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DNA Separations by Slab Gel, and Capillary Electrophoresis: Theory and Practice

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DNA SEPARATIONS BY SLAB GEL AND CAPILLARY ELECTROPHORESIS: THEORY AND PRACTICE

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Abstract

In this review we evaluate existing experimental methods for the electrophoretic size separation of nucleic acids, including both slab gel electrophoresis and capillary electrophoresis (CE). First, we briefly discuss some important applications of DNA electrophoresis in molecular biology. We then focus on the theory and the practice of DNA separations by slab gel electrophoresis and CE, emphasizing the different practical limitations of the two techniques. In particular, we review new developments in CE, a technique which is evolving rapidly.

1. Introduction: Important biological techniques which employ DNA electrophoretic separations

Many of the most important methods in molecular biology require the size-based separation of nucleic acid biopolymers. Examples include DNA sequencing, physical mapping of the restriction enzyme sites in chromosomal DNA, Southern blotting, and DNase footprinting. All of these methods rely upon the use of extremely specific enzymes, in combination with an electrophoretic step to provide the desired information. We begin by briefly discussing each of these techniques, and the resolution and size range of DNA separation which they require.

1. 1. DNA sequencing

Deoxyribonucleic acid molecules (DNA), which serve as the genetic blueprint for all living things, are right-handed helices formed by two anti-parallel strands of nucleotides. The

four different bases which are the foundation of the genetic code (adenine, guanosine, thymine, and cytosine, or A, G, T, and C) are hydrogen-bonded (base-paired) across the axis of the helix [1]. The sequencing of DNA to determine the linear order of the nucleotides is arguably the most important tool in molecular biology [2, 3]. The technique is so successful that the amino acid sequence of a protein is preferentially deduced by sequencing the gene (or a DNA copy of a messenger RNA), rather than performing Edman degradation sequencing of the protein itself [2]. Two DNA sequencing techniques are currently used: the enzymatic method of Sanger *et al.* (the chain-termination method) [4], and, less frequently, the chemical degradation method of Maxam and Gilbert [5]. Although the two methods are quite different in their specifics, the underlying paradigm is the same for both. The DNA fragment to be sequenced is reduced by enzymatic or chemical reactions to produce separate, nested sets of radiolabeled fragments which share a common starting point and terminate randomly at a particular base or combination of bases [2]. The several sets of fragments are then separated, based on their size, by polyacrylamide gel electrophoresis; the nucleotide sequence can be decoded directly from the gel.

For example, in the commonly-used Sanger method (see Figure 1) [4], identical template strands of DNA are divided among four sets of reactions, one for each base (A, G, C, and T). First, an oligonucleotide primer complimentary to the 3' end of the sequence of interest is hybridized to the template. A DNA polymerase then synthesizes a DNA strand which is complementary to the template strand, using the primer as a starting point. However, a 2',3'-dideoxy analog of one type of nucleotide triphosphate (ddATP, ddGTP, ddCTP, or ddTTP), is present at a low concentration in each of the four reaction vessels. Since the 2',3'-ddNTP lacks the 3'-hydroxyl group which is necessary for DNA polymerization, after a ddNTP is incorporated into the chain it cannot be extended further. The ddNTP concentration in each reaction vessel is adjusted such that any position in the new DNA strand is equally likely to be the point of dideoxy termination, and thus a complete distribution of chains terminating at each site is produced. Overall, the Sanger sequencing reaction yields four sets of fragments, the lengths of which are determined by the position of a particular base (*e.g.*, all of the fragments will terminate at a guanosine, if ddGTP was present) on the original template strand relative to a fixed 5' terminus where the primer was annealed. These four sets of single-stranded DNA fragments are then subjected to electrophoresis in a denaturing polyacrylamide gel (to prevent the formation of secondary structure) under conditions which provide *single-base* resolution (this is crucial). The DNA

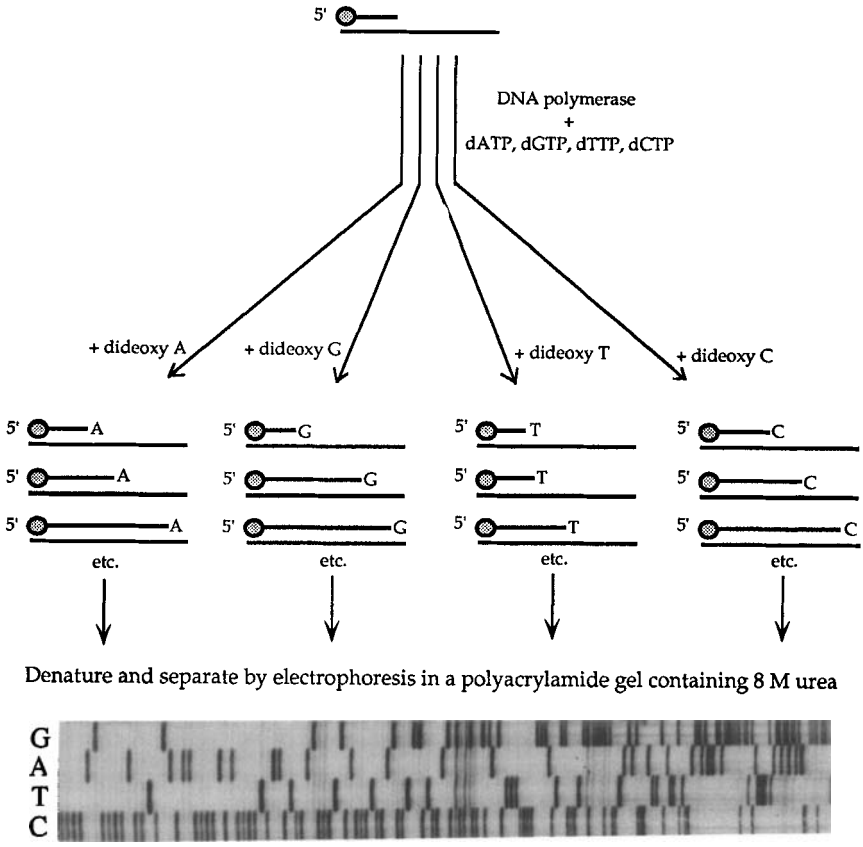


FIGURE 1

DNA sequencing by the Sanger (dideoxy) method. (a) A single strand of DNA to be sequenced is hybridized to a 5'-end-labeled deoxynucleotide primer; four separate reaction mixtures are prepared in which the primer is elongated by a DNA polymerase. Each mixture contains the four normal deoxynucleoside triphosphates plus one of the four dideoxynucleoside triphosphates in a ratio such that about 1 in every 200 residues is a dideoxynucleotide. Since a dideoxynucleotide has no 3' hydroxyl, no further chain elongation is possible when such a residue is added to the chain. Thus, each reaction mixture will produce prematurely terminated chains ending at every occurrence of the dideoxynucleotide. Each mixture is then separated on a sequencing gel. (b) An actual radioautogram from which a DNA sequence can be read. [Reprinted, with permission, from Reference [9], p. 213.]

sequence may be then read directly from an autoradiographic image of the gel in the case of manual sequencing, or from an electropherogram in the case of automated sequencing, which features on-line fluorescence or infrared detection with a gel scanner [6, 7]. Hence, DNA fragments which differ in length by only one base must be resolved by electrophoresis in order for a sequence to be determined. In general, the length of DNA sequence which can be obtained from one set of base-specific reactions is limited by the ability of the gel to provide single base resolution of larger DNA fragments [8]. This size limit may be anywhere between 250 and 1000 bases for a given electrophoretic separation, depending on the method of electrophoresis employed as well as other experimental factors which we shall discuss later in this review.

1. 2. Restriction mapping of chromosomal DNA

It is useful to begin the analysis of a large DNA molecule (such as a chromosome) of interest by constructing a physical map of the restriction sites within the chain, a technique which is called *restriction mapping* (see Figure 2) [2, 9]. This simple and very important tool in molecular biology utilizes bacterial restriction endonucleases which recognize specific short sequences within the DNA, which may be from four to eight base pairs long, and which then cleave the DNA at that site. These enzymes are called "restriction" endonucleases because they function as a primitive type of "immune system" for the bacteria of origin, serving to destroy (or restrict) incoming foreign DNA (*e.g.*, DNA from the bacteriophage particles which prey upon bacteria) by cleaving the DNA at these specific restriction sites. Since they always cleave at the same sequence, restriction enzymes will reproducibly and consistently cut a given DNA sample into a set of fragments of defined length [10]. These DNA fragments are then separated by slab gel electrophoresis alongside a standard set of fragments of known length, and by comparison, their size (which relates to the position of the restriction sites) can be accurately judged [11]. Many hundreds of restriction enzymes, which recognize different restriction sites, are now commercially available. The digestion of a piece of DNA by two or more different restriction enzymes, singly and in tandem, followed by separation and size determination by slab gel electrophoresis, will yield the order of the restriction sites within the DNA sample.

