the absence of effects of field-induced polarization (Fixman and Jagannathan, 1981), a function primarily of the average electrical charge per unit surface area of the particle (Shaw, 1969). Because the charge density of double-stranded DNA is uniform, μ in the absence of a gel (μ_0) should be independent of the extended length of the DNA. The data indicate that this is the case for double-stranded DNAs shorter than 0.17 Mb (Olivera, Baine and Davidson, 1964; more recent data is reviewed in Serwer, 1989). This study has not yet been performed for longer DNAs.

In the following sections, the assumption will be made that, during agarose gel electrophoresis both μ_0 and μ extrapolated to an agarose percentage of zero (μ'_0) do not vary with the length of the DNA. Then, μ is the product of μ'_0 and a factor that describes the retardation of the DNA caused by either steric or hydrodynamic effects of the gel (to be collectively called sieving; *see also* Serwer, 1983; Stellwagen, 1987; Tietz, 1987). During gel electrophoresis, all separations of double-stranded DNA involve sieving. Therefore, in order to understand and optimize the use of PFG electrophoresis, attention must be

turned toward the sieving that occurs during agarose gel electrophoresis. The value of μ'_0 is μ_0 added to a term that quantifies the electric field-induced flow of buffer through the gel (electro-osmosis; reviewed in Serwer, 1983). Electro-osmosis is caused by residual negatively charged groups on the agarose (Cook, 1981), varies with the preparation of agarose and will not be further analysed here.

Structure-based interpretation of sieving

The structural complexity of agarose gels poses the problem of how to develop a structure-based description of sieving, by use of the minimum number of variables. For practical purposes, such a description ought to help rapidly determine conditions for any desired separation.

RADIUS OF THE EFFECTIVE PORE

For spherical particles that have a radius (R) of 200 nm or less, success has been achieved in the use of one variable for structure-based, quantitative description of sieving for both underivatized and mono hydroxyethylated preparation of agarose. The variable is the radius of the effective pore (P_E). As a function of agarose concentration, P_E can be defined in at least three different ways:

1. the largest sphere that can enter a gel (Righetti, Brost and Snyder, 1981; Griess *et al.*, 1989);
2. the radius of the cylinder that would cause appropriate hydrodynamic effects to produce the observed gel-induced retardation of a sphere small enough to migrate; and
3. one-half the length of the longest, rigid rod that can achieve random orientation during gel electrophoresis—random orientation is revealed by the shape of a μ v. agarose percentage plot.

For an underivatized agarose, definitions (1) and (2) produce the same value of P_E (Griess *et al.*, 1989). For the smaller variants of the rod-shaped bacteriophage fd, definition (3) is in agreement with definitions (1) and (2). Disagreement was observed for longer variants of bacteriophage fd; the proposed reason for this disagreement is the effect of flexibility that must become increasingly significant as a rod becomes longer (Griess *et al.*, 1990). Hydrodynamics-based values of P_E [i.e. definition (2), above] are independent of the radius of the particle used to determine P_E (Griess *et al.*, 1989). Thus, P_E values based on hydrodynamics provide an accurate description of the sieving of at least one type of agarose gel. The relationship used for the hydrodynamics-based P_E values was:

$$\mu/\mu'_0 = 1 - 2.104 (R/P_E) + 2.09 (R/P_E)^3 - 0.95 (R/P_E)^5 \quad \text{Eq. (1)}$$

(Bacon, 1936; Renkin, 1954; Cannell and Rondelez, 1980; Griess *et al.*, 1989).

The self-consistency, accuracy and range of use of equation (1) do not necessarily establish the theory used to derive equation (1). A relationship that is practically indistinguishable from equation (1) has been derived by assuming that sieving is an effect of steric exclusion from pores (Ferry, 1936; Griess *et al.*, 1989). Values of P_E in agreement with those of Griess *et al.* (1989) and the shape of μ v. agarose percentage plots have both been derived from sterics (Schnitzer, 1988). Other relationships based on sterics have been used (Tietz and Chrumbach, 1987).

Comparison of values of sieving-based P_E with those determined by the use of electron microscopy (Attwood, Nelmes and Sellen, 1988) reveals agreement for 4% agarose (Griess *et al.*, 1989). However, for the gels compared, the agarose preparations and the conditions of gelation differed. Dependence of gel structure and sieving on the concentration of buffer present during gelation has been observed previously (Waki, Harvey and Bellamy, 1982; Peats, Nochumson and Kirkpatrick, 1986). For underivatized agarose, an increase in the value of P_E by approximately a factor of two was observed when the concentration of sodium phosphate (pH 7.4), present during gelation, was increased from 0.0 to 0.1–0.2 M (G.A. Griess and P. Serwer, unpublished observations). Dependence of turbidity, optical rotation and gelling temperature (studies of sieving were not made) on the type of anion present has also been found (Piculell and Nilsson, 1989). In addition, a significant increase in P_E is observed when the electro-osmosis of the agarose decreases (Griess *et al.*, 1989). Thus, when comparing the sieving of any macromolecule (spheres, rods or random coils), either both the gel and conditions of gelation should be the same or comparison should be made as a function of P_E (not as a function of gel concentration).