When a DNA molecule has been physically mapped with many different restriction enzymes, researchers wishing to isolate certain genes or regulatory sequences of interest can use the map to obtain specific, manageably sized, portions of the genome for study.

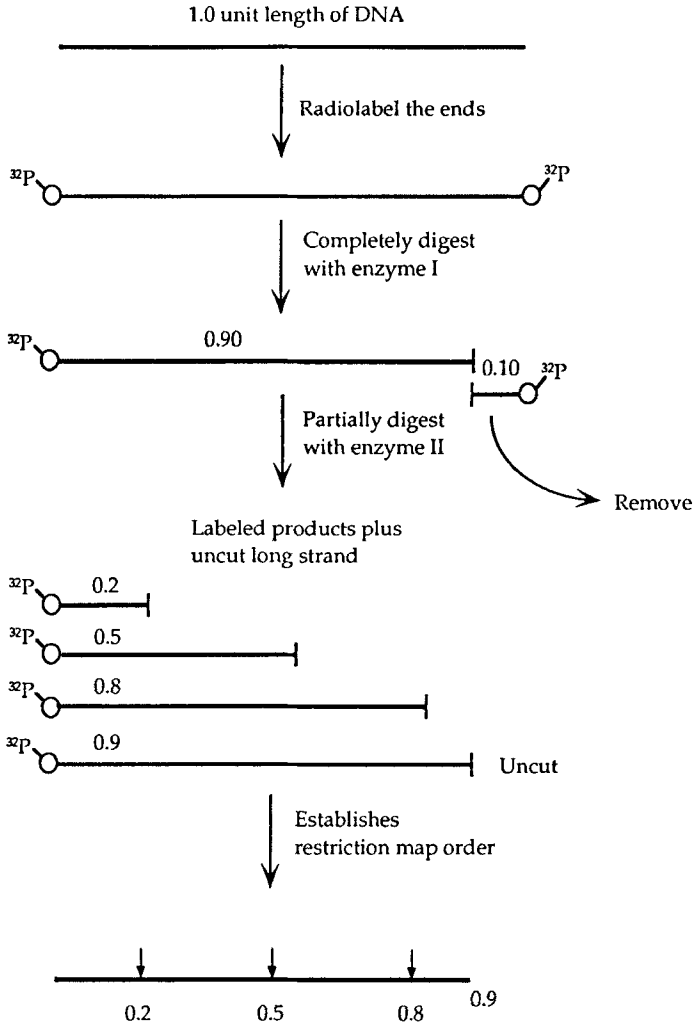


FIGURE 2

Mapping the multiple recognition sites of a restriction enzyme by partial digestion. DNA is labeled at its termini with ^{32}P, and fragments with *one* labeled terminus can be obtained by cutting off one end with an appropriate enzyme. The mapping procedure is applied to the remaining piece with a second enzyme. Complete digestion would produce only one label fragment (here, the 0.2-unit piece), but brief, partial digestion (in which the enzyme cuts each long piece only once, at most) produces a labeled fragment for each restriction site. From the lengths of the labeled pieces, the positions of enzyme II restriction sites can be inferred. [Reprinted, with permission, from Reference [9], p. 209.]

Depending on the size of the genome of interest and the stage of the mapping effort, restriction mapping may require the electrophoretic separation of fragments ranging in size from about 100 base pairs (bp) up to 10 million bp (Mbp) [12]. Different electrophoresis conditions and different types of gels are employed depending on the DNA size range which must be resolved; the required resolution will also vary with the application. Generally, however, the resolution of fragments differing in length by 10 bp is sufficient for the mapping of DNA smaller than ~ 1 kilobase pair (kbp), while 100 bp resolution is acceptable for DNA between 1 kbp and 25 kbp, and even coarser resolution is usually acceptable when very large (chromosomal) DNA is being separated.

1. 3. Southern Blots

A restriction map and one or more restriction enzymes can be used to isolate a DNA fragment containing a sequence of interest, if this sequence can be identified. Thus, an important first step in the search for a gene or regulatory region is to roughly locate the sequence of interest within the genome. This task is accomplished using the Southern blotting technique (outlined in Figure 3) [13], a method to locate and isolate a desired sequence from a complex mixture of DNA sequences. Although the method was developed to locate homologous sequences in genomic DNA and to aid in genetic mapping, it is also used in a variety of DNA cloning techniques, since it allows the detection of a desired DNA fragment or fragments in analytical restriction enzyme digests of cloned material [14]. Southern blotting is also used to analyze polymerase chain reaction (PCR) products.

The first step in a Southern analysis is to purify a nucleic acid sample from bacteria or eukaryotic cells. Following purification, the DNA will usually be digested with a restriction enzyme, generating a mixture of smaller fragments. The mixture of restriction fragments is then separated by slab gel electrophoresis according to its size. The distribution of bands in the gel is preserved as they are then chemically denatured, transferred by blotting, and fixed to a solid support (usually a Nylon membrane). The membrane holding the immobilized DNA is then soaked in a solution containing a radiolabeled single-stranded DNA oligomer (the probe). The radioactive probe will hybridize (by specific base-pairing) to the membrane-bound single-stranded nucleic acid fragments which contain its complementary sequence. The membrane is then washed to remove any unhybridized or non-specifically bound probe, and the location of the probe on the membrane (and hence on the slab gel) is detected by autoradiography. Southern

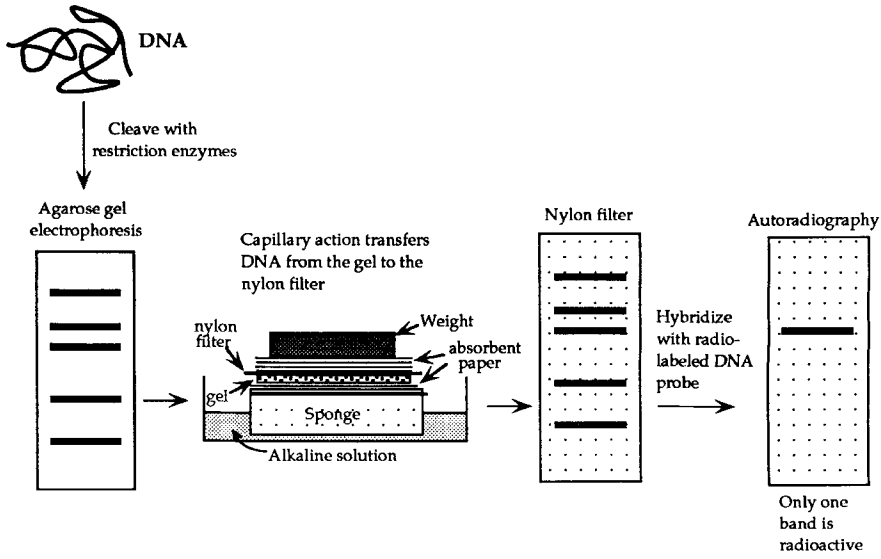


FIGURE 3
The Southern blot technique for detecting the presence of specific DNA sequences (see Reference 13). [Reprinted, with permission, from Reference [9] p. 210.]

blotting with autoradiographic detection is so sensitive that a DNA sequence appearing only once in the human genome (about 1 part in 10^6) can be detected in only 5 μg of DNA (the DNA content of about 10^6 cells) [9]. A Southern blot requires roughly the same level of DNA resolution as restriction mapping. DNA separated by slab gel electrophoresis for a Southern blot may be anywhere between 100 bp and 10 million bp long, as in restriction mapping. However, the majority of Southern analyses require the electrophoretic resolution of DNA ranging from 200 bp to 20 kbp [14], which means that 10 bp resolution is usually sufficient.