HETEROGENEITY OF RADII OF PORES

For a sphere with a radius of either 520 nm or (presumably) greater, the following observations indicate that a single variable (i.e. P_E) is not sufficient to describe sieving. When electrophoresis is conducted at 2 V cm^{-1} through a 0.1% agarose gel, spherical particles, observed by use of phase-contrast light microscopy, initially migrate through the gel. However, with a half-life of 20–60 s, the spheres abruptly undergo a cessation of all motion. Arrested spheres do not move again until E is reduced to 0, after which Brownian motion resumes within 1–2 s (Griess and Serwer, 1990). The interpretation of this study is that, in agreement with electron microscopy, the radius of pores varies and the spheres are sterically trapped in the smaller pores. Presumably, the Brownian motion that prevents this trapping for the smaller spheres is insufficient for the larger spheres. Thus, to describe the sieving of spheres ≥ 500 nm in radius, heterogeneity of pore radius must be considered, in addition to P_E .

The arrest described in the previous paragraph is reversed both microscopically and macroscopically by periodically either reducing E to zero or inverting the direction of E (Griess and Serwer, 1990). The latter is a procedure of PFG electrophoresis used to improve fractionation of DNA.

These results indicate that inverting fields during gel electrophoresis should also be a method for fractionating whole (folded) chromosomes. During electrophoresis in the absence of a gel, chromosomes are stable, but are not separated from each other (Bier *et al.*, 1989). Micro-organisms should be and, in the case of *Escherichia coli*, have been (Serwer, Moreno and Griess, 1988) also separable by agarose gel electrophoresis.

THREADING OF DNA

During agarose gel electrophoresis, an elevated E -induced arrest of 0.001–0.05 Mb open circular, but not linear, DNA (Levene and Zimm, 1987; Serwer and Hayes, 1987) indicates that DNA can be threaded by projections from the matrix of an agarose gel. This arrest is prevented by periodically either reducing E to zero (Serwer and Hayes, 1987) or inverting E (Levene and Zimm, 1987). Although the structural component that threads open circular DNA has not been identified for agarose gels, during field inversion the effects of threading increase as the concentration of agarose decreases (Serwer and Hayes, 1989b). Remarkably, for some conditions, μ v. agarose percentage plots become inverted, i.e. mobility decreases as agarose concentration decreases. Thus, in addition to P_E and a variable that describes heterogeneity of the radius of pores, at least one variable that quantifies threading is needed to produce a complete, quantitative description of the sieving of DNA during agarose gel electrophoresis.

The disadvantages of complications introduced by the need for additional variables are accompanied by advantages. That is, more types of separation can be made. For example, after a first electrophoresis that includes pulsing, open circular DNA molecules present among linear molecules can be identified by a second, orthogonally oriented, invariant E electrophoresis (Serwer and Hayes, 1987; Louie and Serwer, 1989). This procedure has also been used to identify a prokaryotic chromosomal DNA that, atypically, is linear (Ferdows and Barbour, 1989).

As the length of linear DNA increases toward 1 Mb, partial and then total arrest of linear DNA is also observed during invariant field electrophoresis (Ferdows and Barbour, 1989; Turmel *et al.*, 1990). The minimum length of the DNA arrested decreases as E increases (Turmel *et al.*, 1990), as was also found for open circular DNA (Levene and Zimm, 1987; Serwer and Hayes, 1989b). Turmel *et al.* (1990) have included among possible explanations: threading, entry into culs-de-sac (i.e. the event that causes trapping of the larger spheres) and competing bulges in the random coils. The trapping of DNA appears to be the primary limitation to PFG electrophoresis and will be further discussed in subsequent sections.

Types (modes) of PFG electrophoresis

INITIAL MODE

The type of field variation used during PFG electrophoresis will be called a

mode. The mode of PFG electrophoresis first discovered (initial mode) consists of electrophoresis in one direction periodically interrupted by electrophoresis of equal duration in a second direction. The discovery of the initial mode was made by Schwartz and Cantor (1984). Additional experiments revealed that the improved separations obtained required that the two