1. 4. DNase Footprinting

A particularly elegant molecular biology technique which relies on DNA electrophoresis is DNase footprinting (illustrated in Figure 4) [9]. A *DNase* (short for deoxyribonuclease) is an enzyme which will attack and degrade naked double-stranded DNA. This property has been exploited to deduce the regions within a DNA regulatory sequence which are covered

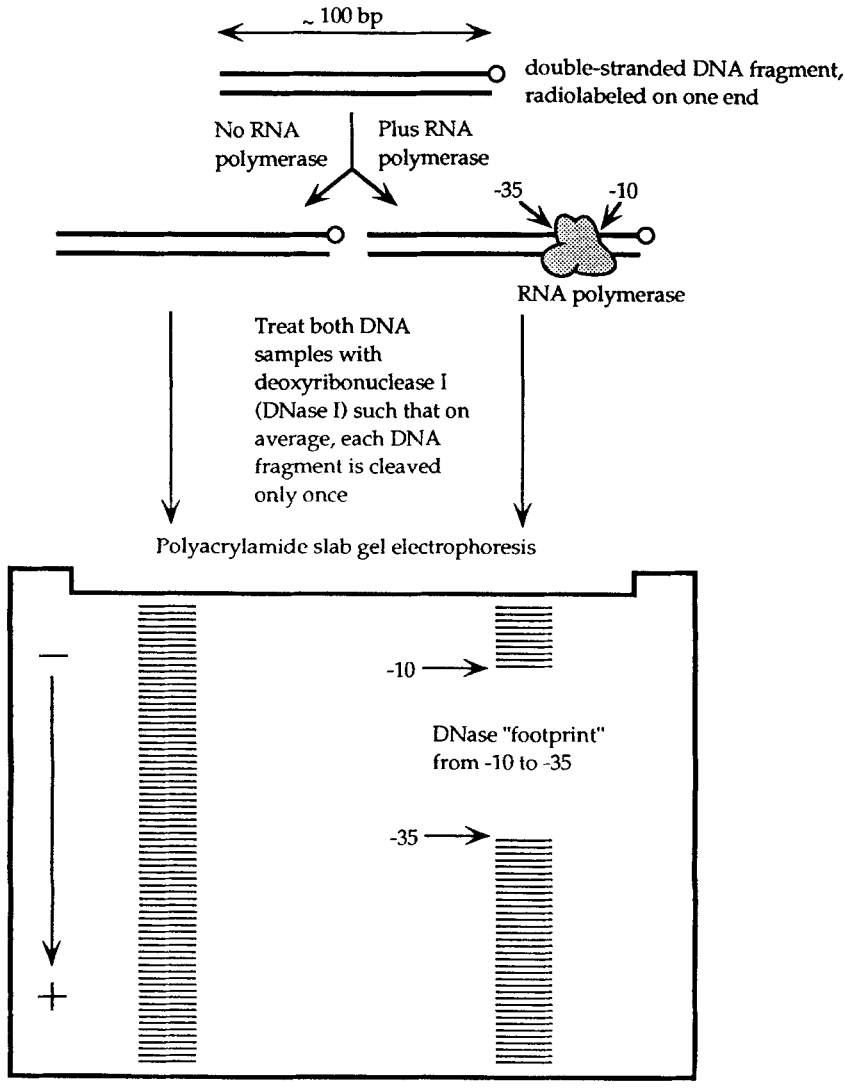


FIGURE 4

Footprinting experiments are used to determine the sites of interaction between DNA and a protein. In this case, a purified double-stranded DNA fragment that is labeled at the 5' end of one strand is allowed to interact with RNA polymerase. The DNA-protein complex is then subjected to DNase attack in such a way that the chain is cleaved at all bases which are not in contact with the protein; the DNase concentration is such that each individual DNA strand is cut at one position only, at the most. Examination of the band pattern of the denatured DNA after electrophoresis indicates which of the bases on the labeled strand of DNA were protected by protein. In this diagram, the region between -10 and -35 bases away from the start of transcription were protected. [Reprinted, with permission, from Reference [9], p. 233.]

or protected when a protein (for example, RNA polymerase) is bound. In this method, a purified double-stranded DNA fragment which contains the regulatory sequence of interest (for example, a promoter sequence for RNA polymerase) is radiolabeled at one end and incubated with the DNA binding protein. The DNA-protein complex is then exposed to DNase attack under conditions such that any individual DNA molecule is degraded at one position only, but always at a nucleotide which is not protected by intimate contact with the protein. Thus the DNA fragment will remain uncut only at the positions which the protein is covering, and fragments terminating at protected positions will not be represented in the population of DNA fragments. The mixture of single-cut DNA fragments is then electrophoresed on a slab gel providing single base-pair resolution, adjacent to a "control" lane containing a similarly digested DNA strand which was not complexed with the protein. When the patterns of DNA bands in the two lanes are compared, it is seen that a gap, or "footprint," is left for fragment lengths which would correspond to termination at residues which are protected from the DNase by contact with the DNA binding protein. Interestingly, the "footprint" is often somewhat different for the two DNA strands (the "upper" and "lower" strands), so that even some three-dimensional information about how the protein binds to the DNA can be gleaned from this experiment [15]. DNase footprinting is usually performed using relatively short DNA fragments, and single base-pair resolution provides optimal conditions for the accurate determination of important bases for protein binding to DNA. Thus, the demands upon the electrophoresis system for DNase footprinting are similar to those required by DNA sequencing.

DNA electrophoresis also finds important applications in forensic science, for such techniques as DNA fingerprinting to establish identity. Although we do not discuss them here, these methods have been recently reviewed elsewhere [16-19]. In the remainder of this review, we will discuss the theory and practice for the electrophoretic separation of nucleic acids. Throughout this discussion, we will refer to the molecular biology methods discussed above, pointing out the suitability of certain electrophoresis systems for carrying out various techniques effectively. We begin with a general discussion of the principles of electrophoresis and of the important theories for the mechanism of DNA separation by electrophoresis in gels.

2. Principles of electrophoresis

The basic relationships which are necessary for the interpretation of DNA electrophoresis data are given below. They are equally applicable both for slab gel electrophoresis and CE.

2. 1. The electrophoretic mobility

Electrophoresis is the migration of a charged analyte within an electric field. In a typical electrophoresis experiment, the movement of an analyte is quantified in terms of its *electrophoretic mobility*, μ_e, defined as:

$$\mu_e = \frac{v}{E} = \frac{L_d L_t}{t_d V} \quad (1)$$

where v is the migration velocity of the analyte, E is the strength of the potential field (in volts per unit length), t_d is the time required for the analyte to reach the detector, L_t is the total length across which the voltage is applied, V is the applied voltage, and L_d is the *effective length*, as the length to the detector or the distance migrated is called (the former for an electrophoresis system with a built-in detector, and the latter for a manual system). The total length across which the field is applied will generally be greater than the length to the detector or the distance migrated. Note that the *apparent* strength of the potential field (the total voltage applied divided by the distance between the electrodes) is not necessarily the true *voltage gradient* which prevails in the electrophoresis instrument [14]. The voltage gradient is the actual driving force for electrophoresis, and will depend upon the geometry of the electrophoresis chamber, the electrical resistance of the gel (which in turn depends on gel composition and geometry), as well as the electrical resistance of the buffer (dependent upon buffer ionic strength and volume). Although the actual voltage gradient can be measured with a high-resistance voltmeter, in practice it is typical for the apparent voltage gradient to be reported instead. The consequence of this is that nucleic acid electrophoretic mobilities can only be directly and quantitatively compared for systems having the same total resistance; this is not usually a problem, since exact comparisons between results obtained in different electrophoresis systems are rarely required. Electrophoretic mobilities are usually reported in the units of cm^2/V sec.

2. 2. Resolution, efficiency, and selectivity

The electrophoretic mobility of an analyte depends on its charge density; hence, not only differences in net charge, but also differences in size and shape can be distinguished by electrophoresis. The dielectric constant and the viscosity of the medium are additional factors which, in a given experiment, will affect the electrophoretic mobilities of all analytes to the same extent. A complex mixture of charged molecules subjected to electrophoresis will generally separate into several different zones, or "bands," based on differences in the electrophoretic mobilities of the molecules. If the peak shapes are Gaussian or nearly so, a

useful expression for the *resolution* R_s between two peaks is [20]

$$R_s = 1.18 \frac{\Delta x}{w_{1/2}} \tag{2}$$

where Δx is the distance between adjacent peaks and $w_{1/2}$ is the peak width at half of its maximum height. More generally, however, the resolution R_s of two bands is defined as the distance between their centers, Δx, divided by $4\bar{\sigma}$, where $\bar{\sigma}$ is the variance of the peaks from their centers. Algebraic substitution for Δx and an approximate expression for $\bar{\sigma}$ in terms of the *height equivalent of a theoretical plate* (HETP) [21] lead to the following expression for the resolution

$$R_s = \frac{1}{4}\left[\frac{\Delta\mu_e}{\bar{\mu}_e}\right]\bar{N}^{0.5} \tag{3}$$

where $\bar{N}$ is the *number of theoretical plates*, a parameter drawn from chromatography theory; $\Delta\mu_e$ is the difference in mobility between the two peaks; and $\bar{\mu}_e$ is the average mobility. In the ideal case when molecular diffusion is the only source of band broadening [21], $\bar{N}$ is given by:

$$\bar{N} = \frac{\mu_e V}{2D} \tag{4}$$

where D is the molecular diffusion coefficient, which one must estimate or measure experimentally. The expression for the resolution given in Equation (3) contains two factors -- the system "efficiency" as given by $\bar{N}^{0.5}/4$, and the selectivity between bands as given by the relative mobility difference, $\Delta\mu_e/\bar{\mu}_e$ [21]. When $R_s = 1.5$, the separation of the two peaks is essentially complete and the peaks are said to be "resolved to base-line" [22]. Note that the smaller the diffusion coefficient of the analyte, the higher will be the number of theoretical plates and the resolution; high-molecular weight species such as DNA have relatively small diffusion coefficients, implying that extremely high efficiencies and selectivities are possible. The height of a theoretical plate will be given simply by the total effective length divided by the number of theoretical plates. Equation (1) can be rearranged to give the solute migration time in terms of the electrophoretic mobility, potential field, and effective length:

$$t_d = \frac{L_d L_t}{\mu_e V} \tag{5}$$

Thus, other factors being held constant, increasing the applied voltage will reduce the required analysis time. Increasing the voltage will also increase the number of theoretical plates and hence the system efficiency. However, depending upon the resistance of the electrophoresis medium, high voltages can produce large electric currents, which lead to the undesired creation of Joule heat. Temperature gradients caused by Joule heating can lead to natural convection within the solution and result in band broadening, which will reduce the resolution of the analytes. In the case of excessive Joule heating, analyte biopolymers may be denatured or degraded. Hence, there will be an optimum applied voltage for any given electrophoresis system, which will give the greatest resolution and efficiency with the least amount of Joule heating and resultant band broadening [23-25].