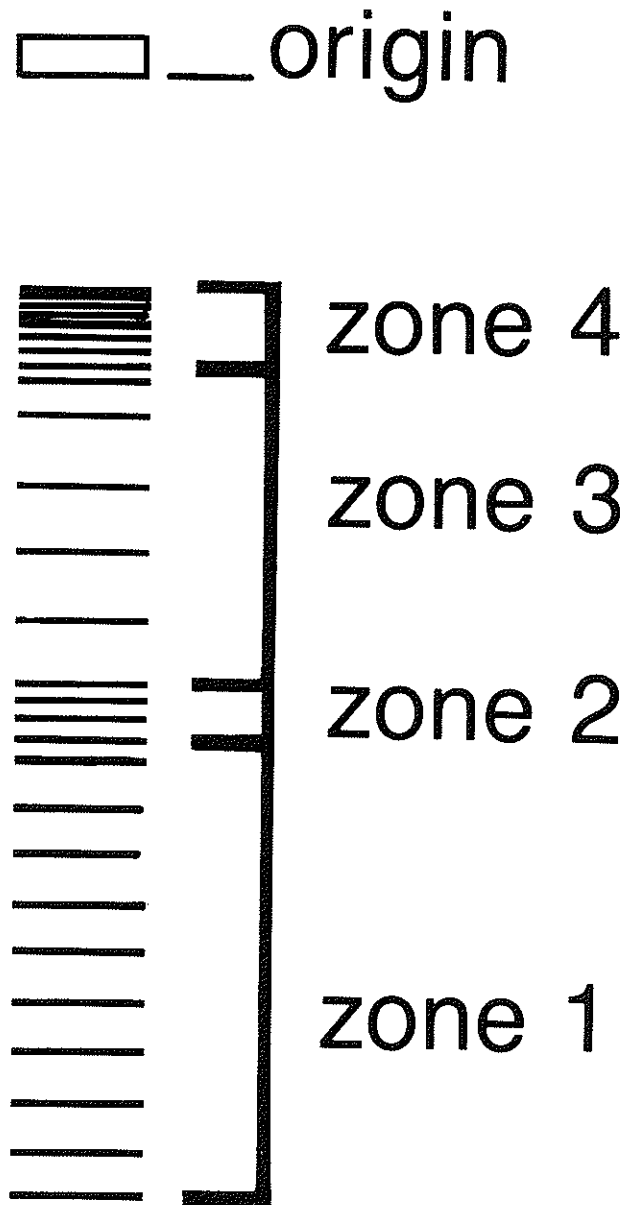


Figure 2. Dispersion of a DNA ladder during PFG electrophoresis, initial mode. Assuming that the rungs of a DNA ladder have equal intensity, the distribution of rungs is shown. (Reproduced from Serwer, 1988.)

directions of the electrical field be separated by an angle (ψ) that was between $\pi/2$ and π radians (Chu, Vollrath and Davis, 1986; Serwer, 1987b; Southern *et al.*, 1987; Birren *et al.*, 1988; Cantor, Gaal and Smith, 1988).

For the initial mode of PFG electrophoresis, the distance migrated v . length relationship usually has the pattern indicated in *Figure 2* for a collection of DNAs that differ in length by a constant amount (called a ladder; for details, see p. 334) (Southern *et al.*, 1987; Vollrath and Davis, 1987; Birren *et al.*, 1988; Mathew, Smith and Cantor, 1988a). A region of equal spacing between bands (zone 1) is followed by a region of lesser (zone 2) and then greater (zone 3) spacing. For the longest DNAs, resolution is lost (zone 4). As the time of uninterrupted electrophoresis (to be called the pulse time) increases, the spacing in zone 1 decreases and longer DNAs move out of zone 4 into zone 3. The same pattern is observed as the electrical field increases.

For some, but not all, conditions the dispersion of a λ ladder is a function of the product of E and pulse time (Birren *et al.*, 1988; Cantor, Smith and Mathew, 1988). However, for linear DNAs longer than about 3 Mb, arrest and band spreading force the use of progressively lower E values as the DNA becomes longer (Snell and Wilkins, 1986; Vollrath and Davis, 1987; Birren *et al.*, 1988). Solvent-induced and temperature-induced changes in viscosity do not have the same effect on separations (Mathew, Smith and Cantor, 1988b), thereby preventing the integration of viscosity into a quantitative description. Effects of gel concentration have been studied (Birren *et al.*, 1988), but have not been expressed as a function of P_E . In the absence of a quantitative description that includes all relevant variables, the author has used the following guidelines for achieving separations.

1. Choose an agarose preparation rated for use during PFG electrophoresis. These preparations have comparatively low values of electro-osmosis. Manufacturers are continually attempting to produce preparations with lower values of electro-osmosis. Although lowering electro-osmosis does reduce running time during PFG electrophoresis, it also has, thus far, increased the price of the agarose preparation.
2. Find a separation in the literature that has approximately the range of resolution desired. Prepare a gel according to the protocol of the previous experiments. Typically, the gel will have a concentration between 1.0 and 1.5% for 0.1–2.0 Mb long DNA. Use one-half the E recommended and twice the pulse time. If the bands are of adequate quality, progressively raise E and lower pulse time, keeping the E , pulse time product constant, until the quality of bands becomes unacceptable. By use of the highest E that produces acceptable bands, lower the pulse time until zone 4 engulfs any linear DNA longer than a DNA about 25% longer than the longest DNA that you want to resolve. By thus adjusting the pulse time, the separations of all shorter DNAs are maximized. This process was used to develop conditions for the separation of DNAs from size variants of bacteriophage T4 (Lane *et al.*, 1990).

FIELD INVERSION MODE

Periodic inversion of the electrical field (i.e. $\psi = \pi$ radians) has been used to improve the separation by length of linear DNA, by use of either a time (Carle, Frank and Olson, 1986) or an E (LaLande *et al.*, 1987) greater in the forward direction than it is in the reverse direction. Both of these procedures for use of the field inversion mode improve separations for DNAs 0.1–2.0 Mb long. However, the dispersion of ladders (i.e. *Figure 2*) is not as uniform for the field inversion mode as it is for the initial mode of PFG electrophoresis (Bostock, 1988; Heller and Pohl, 1989). In addition, as the zone of no resolution (equivalent to zone 4 in *Figure 2*) is approached, non-monotonic μ v. DNA length relationships are observed. This effect can, however, be reduced by use of pulse frequency ramps (Carle, Frank and Olson, 1986; Heller and Pohl, 1989; reviewed by Olson, 1989b).

The field inversion mode (asymmetric E) has been used to improve the separation of 0.001–0.05 Mb, in addition to the longer double-stranded DNAs (Birren, Simon and Lai, 1990). The application to single-stranded (1–500 bp) DNA in polyacrylamide gels has also yielded significant results (Lai, Davi and Hood, 1989; Birren, Simon and Lai, 1990). The field inversion mode has the advantage of being applicable with any electrophoresis apparatus used for invariant-field gel electrophoresis.

ROTATION-BASED MODE

When the direction of E changes by either $2\pi/3$ radians or less during a single pulse and at least three successive pulses are delivered in the same direction, E appears to be rotating and improvements in the separation of linear DNA have been observed. The average pulse delivered in the direction of net motion has to be either stronger or more numerous than the other pulses. Variations of this procedure include both discontinuous rotation by $2\pi/3$ radians (Clark *et al.*, 1988), discontinuous rotation by $\pi/2$ radians (Bancroft and Wolk, 1988) and continuous rotation (Serwer and Hayes, 1989c). In the absence of data that distinguish the mechanisms of these procedures, they will all be classified here as a rotation-based mode.

The mobility v. DNA length relationship for the rotation-based mode is not as simple as that for the initial mode and, for routine separations, the rotation-based mode has not yet been competitive with either the initial mode or the field inversion mode. However, because the rotation-based mode delivers pulses in a direction opposite to that of the direction of net motion, the arrest of open circular DNA (and, presumably, other arrested DNAs) is reversed (Serwer and Hayes, 1989a,b,c). By merging the rotation-based mode with the original mode, Louie and Serwer (1989) have achieved the separation shown in *Figure 2* with a linear λ DNA ladder (0.048502 Mb between rungs) and have also resolved an open circular λ DNA ladder present (as expected: Wang and Davidson, 1966) together with the linear ladder. Four rungs of the open circular DNA ladder were observed in Louie and Serwer (1989); seven rungs have subsequently been observed by these

authors (unpublished). The upper limit for the length of resolvable open circular DNA is, therefore, at least 0.3 Mb and probably higher.

Mechanisms of PFG electrophoresis

In the case of the particles that become arrested during the time of a pulse, reversal of the arrest is a mechanism for separation by PFG electrophoresis. If the average time needed for arrest is much smaller than pulse times, this mechanism would probably be the most predominant. That is, sieving would be predominantly steric. In addition to micron-sized spheres and open circular DNA, DNA-protein complexes have also been found to undergo an elevated E -induced arrest in agarose and polyacrylamide gels. These complexes include: (1) a complex of a bacteriophage T7 capsid, 30 nm in radius, and 0.04 Mb linear T7 DNA—agarose gels were used (Serwer and Hayes, 1989a); and (2) complexes of single-stranded DNA and streptavidin—polyacrylamide gels were used (Ulanovsky, Drouin and Gilbert, 1990).

To model the sieving of reptating, linear, double-stranded DNA during invariant field agarose gel electrophoresis, the DNA was represented as stretched (i.e. no loops within a pore) and the gel was modeled as a tortuous, smooth cylindrical tube (Lumpkin, Déjardin and Zimm, 1985; Slater and Noolandi, 1986). For this model, the loss of length resolution induced by progressively higher E is caused by progressively greater bias, quantified by use of the Boltzmann distribution, for forward orientation when the leading end enters a new pore. Although this theory correctly predicts some of the observed sieving of DNA (Lumpkin, Déjardin and Zimm, 1985; Hervet and Bean, 1987), incorrect predictions are also made (Lumpkin, Déjardin and Zimm, 1985; Hurley, 1986; Hervet and Bean, 1987; Jamil, Frisch and Lerman, 1989). In addition, computer-based modeling of the motion of a random coil through a regular network of static obstacles (Deutsch, 1988) indicates collisions that cause accumulations of DNA segments, multiple leading ends, hooking around obstacles and the generation of new leading ends by way of herniation (i.e. steric sieving). These effects have been confirmed during the observation of individual DNA molecules by use of light microscopy (Schwartz and Koval, 1989; Smith, Aldridge and Callis, 1989; Gurrieri *et al.*, 1990). In addition, reptation in invariant fields can occur in the absence of complete stretching (Serwer and Allen, 1984) and DNA is usually not completely stretched during PFG electrophoresis (Gurrieri *et al.*, 1990). Conversely, rod-shaped viruses longer than 900 nm (bacteriophage fd, for example; Herrmann *et al.*, 1980; Day *et al.*, 1988) do not lose resolution by length as they are increasingly forced to reptate by lowering P_E (to 60 nm) and raising E (to 4 V cm⁻¹) (P. Serwer, E.T. Moreno and G.A. Griess, unpublished observations). This tempts the proposal that, if E values high enough and P_E values low enough to completely stretch DNA were used during invariant field agarose gel electrophoresis, resolution by length would be regained.