3. Why is the free-solution electrophoretic mobility of double-stranded DNA independent of molecular size?

At neutral pH, nucleic acid polymers are negatively-charged, due to the phosphate groups which alternate with pentose sugars in the polymer backbone [1]. Thus, DNA molecules will migrate toward the positively-charged electrode (the anode) when placed in a potential field. It was first noted by Olivera *et al.* that during electrophoresis in free solution, DNA molecules display virtually identical electrophoretic mobilities regardless of their size [26]. One reason for this is that double-stranded DNA molecules are very stiff (more than 50 times stiffer than a typical random-coil polymer [27]), and hence approximate the behavior of hydrodynamically *free-draining coils* during electrophoresis [28, 29]. This means that DNA molecules adopt an open, extended conformation in solution which allows solvent to stream around all segments of the polymer equally [30]. Neglecting relaxation effects, the electrophoretic mobility of free draining coils may be approximated by the simple expression:

$$\mu_e = \frac{q}{f} \tag{6}$$

where q is the net charge on the molecule and f is the molecular friction coefficient [31]. Since DNA is a repetitive polymer of phosphate-pentose-nitrogenous base monomers (nucleotides) [1], and the difference in charge on the various nucleotides is relatively small (the phosphates account for most of the charge), the net charge q on a DNA polymer is roughly proportional to the number of base pairs in the chain, N [32]. That is,

$$q_{DNA} \sim N^1 \tag{7}$$

(Sequence-dependent differences in the net charge of DNA are generally only distinguishable for oligonucleotides shorter than 20 bp [33, 34].) Since DNA acts as a free-draining coil during electrophoresis, the molecular friction coefficient also scales with the number of base pairs in the DNA molecule [35]:

$$f_{DNA} \sim N^1 \tag{8}$$

Therefore, both the net charge on the DNA molecule and its molecular frictional coefficient increase linearly with the number of base pairs. According to Equation (6), then, the free-solution electrophoretic mobility of DNA is independent of its size [35]:

$$\mu_{e,\,DNA} \sim \frac{N^1}{N^1} \sim N^0 \tag{9}$$

Some highly-charged synthetic polymers exhibit electrophoretic behavior similar to that of DNA, in particular, polystyrene sulfonates [36-39]. Proteins which have been complexed with sodium dodecyl sulfate (SDS-proteins) also behave as free-draining polyelectrolytes during electrophoresis.

It has been suggested by Noolandi [40] and by Mayer *et al.* [41] that this symmetry of constant charge density with increasing chain length, which prevents the separation of DNA by free-solution electrophoresis, may be overcome by the attachment of "perturbing entity," such as a protein, virus, or charged sphere, to one end of the DNA chain. Experimental tests of the potential of this strategy of DNA separation have not yet appeared in the literature, although the theory has been explored [42, 43].

Another way to explain of the lack of dependence of DNA electrophoretic mobility upon molecular size was discussed by Schellman and Stigter [32]. They point out that during electrophoresis, the negatively-charged DNA chain is surrounded by a sheath of positive counter-ions, which are streaming in the opposite direction by electrophoresis. The sheath has a thickness approximately equal to the Debye-Hückel screening length, which is less than 10 nm for the salt concentrations typically employed for electrophoresis. The counter-ion sheath effectively screens hydrodynamic interactions between any two parts of the chain which are separated by more that 10 nm. Thus, there is no mechanism to create a dependence of DNA velocity on size over a length scale of greater than 10 nm [32, 44].

4. Theoretical approaches to modeling the electrophoresis of DNA in gels

4. 1. Approaches to understanding the mechanism of separation

It was first realized in 1966 that if DNA electrophoresis is performed within a gel, size-based separation of DNA molecules will occur [45]. Gels became commonly used as support matrices for electrophoresis for two reasons: (1) by virtue of their rigid, yet highly porous structure, they allow the passage of analytes, yet dramatically reduce the thermal and gravitational convection which would destroy fine resolution, and (2) they provide the size-based separation of free-draining polyelectrolytes with nearly equal free-solution electrophoretic mobilities, such as DNA fragments and proteins complexed with sodium-dodecyl sulfate (SDS-proteins).

Since there is no change in the distribution of charge on the DNA molecule during electrophoresis, it is clear that DNA separation in gels must arise because the molecular friction coefficients are changed in a size-dependent manner when the DNA molecules are forced to migrate through the gel. The dependence of electrophoretic mobility on DNA size, gel concentration, and field strength is relatively complex, however. This interesting dependence is illustrated in Figure 5, in which DNA electrophoretic mobility in agarose slab gels is plotted as a function of DNA size (in base pairs) at a fixed applied voltage, for several different gel concentrations. The mobility of small DNA fragments shows a relatively weak on size, and approaches a common limit as gel concentration tends to zero: this is the free-solution electrophoretic mobility, which is a constant practically independent of chain length for DNA longer than 20 bp [33, 34]. For intermediate-sized DNA, the dependence of electrophoretic mobility on DNA size is greater, giving a slope of -1 on a log-log plot of μ vs. N (in the limit of high gel concentrations). For large DNA, the slope of the log μ vs. log N curve tends to zero as the size-dependence of mobility disappears. Although it is not shown in Figure 5, there is also an increasing dependence of mobility on field strength as DNA size increases. These three regimes differ not only in the dependence of electrophoretic mobility, but also in the orientational behavior of the DNA as measured by linear dichroism spectroscopy [46].

When these regimes of DNA electrophoretic behavior had been mapped out experimentally, theorists in the fields of biophysical chemistry and polymer physics turned their attention to modeling this problem, for it was not immediately clear how the size- and field-dependences of DNA electrophoretic mobility arose. To model the mechanism of DNA separation by gel electrophoresis, two things have to be understood: (1) the

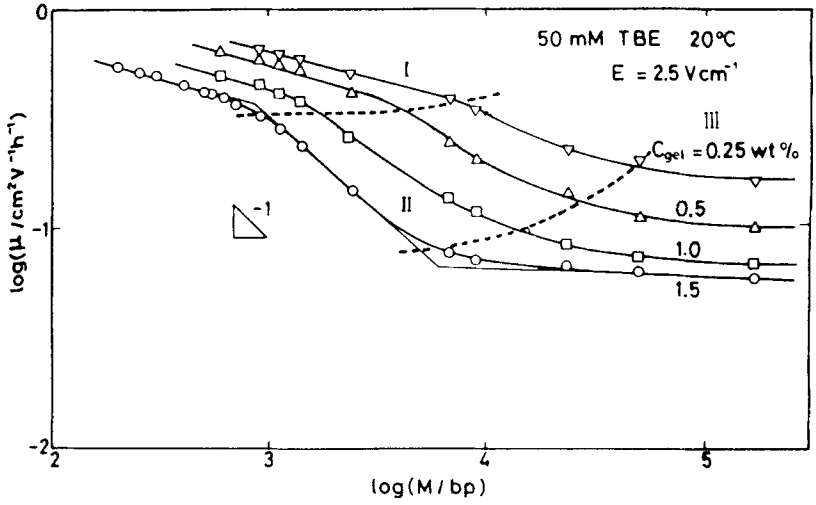


FIGURE 5

Dependence of electrophoretic mobility (μ) on molecular mass (M) for systems with gel concentrations (C_{gel}) of 0.25, 0.5, 0.7, 1.0, and 1.5 wt % and with 50 mM TBE buffer under a steady electric field of strength E = 2.5 V/cm at 20°C. The dotted lines indicate the boundaries between regimes I (Ogston-Rodbard-Chrambach), II (reptation without DNA stretching), and III (reptation with DNA stretching). [Reprinted, with permission, from Reference [138].]

conformation assumed by nucleic acids during electrophoretic migration through the gel, and (2) the structure of the gel and how it influences DNA motion. During the twenty years following the advent of DNA gel electrophoresis [45], two important theories were developed to explain the mechanism of DNA separation: the Ogston-Rodbard-Chrambach model, and the reptation model.

4. 2. The Ogston-Rodbard-Chrambach model of DNA gel electrophoresis

In 1958, Ogston modeled the motion of a spherical penetrant in a static network of long, non-interacting, randomly distributed linear fibers [47]. The spaces in this random fiber network are mathematically characterized with a certain size distribution as well as an average size. Ogston calculated the probability that, in a given random fiber network, an undeformable spherical object of a given size would encounter a space through which it could pass. This general theory was first applied to DNA electrophoresis in 1970 by

Rodbard and Chrambach [48-50]. In the Ogston-Rodbard-Chrambach model of DNA electrophoresis, the spaces in the random fiber network are held to be equivalent to the "pores" of a crosslinked polyacrylamide gel. A DNA chain is assumed to electrophorese through this random network of linear fibers as though it were a spherically-shaped analyte, the assumption being that if the network is not too constrictive, the DNA is able to move through the network in any orientation. Thus, a DNA molecule with constantly changing orientation sweeps out an "equivalent sphere," that must diffuse laterally until it encounters a pore large enough in volume to permit its passage. Figure 6 is a schematic representation of the physical picture on which the Ogston-Rodbard-Chrambach model is based.

The electrophoretic mobility is assumed to be proportional to the probability that a DNA molecule with a certain equivalent spherical radius will fit through an average-sized pore; and, in turn, this probability is held to be proportional to the volume fraction of the gel which is available to the migrating analyte, by virtue of its size. According to this purely geometrical view, "equivalent spheres" of DNA which are much larger in radius than the gel pores would not be able to enter the gel at all. Yet it is well known that DNA molecules much larger in coil radius than the estimated average size of the gel pores will freely enter the gel matrix and migrate through it rapidly [51]. Hence, until recently when it underwent a rebirth as "The extended Ogston model" [52-55], the Ogston-Rodbard-Chrambach model was considered to be useful only for fitting experimental data for small DNA fragments (with radii of gyration comparable to or smaller than the average pore size of the gel), at low electric fields to ensure that field orientation effects are negligible [56, 57].

The applicability of the Ogston-Rodbard-Chrambach model is typically ascertained by use of a semi-logarithmic plot of DNA electrophoretic mobility vs. gel concentration (a Ferguson plot [58]). Accord to the theory, this type of plot should be linear, with a slope of $K_r = k(r + R_g)^2$, where K_r is the "retardation coefficient," k is a constant of proportionality, r is the radius of the gel fiber, and R_g is the radius of gyration of the DNA molecule [48-50, 57]. A Ferguson plot does appear to be approximately linear for small DNAs over a relatively wide range of agarose concentrations [59]. But Ferguson plots were found to be non-linear (usually concave) for gel electrophoresis of DNA molecules larger than a few hundred base pairs [53]. To explain this non-linearity, it was reasoned by Tietz *et al.* [54, 55] that the slope of the Ferguson plot decreases with increasing gel concentration because the equivalent spherical radius of the DNA is a function of the gel concentration; *i.e.*,

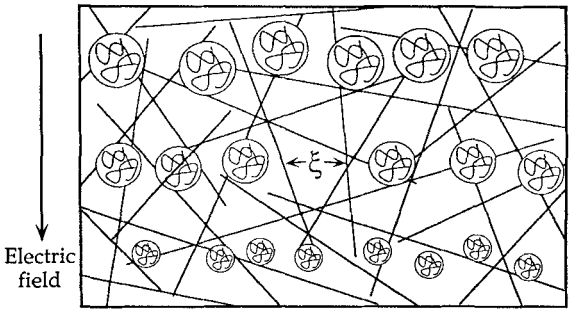


FIGURE 6
A schematic diagram illustrating the assumptions upon which the Ogston-Rodbard-Chrambach model of DNA gel electrophoresis is based. DNA molecules are assumed able to adopt any conformation as they move through the gel, so that each sweeps out an "equivalent sphere" with a radius smaller than the average pore size of the random fiber network (ξ). A spherically-shaped DNA analyte can only proceed in the direction of electrophoresis if it finds a pore large enough to permit its passage. The electrophoretic mobility of DNA molecules is assumed to be proportional to the volume fraction of the fiber network which is available to the spherical penetrant. As larger DNA molecules will be excluded from a larger fraction of the pores in the network, DNA molecules are "sieved" on the basis of their size, with electrophoretic mobility decreasing as DNA size increases.

orientation of the DNA becomes important when the gel matrix is constrictive. Yet even when a dependence of DNA radius of gyration on gel concentration is permitted, the model does not provide a satisfactory fit to the experimental data. It was also hypothesized, specifically for agarose gels, that as gel concentration increases, the radius of the supercoiled agarose gel fiber decreases and the total fiber length increases to approach the dimension of the single-stranded agarose double helix [60]. With these added provisions, the Ogston-Rodbard-Chrambach model became the "extended Ogston model," [52, 54, 55, 60], which allows the retardation coefficient to depend on the gel concentration, as well as the original variables of DNA radius of gyration and gel fiber radius. Furthermore, the extended Ogston model applies non-linear curve-fitting to concave Ferguson plots to determine "local retardation coefficients" for large DNA molecules (up to thousands of base pairs long) (*e.g.*, [61-63]). Except for the provisions allowing both the DNA radius of gyration and the gel fiber radius to vary with gel concentration, the basic geometrical assumptions of the model concerning the separation matrix and the mechanism of DNA separation remain the same as in the original Ogston-Rodbard-

Chrambach model. Intrinsic properties of the gel fibers (such as flexibility) are not considered.

4. 3. The reptation model of DNA gel electrophoresis

The basic geometrical assumptions of the original Ogston-Rodbard-Chrambach model are invalid for larger DNA, since DNA with average radii of gyration much larger than the estimated average pore radius can still migrate through the gel during electrophoresis. It is clear, then, that DNA molecules must stretch and change shape under certain conditions to move through constrictive spaces in the gel. The *reptation model* was developed to explain these experimental findings [56, 64-72]. This model is based on the assumption that a randomly-coiled DNA molecule too large to fit through a pore while maintaining a coiled conformation will migrate head-first, snake-like, through tight "tubes" formed by the gel pores surrounding the DNA chain, as shown schematically in Figure 7. In the classic reptation model, the tube is assumed to be the same length as the DNA chain and only end-on motion is allowed. Indeed, the reptative motion of DNA has been observed by fluorescence microscopy [73, 74]; but the electrophoretic motion through a gel appears to be more complicated than that described by the classical reptation model, as we will discuss. The major success of the reptation model is its ability to provide a theoretical explanation for the experimentally observed regime in a log-log plot of electrophoretic mobility vs. DNA size, in which mobility is inversely related to size [56, 75, 76].

Based on both physical and theoretical evidence, it was realized in 1988 that DNA molecules alternately stretch and relax during gel electrophoresis, as a consequence of their viscoelastic character [77]. Such behavior was predicted by computer simulations using a Langevin equation [78, 79] and also by the Monte Carlo method using the Smoluchowski equation [80]. Schwartz *et al.* [73] observed the electrophoretic motion of fluorescently-labeled DNA under an epifluorescence microscope and reported that a DNA molecule cycles between extended and compact configurations as it becomes caught on obstacles, stretches, and then frees itself. Concomitant fluctuations in the length of the theoretical reptation tube were incorporated into the reptation model by Duke *et al.* to create the more realistic *biased reptation model with fluctuations* [81, 82].

DNA larger than about 40 kbp cannot be separated in a steady DC electric field, but migrates at a single, field-dependent mobility [51]. To explain these experimental findings, the authors of the reptation model postulated that at high fields, and/or for DNA larger

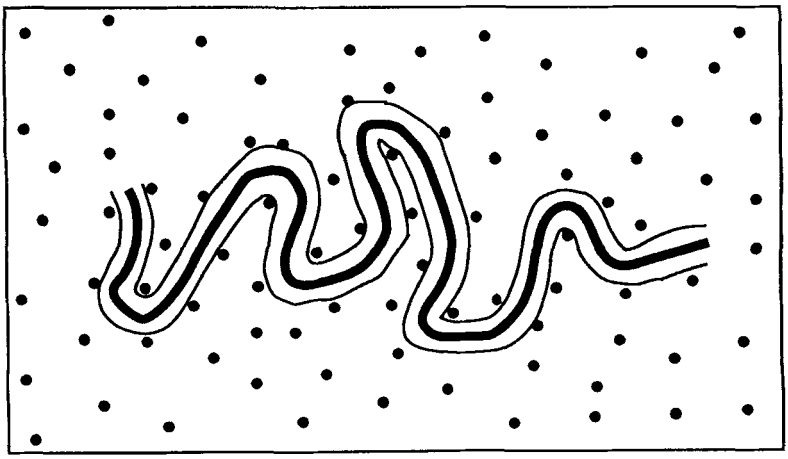


FIGURE 7
A schematic representation of reptative DNA motion in a "tube" formed by gel structure obstacles, shown as black dots in the drawing. This type of end-on, field-biased migration is assumed to occur for DNA with a radius of gyration larger than the average pore size of the gel.

than ~ 40 kbp, field-induced orientation extends the stretching periods of the DNA, causing their random walk to become strongly biased in the forward direction (the *biased reptation* regime [56, 68, 70, 71]), so that DNA is stretched to a rod-like conformation. Consequently, the electrophoretic mobility increases to a maximum, or "saturated" level [65], and size-based separation in a constant field diminishes dramatically.