Because of the complexities of the motion of DNA in gels, no mode of PFG electrophoresis is as yet understood in detail. However, the use of simplifying

assumptions has produced formalisms with some capacity for prediction. Attempts to explain the effects of PFG electrophoresis are intertwined with attempts to develop more demanding separations. Both of these topics will be discussed in the present section.

INITIAL MODE

In an attempt to explain the observation that improved separation of linear DNA by length was achieved only for ψ greater than $\pi/2$ radians, Southern *et al.* (1987) assumed: (1) reptation in a smooth-walled tube; (2) front end-to-back end interchange at the beginning of each pulse for $\psi > \pi/2$ radians, but not for $\psi \leq \pi/2$ radians; and (3) constant velocity of migration. Assumption (2) for $\psi > \pi/2$ radians implies that, microscopically, a reptating random coil migrates a distance greater than that migrated by a sphere for any given macroscopic migration. If the entire random coil can change direction during a pulse, the increased distance is proportional to the length of the random coil and the number of pulses. Thus, both the existence of zone 1 (*Figure 2*) and, at any given ψ , the dependence of rung spacing on pulse time are explained. Zones 2 and 3 are not explained. By the hypothesis of Southern *et al.* (1987), DNA in zone 4 is longer than the shortest DNA that can change directions during a single pulse. Independent of its accuracy at the molecular level, the hypothesis of Southern *et al.* (1987) is useful for guiding some procedures for optimization, for example, the procedure used for the initial mode in the previous section.

Light microscopy of individual molecules undergoing the initial mode of PFG electrophoresis at $\psi = \pi/2$ reveals some molecules that do undergo the front end-to-back end exchange assumed in the hypothesis of Southern *et al.* (1987) and some that don't (Gurrieri *et al.*, 1990). Because zone 4 is not at the origin of electrophoresis, at least some changes in direction must be accomplished by herniation of the DNA random coil. As discussed in a previous review (Cantor, Smith and Mathew, 1988), resolution by length can be achieved during herniation, by DNA length-dependent differences in the average DNA velocity during change of direction. Hysteresis in the orientation of fibrous projections from the gel has been proposed to be a mechanism for this latter effect (Serwer, 1988). Evidence for gel-based hysteresis in the response of DNA to successive unidirectional pulses has been obtained (Akerman *et al.*, 1989).

The observations made thus far indicate that the improved separations of linear DNA, achieved by use of the initial mode, are derived from a combination of DNA path length-based (i.e. hernia-free) and DNA velocity-based effects. In agreement with this proposal, zone 4 moves closer to the origin of electrophoresis as ψ changes from 0.5π to 0.9π (Louie and Serwer, 1989). This is the result predicted if, as appears reasonable, herniation of DNA only sometimes occurs, but decreases in frequency as ψ changes from 0.5π to 1.0π radians.

Human chromosomal DNA, presumed intact, does not move during application of the original mode, when released from cells in agarose plugs

(Gardiner, Laas and Patterson, 1986). As the length of DNA increases, during use of the initial mode, sharp bands become increasingly difficult to achieve; lowering E sharpens bands, but also increases the time of electrophoresis (Snell and Wilkins, 1986; Vollrath and Davis, 1987). Systematic attempts to overcome these limitations for linear DNAs longer than 3–6 Mb, by use of the initial mode of PFG electrophoresis, have failed (Birren, Hood and Lai, 1989; Chu, 1989). Because fractionation of linear DNA as long as 50–250 Mb is needed for the low resolution mapping of eukaryotic genomes (Smith and Cantor, 1987), procedures for overcoming these limitations are desirable. Three possible procedures are the use of: (1) an alternative gel matrix, perhaps one that has either a less variable radius of pores or less threading; (2) an alternative mode of PFG electrophoresis; or (3) a non-gelled, sieving polymer (*see*, for example, Laurent *et al.*, 1963; Ogston, Preston and Wells, 1973; Tietz *et al.*, 1986; ungelled agarose solutions that have undergone partial phase separation are possibly useful).