Typically, the logarithm of DNA electrophoretic mobility (extrapolated to zero field strength) is plotted as a function of the logarithm of inverse DNA size (in bp) to test for adherence to the reptation model. The curves generally have a sigmoidal shape, with a linear region in the center (*i.e.*, for intermediate DNA sizes) having a slope of approximately -1.0. As we have mentioned, this linear region is attributed to the reptation (without DNA stretching) regime, and is observed experimentally [75]. The non-linear ends of the curve have been thought to be the result of Ogston-Rodbard-Chrambach-type sieving (for small DNA) and biased reptation with DNA stretching (for large DNA) [56].

4. 4. A non-pore-based model of DNA gel electrophoresis

H.-J. Bode [83, 84] was skeptical of the accuracy of the static, pore-based concept of a gel which is employed by the Ogston-Rodbard-Chrambach model. He pointed out that this type of model seemed particularly inappropriate for polyacrylamide, a vinyl polymer which would be expected to form a highly flexible and thus easily deformable gel network.

He was guided by the results of his experimental work, in which he found that the crosslinking of a gel to form a semi-rigid structure (a "porous" network) was not a prerequisite for DNA separation, and that solutions of linear polymers could also resolve free-draining polyelectrolytes during electrophoresis [85-88]. Later, Horowitz *et al.* showed that acrylamide-agarose mixed gels lacking covalent crosslinking would separate free-draining polyanions (DNA and SDS-proteins) [89]. Bode showed in 1979 that experimental results from the polyacrylamide gel electrophoresis of macro-ions, which had been interpreted earlier as substantiation of the "rigid-pore" concept of a gel, could be equally well-explained by a *microscopic viscosity model* based on the assumption that solvated gel polymer molecules represent obstacles which must either be deformed or cleared aside by the electrokinetic pressure of the migrating macro-ions [84]. A polyacrylamide gel is modeled as a "viscosity emulsion" consisting of two types of interpenetrating regions, one of which allows DNA to migrate at its free-solution mobility, and the other of which presents more resistance to straight-on DNA motion; this additional resistance is modeled as an increased viscosity in the latter region. Bode's viscosity model did not gain wide acceptance, primarily because it was eclipsed by the successes of the reptation model of DNA electrophoresis, the concepts of which were first introduced in 1982 [64].

In recent years, however, it has become apparent that some of Bode's ideas have a great deal of merit. As we will discuss in Section 6. 8. 2., it has become common for researchers to perform the electrophoretic separation of DNA fragments in solutions of linear, hydrophilic polymers, using very high electric fields (250 V/cm) and the technique of CE (electrophoresis within a microbore capillary, with a 25-100 μm inner diameter). In fact, DNA separation will occur even when the polymer solution is quite dilute [90, 91]. Thus, the existence of "pores" or "tubes" in a rigid gel cannot entirely explain the mechanism of electrophoretic DNA size separation, and new approaches have been taken to understand separation in dilute, uncrosslinked polymer solutions [90, 91]. For the case of concentrated polymer solutions, Viovy *et al.* have adapted the biased reptation with fluctuations model to allow the polymers which form the "tube" around the DNA molecule to reptate away,

releasing the constraints on the DNA motion (constraint release) [57, 92-94]; but this model is applicable only to fully entangled polymer solutions, when electrophoresis is performed under low electric fields.

4. 5. Critique of current models

The development of a physically realistic model for DNA electrophoresis is an exceedingly difficult task. As recently pointed out by Zimm and Levene [44], several aspects of the problem complicate the development of a model. The energies which result from the electric fields commonly employed may be both small and large in comparison to the thermal energy, k_BT. The electric fields applied may be either steady DC fields, alternating fields applied at angles between 90° and 180°, or an AC field superimposed over a DC field. The gels employed for electrophoresis may represent either a tightly constrictive or a very loose structure around the migrating DNA; and the DNA molecules themselves may range in size from very small fragments to enormous chromosomes. For a model to be strictly correct, it would need to consider the polymeric character not only of the DNA molecules, but also of the gel or uncrosslinked polymers which form the sieving matrix. The two theories which have become accepted for steady-field electrophoresis reflect different approaches to reducing the number of degrees of freedom in this complicated theoretical problem.

The purely geometrical Ogston-Rodbard-Chrambach model treats DNA as a sphere, and gel fibers as straight, infinitely long rods. Neither the visualization of DNA as an equivalent sphere, nor of the gel as a random network of non-interacting, infinitely long, straight fibers seems to capture accurately the essential physics of the problem. The Ogston-Rodbard-Chrambach model has been used to estimate gel properties such as pore size or to gain information about DNA size and conformation during electrophoresis, by use of Ferguson plots [49, 52-55, 95, 96]. It is difficult to confirm the accuracy of these estimates by direct measurements. It has been accepted within the physics community that the model is only useful in modeling electrophoresis data for small DNA under low electric fields, since for larger DNA, experimental findings are in direct contradiction to the physical assumptions of the model.

Furthermore, some new findings of physicists Slater and Guo [97] may result in the complete abandonment of the Ogston-Rodbard-Chrambach model as a useful method for the analysis of gel electrophoresis data. In a recent paper, these authors have re-examined

the basic assumptions of the Ogston-Rodbard-Chrambach model, using a Monte-Carlo computer simulation algorithm to study the electrophoretic sieving of particles in gels [97]. They chose the simplest possible system for study: the motion of a simple particle in a "periodic gel" (a regular array of obstacles surrounded by free volume), where the only areas unavailable to the particle were the obstacles themselves. In this work, they showed that the most basic assumption of the Ogston-Rodbard-Chrambach model is incorrect. They found that it is not true, even in the simplest case, that the electrophoretic mobility of a particle in a gel is proportional to the volume fraction of the gel which is available for its passage. In fact, they found that a Ferguson plot is *inherently non-linear*. According to Slater and Guo, the failure of the most basic assumption of the Ogston-Rodbard-Chrambach model for gel electrophoresis of simple particles can be ascribed to the effects of three complicating factors, which are prevalent in electrophoresis but were overlooked in the formulation of the model: (1) the effect of electric field intensity, (2) the nature of the interactions between the particles and the obstacles, and (3) the nonuniform probability of occupation of the different available volumes [97].

The classical reptation model accounts for the polymeric nature of the DNA but models the gel only as a rigid, constrictive tube around the DNA. As for the Ogston-Rodbard-Chrambach model, the physical assumptions of the model are assumed to be violated when moderate to high electric fields are applied, so quantitative application of the model to electrophoresis results obtained at experimentally useful field strengths is not yet possible. The main success of the reptation model has been to elucidate and confirm the validity of the currently accepted picture for the physical mechanism of DNA separation in the most useful electrophoresis regime. An understanding of the mechanism has helped researchers to improve upon the technique and expand its usefulness; for example, the idea for pulsed-field electrophoresis, which now allows valuable studies of intact chromosomal DNA, arose from the understanding that orientation of large DNA molecules during electrophoresis was a limiting factor in the size of DNA which could be separated. However, recent experimental results have shown that the physical assumptions of the classical reptation model were far too simple. Several important features are not included in the model. First, there is the observed cycling of DNA molecules between tightly compact, and extremely extended conformations, in which the DNA chains often appear to be hung up on one gel obstacle in a U shape. Second, frictional drag in the classical reptation model arises only from DNA friction against the solvent in the tube; no direct polymer-polymer interactions between the DNA and the gel are considered, although

epifluorescence microscopy indicates that it is necessary to model the catching of DNA on gel obstacles. In the case of electrophoresis in flexible gels (such as polyacrylamide), polymer-polymer entanglement interactions and the deformation of the gel by DNA are expected to be very important. As of yet, a mathematically feasible way to model polymer-polymer interactions and include them in the reptation model has not been found, although Deutsch has used computer modeling to examine the sliding of DNA chains around solid obstacles [78, 79].