FIELD INVERSION MODE

Explanation of the improved linear DNA separation induced by field inversion cannot be based on motion of an elongated, symmetrical random coil in a cylindrical tube. In this case, the free-draining character of DNA (Olivera, Baine and Davidson, 1964; Cantor and Schimmel, 1980) would prevent the interaction between DNA segments necessary to promote improved fractionation. Previously proposed explanations have been based on: (1) head–tail asymmetry of the random coil and DNA length-dependent time of inversion of this asymmetry (Noolandi *et al.*, 1989; Olson, 1989b); (2) heterogeneity of the pore radius and DNA length-dependent trapping of pools of DNA in the larger pores (Zimm, 1989); and (3) invertible asymmetry of the gel, transmitted by and to the DNA (Serwer, 1988). Observation of individual DNA molecules, by use of fluorescence light microscopy, has revealed head–tail asymmetry (Schwartz and Koval, 1989; Smith, Aldridge and Callis, 1989; Gurrieri *et al.*, 1990) during agarose gel electrophoresis. The source could be either repeated collision of DNA segments with an immobile network of gel fibers (Deutsch, 1988) or a more continuous contact with projections, possibly mobile, from the main network of fibers. In addition, both heterogeneity of pore radius (*see* pp. 321, 324) and field-induced mobile asymmetry of agarose gels (Akerman *et al.*, 1989; Holmes and Stellwagen, 1989; Stellwagen and Stellwagen, 1989) have been observed. Thus, a basis in experiment exists for all three of the above hypotheses. Additional experiments are needed to discriminate among these hypotheses; all may describe events that contribute to the final separation observed.

An observation useful for developing both theory and practice is the finding of separations more dependent on the time of reverse pulse than they are on the time of forward pulse (P. Serwer and E.T. Moreno, unpublished observations). For example, for field inversion at constant E , by use of the other conditions of *Figure 3*, when the time forward/time reverse ratio is 3,

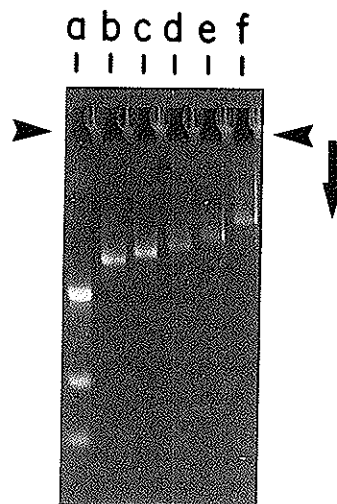


Figure 3. Reverse pulse resonance observed for the field inversion mode (symmetric E) of PFG electrophoresis. The ratio of distance migrated for 0.039936 Mb T7 DNA and 0.048502 Mb λ DNA was measured as a function of pulse time. When the forward time/reverse time ratio was 3.0, electrophoresis at 2 V cm^{-1} , 15°C in a 1.5% agarose gel (Seakem LE, from FMC Bioproducts) cast in 0.01 M sodium phosphate, pH 7.4, 0.001 M EDTA, yielded an optimum forward pulse time of 4.5 s (ratio of distance migrated = 1.11). When the forward time/reverse time ratio was 1.2: (1) use of a forward time of 4.5 s yielded a ratio of distance migrated that was comparatively low (1.05); (2) use of a reverse time of 1.5 s yielded an unusually high ratio of distance migrated (1.36). Shown for condition (2) are the profiles of the following DNAs, after electrophoresis for 100 h (DNA lengths are indicated in Mb): (a) a restriction endonuclease *Hind*III digest of λ DNA (0.0231, 0.00941, 0.00656), (b) bacteriophage T7-C5, LG3, a T7 deletion mutant (0.0366), (c) bacteriophage T3 (0.0381), (d) bacteriophage T7 (0.0399), (e) bacteriophage P22 (0.0421), and (f) bacteriophage λ (0.0485). The gel was stained with ethidium. The arrow indicates the direction of electrophoresis; the arrowheads indicate the origins of electrophoresis. The bands obtained for a forward time/reverse time ratio of 1.2 were less sharp than those obtained for a ratio of 3.0.

the best separation of DNA about 0.04 Mb long is achieved for time forward = 4.5 s (legend to *Figure 3*). When the time forward/time reverse ratio was reduced to 1.2, use of a time forward of 4.5 s yielded a comparatively poor separation (legend to *Figure 3*). However, a time forward/time reverse ratio of 1.2 yielded a surprisingly good separation when the reverse pulse was 1.5 s (*Figure 3* and legend). Further probing of reverse pulse times around 1.5 s yielded a sharp peak of highly resolving pulse times that is probably no more than 0.5 s wide. Quantification of this peak has been prevented by scatter in the results. The scatter is presumably caused by the imprecision of the control of conditions of electrophoresis. None the less, the data of *Figure 3* are an example of an unusually sharp and high reverse pulse resonance peak of resolution (for further discussions of resonance during field inversion, see Noolandi *et al.*, 1989; Olson, 1989b).

At present, the limits of resolution that can be achieved by searching for higher (and, presumably, sharper) peaks of reverse pulse resonance are not

known. One procedure for increasing resonant field inversion behavior for single-stranded DNA in polyacrylamide gels is to attach a protein (streptavidin) to an end of the DNA (Ulanovsky, Drouin and Gilbert, 1990). In the case of DNA sequencing, the hope is that a resonant, high resolution pulse time window can be opened initially on the shortest DNAs and, subsequently, on progressively longer DNAs. The longer DNAs are held either near (in the case of resonance for pure DNA) or at (in the case of a DNA-protein complex) the origin of electrophoresis during the resolution of the shorter DNAs. Optical monitoring of DNA bands is the most efficient way to analyse this type of experiment. For this reason, and because raising E and controlling both E and temperature will be critical for sharpening and staying on peaks of resonance, the most promising vehicle for this type of experiment appears to be capillary electrophoresis (reviewed by Karger, Cohen and Guttman, 1989). Invariant field (200–300 V cm⁻¹) polyacrylamide gel capillary electrophoresis has already been used to obtain one base pair resolution for single-stranded DNA as long as 300 base pairs (Guttman *et al.*, 1990; Swerdlow and Gesteland, 1990).

In analogy with results obtained by attaching streptavidin to single-stranded DNA, the arrest of the T7 capsid-DNA complex is overcome by use of field inversion (Serwer and Hayes, 1989a). Thus, if methods for attaching sufficiently large objects to double-stranded DNA can be developed, then attachment of these objects should also be useful to enhance resonance during PFG electrophoresis in agarose gels. The arrest of such complexes should be useful for detecting and isolating complexes that are the product of the nucleotide sequence-specific binding of proteins (*see*, for example, Ceglarek and Revzin, 1989; Fried, 1989). That is, such binding can be used to select DNA fragments with the binding site. The inverted μ v. agarose percentage plots observed during field inversion for open circular DNA are not observed during field inversion for the T7 capsid-DNA complex (P. Serwer and S.J. Hayes, unpublished observations). Thus, the mechanism for arrest of the capsid-DNA complex at constant E apparently does not require the projections postulated for the arrest of open circular DNA.

To sharpen bands formed by megabase-sized, linear DNA during PFG electrophoresis in the field inversion mode (asymmetric E), comparatively short reverse pulses have been superimposed on the longer pulses that are used to obtain resolution of linear DNA as long as 3–6 Mb. The shorter pulses are designed to prevent trapping (Turmel *et al.*, 1990). Although successful with 3–6 Mb yeast chromosomal DNAs, the field inversion mode has, like the original mode, not been useful for longer DNAs.

ROTATION-BASED MODE

Because successive pulses differ by either $2\pi/3$ radians or less for the rotation-based mode, the assumption is made that front end-to-back end interchange is not the reason for the improved resolution by length. More likely, the pulling of DNA into circular arcs is part of the reason. Dependence of forward mobility on the length of DNA in an arc could be the result of

greater frictional resistance of, herniation of, or decreased forward net electrical force on DNA in the arc. These events may all occur. Analysis of the motion of individual DNA molecules for the rotation-based mode has not yet been performed by either computer-based modeling or light microscopy. Attempts to break through the 3–6 Mb length resolution limit for linear DNA have, apparently, not been made by use of the rotation-based mode of PFG electrophoresis.

Length standards: linear and circular

Three procedures have been used to provide length standards for 0.1–6.0 Mb linear DNA:

1. joining by complementary base pairing of terminally repetitious bacteriophage DNAs; the result is a collection of end-to-end joined oligomers (concatemers) called a ladder (*see Figure 2*);
2. release of intact yeast chromosomal DNAs that have been sized by either restriction endonuclease analysis or comparison with DNA ladders; and
3. formation of ladders by use of blunt-ended ligation.

The formation of ladders by use of DNAs with naturally single-stranded terminal repeats (spontaneous ladders) and the release of yeast DNAs have been previously reviewed (Anand, 1986; Smith and Cantor, 1987; Olson, 1989a).

The even spacing of ladders and the unambiguous length separating rungs is advantageous. However, spontaneous ladders have the disadvantages that growth can't be stopped and the comparatively short base-paired overlap (12 for bacteriophage λ DNA) makes the ladders comparatively unstable to lowered ionic strength and elevated temperature (for quantitative details, *see* Wang and Davidson, 1966). The λ ladders have a rung spacing of 0.048502 Mb and can be grown to 25–35 rungs; they are, therefore, useful to about 1.5 Mb. However, even if longer λ ladders could be made, their usefulness would be compromised by rungs that are too close to be resolved during separation of DNAs longer than 2–4 Mb. The more recent use of blunt-ended ligation to form a ladder from the 0.17 Mb DNA of bacteriophage T4 (thus far, 6–7 rungs; Louie and Serwer, 1990) is a procedure with promise for producing useful ladders long enough to reach the size of human chromosomal DNAs. For stable ladders as long as 0.6 Mb, enzymatic, complementary base pair-joined, 0.039936 Mb bacteriophage T7 ladders can be used (Son, Hayes and Serwer, 1988).

The development of open circular λ DNA ladders has reached the level of a heptamer (p. 328). These open circular ladders can be used as length standards for open circular DNA. Alternatively, the length of a circular DNA can be determined either by linearizing the circular DNA and then comparing its mobility to linear DNA standards (van der Blik, Lincke and Borst, 1988) or by restriction endonuclease analysis.