So far, the most ambitious and realistic attempt at modifying the classic reptation model to capture the essential physics of the problem is the biased reptation with fluctuations model including constraint release [93]. Like the classic reptation model, however, this model only applies in the limit of very low electric fields and is useful primarily as a tool for understanding the mechanism of separation, rather than for direct modeling of typical experimental data. For DNA electrophoresis at high electric fields (with CE in mind), Schönherr and Noolandi [98] have developed a "fluctuating bond model" based on Monte Carlo simulations, which takes into account both the persistance length of DNA and the random structure of the gel; but to our knowledge this computer model has not yet been applied by experimentalists to the quantitative interpretation of experimental data. To date no theory is successful in predictive modeling of DNA electrophoretic mobility in a gel under experimentally important field strengths, and as a function of DNA size, gel concentration, and temperature. Although the classic reptation model and its modified forms have demonstrated several qualitative successes, and it seems that the basic physical picture upon which they are based is accurate for DNA electrophoresis in a rigid gel, there is a long list of situations in which the models fail to predict the correct qualitative behavior, and in general, they are only quantitatively useful in a narrow range of conditions. These failures (and successes) are detailed in Zimm and Levene's recent review of the problems and prospects in the theory of DNA gel electrophoresis [44].

5. DNA electrophoresis in slab gels

It is typical for DNA electrophoresis to be performed in slab gels. CE, a new technique, is not yet in routine use by molecular biologists. In this section we discuss the practice of DNA electrophoresis in slab gels, so that the advantages and disadvantages of this ubiquitous technique may be compared to those of CE, which will be the subject of Section 6.

5. 1. Advantages of slab gel electrophoresis

The casting of gels in the form of thin slabs has several important advantages. First, relatively large samples can be loaded in each sample well (up to 10 μg of DNA), allowing slab gels to be used for preparative electrophoresis. Second, after electrophoresis, desired DNA bands can be cut out of the gel and purified for further manipulation [2]. Third, there is room for many adjacent sample wells in a single gel (usually 20 or more), allowing many lanes to be run in parallel. The ability to compare, side-by-side, the electrophoresis patterns of related samples is necessary for such techniques as DNA sequencing and DNase footprinting, as well as for studies of DNA tertiary structure (*e.g.*, the resolution of DNA topoisomers; the study of DNA structural polymorphisms [99]), and for mobility shift assays which are used to determine if a protein binds to a certain fragment of DNA. Fourth, Southern blotting can then be performed on slab gels, by binding DNA fragments from the gel onto a nylon filter which then be probed for a desired DNA sequence. We will first discuss the general practice of slab gel electrophoresis, and then we will compare the specific properties and electrophoresis applications of different types of gels.

5. 2. The practice of slab gel electrophoresis

5. 2. 1. General procedures for casting, loading, and running a slab gel

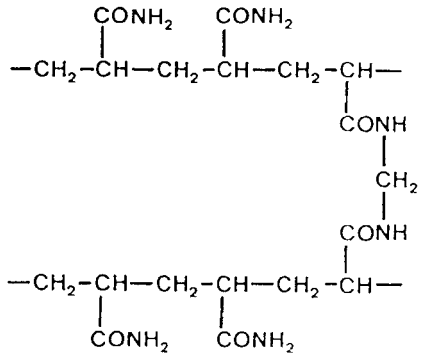
The technique of slab gel electrophoresis is one of the workhorses of a modern molecular biology laboratory. The protocols are relatively simple and have been well established for many years [2]. Two types of gels are routinely used: crosslinked polyacrylamide and agarose. The specific protocols for casting the two types of gels differ somewhat. In general, however, a slab gel is formed by pouring a liquid gel solution into either a horizontal or vertical mold and inserting a teflon comb at one end, the teeth of which will form the sample wells. The gel is then allowed to solidify, and the comb is removed. For polyacrylamide gels, the sample wells are then immediately rinsed out with water so that no gel fragments remain within them, to prevent uneven loading and distorting of DNA bands [2]. The slab gel is then immersed in buffer solution. Typically, nucleic acid samples are mixed with a 40% sucrose "loading buffer" containing a marker dye, such as bromophenol blue, which will migrate with an electrophoretic mobility close to that of small DNA fragments (*e.g.*, ~300 bp). This loading buffer adds color to the sample and also increases its density, making the loading process easier and allowing the progress of electrophoresis to be visually monitored [2]. The mixed samples are manually pipetted into the sample wells as rapidly and accurately as possible to minimize sample diffusion.

The slab gel is then subjected to electrophoresis in a safety-interlocked plexiglass box, to prevent electric shock. Preparing, pouring, and loading a slab gel usually takes 1.5 - 2 hours, including the time it takes for the gel to set. The electrophoretic separation of DNA in slab gels requires anywhere from 2 hours to 2 days, depending on the type of gel used, the size range of the DNA fragments to be separated, the resolution required, and whether steady-field or pulsed-field electrophoresis is used.

5. 2. 2. Polyacrylamide gels

Polyacrylamide gels are usually polymerized from acrylamide monomers and the crosslinker *N,N'*-methylenebisacrylamide (Bis), using two catalysts, persulfate (ammonium or potassium) and *N,N,N',N'*-tetramethylethylenediamine (TEMED) [50, 100]. The structure of polyacrylamide is shown in Figure 8(a). Oxygen is a potent inhibitor of the acrylamide polymerization reaction. To minimize contact with the air during polymerization, polyacrylamide gels are typically cast between two sheets of glass held apart by teflon spacers. Usually, polyacrylamide gels containing between 3.5% and 20% acrylamide monomer are crosslinked with 3 - 5% Bis; the pore size of the gel decreases linearly with increasing percentage of acrylamide monomer added. Upon polymerization (which requires about 60 minutes), a dense, crosslinked network of flexible polymer chains is formed which, because of its intrinsic structure, has pore sizes ranging from a few nm up to about 20 nm [100]. This tight pore network is effective for high-resolution separations of DNA ranging from 6 bp to about 1000 bp long, depending on the gel concentration and the level of crosslinking. Polyacrylamide gels are generally run in a vertical configuration in a constant electric field. Under a steady field, single base resolution can be routinely obtained for DNA fragments up to approximately 500 bases long [2]. It has recently been shown that if pulsed electric fields are used, the size limit for single base-pair resolution can be increased to 900 bases and the bands sharpened considerably, at the expense of a longer per-base analysis time [7, 101, 102]. The primary advantages of polyacrylamide gels are the following: (1) they have extremely high resolving power, and the pore size can be adjusted easily up to 20 nm; (2) relatively large DNA samples can be loaded (up to 10 μg); (3) they are optically transparent, particularly to UV light (which is often important for detection purposes); (4) they are electrically neutral, having no charged groups, so they neither bind charged analytes nor engender electroosmotic flow; (5) DNA samples isolated from polyacrylamide gels are extremely pure, since the gels contain no contaminants. On the other hand, polyacrylamide gels are more difficult to prepare and handle than agarose gels [2]. A particular disadvantage is the fact that the acrylamide monomer is a potent

(a)



(b)

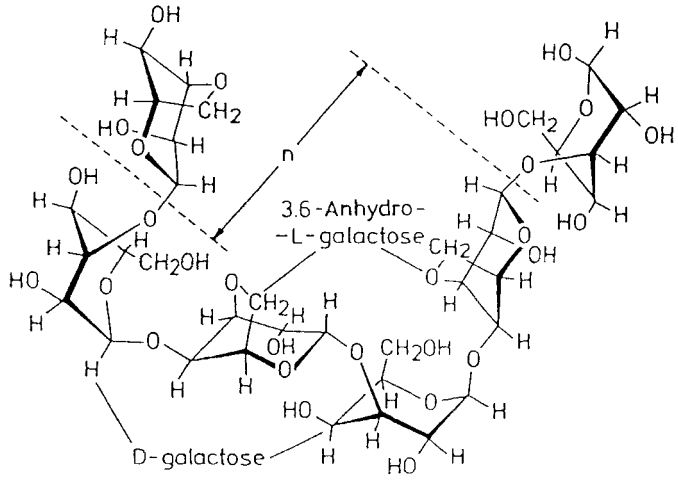


FIGURE 8
Chemical structures of (a) polyacrylamide and (b) agarose. [Reprinted, with permission, from Reference [394].]

neurotoxin which can be absorbed through the skin, and has cumulative affects. Furthermore, if the polymerization reaction is not driven to completion, reactive monomers which remain in the gel can react with the sample or the buffer components [103]. One-half hour of pre-electrophoresis before the sample is loaded can minimize this problem, as the charged monomers will rapidly migrate out of the gel.

Polyacrylamide gel electrophoresis can be used to resolve single-stranded DNA fragments, if a denaturant such as 8 M urea or formamide is added to the buffer to prevent the formation of DNA secondary structure [2]. Since polyacrylamide gels provide single base resolution, they are the most appropriate separation matrix for DNA sequencing (which requires the separation of single-stranded DNA) and DNase footprinting (in which the DNA is double-stranded), as well as for other techniques such as analysis of PCR products, which require the fine resolution of small DNA fragments (< 1000 bp).