Manipulation of 0.1 Mb and longer DNA

To prevent breakage of linear DNA caused by shear, 0.1–6.0 Mb DNAs are usually released from cells and handled in gelled agarose plugs (reviewed by Anand, 1986; Smith and Cantor, 1987; Olson, 1989a). Shear forces generated by rapid pipetting are sufficient to break the 0.17 Mb T4 DNA. The expectation is that, as DNA becomes longer, sensitivity to shear increases (Adam and Zimm, 1977). However, neither the shear forces generated by the various means of pipetting nor the shear sensitivity as a function of length has been studied in conditions appropriate for the handling of megabase paired DNA before PFG electrophoresis. The following observations indicate that manipulation of such DNA in solution is possible:

1. Although not pipetted, DNA as long as 80 Mb has been maintained outside cells without breakage (Kavenoff and Zimm, 1973).
2. DNA probably 10 Mb long has been subjected to rate zonal centrifugation (Lange *et al.*, 1977).
3. An approximately 1 Mb long λ ladder has been loaded by pipetting (i.e. without an agarose plug) and displayed by PFG electrophoresis, without detectable breakage (Lane *et al.*, 1990; Louie and Serwer, 1990).
4. The chromosomal DNAs of *Saccharomyces cerevisiae* (2.2 Mb = length of longest DNA) have also been loaded by pipetting and displayed by PFG electrophoresis, without detectable breakage (E. Lai, personal communication).

The breakage of DNA in solution needs further study before the limitations of handling in solution are known.

Apparatus

The details of apparatus used to achieve the various modes of PFG electrophoresis have been reviewed extensively (Lai *et al.*, 1989; Olson, 1989a; Birren and Lai, 1990). Thus, only the most important aspects of apparatus will be reviewed here.

APPARATUS WITH VERTICAL ELECTRODES AND ELECTRONIC PULSING

The initial apparatus of Schwartz and Cantor (1984) had two vertical sets of opposing electrodes arranged in a square array. Change of the direction of E was accomplished by changing the set of electrodes that was activated. Vertical (instead of horizontal), diode-isolated electrodes were used to prevent short-circuiting of one set of electrodes by the other set. The electrical field produced by this apparatus is inhomogeneous, thereby causing non-linear motion of DNA and loss of control over ψ . Although this non-linear motion is aesthetically unappealing, subsequent versions of this apparatus have been improved and successfully used for 0.1–6.0 Mb linear DNA (*see*, for example, Mathew, Smith and Cantor, 1988a,b).

A hexagonal array of vertical electrodes has been clamped at voltages

calculated to be those at the perimeter of an apparatus with a uniform E . From basic electrostatics, this clamping is sufficient to produce a uniform E . This apparatus achieves ψ values of $2\pi/3$ and $\pi/3$; the former is effective, the latter is not (Chu, Vollrath and Davis, 1986). The uniform E eliminates the non-linear motion of DNA.

To obtain control over ψ , the electrical potential of electrodes in the apparatus of Chu, Vollrath and Davis (1986) has been made programmable (Clark *et al.*, 1988). This programmable apparatus and the other vertical electrode apparatus described here have been effective in separating megabase-sized DNAs.

APPARATUS WITH HORIZONTAL ELECTRODES AND ELECTRONIC PULSING

One advantage of the field inversion mode is that it can be performed by use of any electrophoresis apparatus. Typically, for agarose gels the apparatus contains horizontal electrodes and a horizontal gel submerged beneath electrophoresis buffer. Inversion of electrical fields can be performed by use of either electronic- (Carle, Frank and Olson, 1986) or computer-driven switching. Several gels can be run in series in a single electrophoresis apparatus and one computer can control the switching for several sets of apparatus. Thus, equipment for the field inversion mode is less expensive and less space-consuming than equipment for the other modes, when multiple gels are being run.

APPARATUS WITH HORIZONTAL ELECTRODES AND MECHANICAL PULSING

Horizontal electrodes in an apparatus either without (Southern *et al.*, 1987) or with (Serwer, 1987) buffer tanks has been used, together with a rotating disk, to change the direction of E in relation to an agarose gel. Either a simple electrical motor (Southern *et al.*, 1987), a stepping motor (Serwer, 1987) or a pneumatic motor (Sutherland, Emrick and Trunk, 1989) can be used to rotate the disk. The use of a stepping motor opens the possibility of programmable motion. Apparatus for completely programmable motion of the disk have been constructed (P. Serwer and F. Dunn, unpublished). For the latter apparatus, the magnitude of E is also programmable. The programmable apparatus can be used to search for new modes of PFG electrophoresis.

Although the apparatus for PFG electrophoresis typically consists of simple boxes without buffer tanks, the use of buffer tanks results in improved control of pH, E and temperature. Attachment to buffer tanks of a thermoelectric (Peltier) cooling device and a heat sink can be used to control temperature (P. Serwer and F. Dunn, unpublished). This type of temperature control is the most accurate ($\pm 0.3^\circ\text{C}$ at worst), and reduces the following practical problems encountered with all apparatus used for PFG apparatus: (1) clutter from wires, tubing and apparatus, which includes a circulating temperature control bath, and (2) heat released into the laboratory.

Conclusion

Although most uses of PFG electrophoresis have, thus far, been for genome mapping, PFG electrophoresis intersects other areas of research. These areas include (among others): (1) physics and biophysics of sieving; (2) morphogenesis of supramolecular particles; and (3) biophysical characterization of macromolecules. To make maximum progress in all of these fields, interdisciplinary research will be essential in the future.

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