5. 2. 3. Agarose gels

Agarose is obtained from seaweed and is a linear, alternating co-polymer of β-D-galactose and 3, 6-anhydro-α-L-galactose units (see Figure 8(b)) [104]. This is an idealized monomer, however, and most commercially available types contain sulfate, methyl, or pyruvate chain substituents, and may also be contaminated to varying degrees by other polysaccharides, proteins and salts [105, 106]. Such contaminants can affect the gelling temperature of the agarose and the mechanical strength of the resultant gel, as well as the electrophoretic mobility of DNA during electrophoresis. Furthermore, contaminants can act as enzyme inhibitors which may prevent further enzymatic manipulation of DNA isolated from agarose gels [2]. Many different types and grades of agarose are sold, however, some of which are carefully purified to minimize these problems [100]. The tendency to engender electroosmotic flow during electrophoresis is another significant, usually deleterious, property of agarose gels [106]. Since they contain charged groups (primarily negatively-charged) the motion of positive ions within the gel is accelerated during electrophoresis, while the motion of negative ions is slowed. As they move, the ions entrain solvent molecules, either as water of hydration or by friction. Since the positive ions move more rapidly, the result is a net electroosmotic flow of the buffer in the cathodic direction. This is counter to the direction of DNA electrophoresis, and retards the motion of DNA molecules. The effect is not noticeable for small DNA fragments, but can be significant for large DNA, particularly during pulsed-field electrophoresis when the net electrophoretic mobility of the DNA is low. "Low electroosmotic flow" agarose containing fewer charged

groups, such as FastLane™ agarose from FMC BioProducts (Rockland, ME, USA), is the matrix of choice for the separation of large DNA [107]. In general, care should be taken to choose the correct type of agarose for the separation at hand. A comprehensive review has recently appeared which compares the utility of different types of agarose for the electrophoresis of small and large DNA [108].

Agarose gels are formed by dissolving powdered agarose in the electrophoresis buffer, and then heating this mixture until the agarose melts and a clear solution is formed. The hot solution is then poured into a mold and left to cool and harden (requiring 30-45 minutes at room temperature [2]). Upon gelling, agarose fibers associate into networks of bundled helices; hence agarose gels have a more open and rigid structure than polyacrylamide gels [106]. An important property of agarose gels is their high mechanical strength, which allows the formation of gels with large pores (from the several hundreds of nm up to μm size) that can still be easily handled [100]. Highly pure samples of agarose show the greatest mechanical strength [106]. Hydroxyethylated agarose, which has a lower melting temperature (and lower mechanical strength), can also be obtained; this type of gel can be useful if DNA is to be isolated from the gel after electrophoresis.

Notwithstanding their lack of purity, agarose gels are highly useful for electrophoresis, as was first discovered by Hjertén [109]. Although they do not provide the fine resolution of polyacrylamide gels, they are nontoxic, easier to pour and handle, and can separate DNA in a much wider size range. In fact, DNA fragments between 200 bp and about 50 kbp long can be separated by steady-field electrophoresis on agarose slab gels. This makes agarose the most appropriate type of gel for the techniques of restriction mapping and for most DNA separations which will be used for Southern blot analyses. The DNA size range which can be separated in a single gel depends on the agarose concentration (which usually ranges between 0.3% and 2.0%) [2]. DNA larger than 40-50 kbp can be separated in agarose if pulsed electric fields are applied, as we shall discuss in Section 5. 3.

5. 2. 4. Temperature effects in slab gel electrophoresis

One of the main problems which occurs in slab gel electrophoresis is the creation of Joule heat by the passage of current through the gel and the buffer solution. Electrophoretic mobility is strongly dependent on temperature, increasing about 2% for every 1°C increase [110]. If a gel is run at too high a current and not sufficiently and uniformly cooled, significant Joule heating can occur and temperature gradients form in the gel. This can

have several affects: (1) often the gel will be cooler near the edges than in the center, causing analytes nearer the edge to run more slowly than those in the center, giving rise to a "smiling" effect which hampers side-by-side comparison of the mobilities of related DNA samples [111-113]; (2) temperature gradients cause band broadening due to natural convection of analytes; (3) if a substantial amount of Joule heat is created and not dissipated, an agarose gel may melt and/or the DNA samples may be denatured or destroyed. The Joule heating problem is solved by applying only low voltage gradients (agarose gels are generally run at no more than 5 V/cm, and polyacrylamide gels at no more than 8 V/cm) and using low-conductivity buffers (such as Tris-borate) [114]. In this way, electric current is kept to a minimum. In many electrophoresis apparatuses, the buffer is also circulated through a heat exchanger which cools it during electrophoresis. Even so, the phenomenon of Joule heating substantially limits the voltages which may be applied to slab gels, which means that DNA electrophoretic mobility is low and the time required for separation is rather long.

5. 2. 5. Detection of DNA in slab gels

For detection purposes, a DNA sample may be enzymatically radiolabeled prior to electrophoresis with ^{32}P, ^{35}S, ^{3}H, or ^{14}C-deoxynucleotide triphospates (dNTPs) [115, 116]. The most often used method for detecting radioactively labeled DNA in gels is autoradiography. After electrophoresis, the gel is exposed to X-ray film, usually overnight but sometimes for several days if the radioactive label is a weak emitter. Other techniques, more rarely used, include the use of scintillation counters, multiwire proportional counters [117], and photostimulable phosphors [118, 119].

Fluorescence detection of DNA is another commonly used technique for slab gel electrophoresis. DNA samples may be labeled either before, during, or after electrophoresis with a fluorescent dye which intercalates between the bases of the DNA, usually ethidium bromide (EtBr) [120]. Other intercalating dyes (such as thiazole orange (TO) and oxazole yellow (YO)) with enhanced spectroscopic properties and DNA binding affinities have recently become available [121-126]). Intercalating dyes, in general, are much more fluorescent when bound to DNA than when they are free in solution. For example, the fluorescence quantum efficiency of DNA-intercalated ethidium measured at 302 nm is 21 times greater than that of the free dye, because fluorescence quenching is reduced when the planar ethidium group resides in the hydrophobic regions between adjacent bases. Intercalating dyes are generally cationic, and affect DNA electrophoretic

mobility by neutralizing some of the DNA's negative charge, as well as by stiffening and lengthening the chain upon intercalation [127]. For analytical work, then, it is best to stain the gel with an intercalating dye *after* separation is complete. After electrophoresis, the fluorescence of ethidium-labeled DNA fragments is photographed under short- or long-wavelength UV light (302 nm or 590 nm) [128]. Alternatively, a charge-coupled device (CCD) may be used to quantitate fluorescence [129]. All of these methods for the detection of DNA are discussed in detail in a recent review of quantitative DNA slab gel electrophoresis [130]. Note that the two most commonly used detection methods involve the use either of radioactive compounds, which pose an obvious danger to the researcher, or of intercalating dyes, which are potent mutagens. Non-radioactive detection methods for nucleic acids (which are relatively new and not yet widely used) have been recently reviewed by Düring [131].

5. 3. Pulsed-field electrophoresis of large DNA in agarose slab gels

The highly porous structure of agarose allows DNA fragments up to 50 kbp in length to be separated under low, steady electric fields (less than 3 V/cm) [132, 133]. At higher electric fields, or even at lower fields for all DNA > 50 kbp in length, the size-dependence of electrophoretic mobility is lost [51], presumably because DNA becomes highly oriented in the field direction and no longer takes a tortuous path through the gel. Pulsed field gel electrophoresis (PFGE) can be used to separate larger DNA molecules, up to 10 Mbp in length, and so it is used for long-range restriction mapping and for the study of intact chromosomal DNA [12]. In this technique, an electric field that is applied alternately in two different directions requires large DNA molecules to change conformation and direction periodically [134, 135]. Separation is achieved because the time required to reorient depends on the length of the DNA molecule. The best results are obtained when field strengths are kept relatively low, and the pulse frequency is such that the molecule can change its conformation about once per pulse [70, 133, 136, 137]. There is a complicated dependence of DNA electrophoretic mobility on molecular size, field strength, and the pulse frequency and duration [138]. Furthermore, it has been shown that the angle between the alternating electric fields is an important parameter in determining DNA resolution, and that better results are obtained when obtuse angles (> 105°) are employed [139]. Because the net forward migration of the DNA molecules is slow, a separation by PFGE typically requires at least 24 hours [140-142], and sometimes as long as 65 hours [143]. Analysis times can be reduced and bands sharpened if a secondary, high-frequency pulsed field is superimposed over the primary pulsed field [144, 145]. Primarily because of

the complex behavior exhibited by DNA in pulsed fields, there has been considerable theoretical interest in modeling this problem [146-149]. Pulsed-field electrophoresis was recently reviewed by Dawkins [150] and by Lai *et al.* [151]; the orientational dynamics of DNA during electrophoresis were reviewed by Nordén *et al.* [152].

5. 4. DNA sequencing in ultra-thin polyacrylamide slab gels

The limits on applied voltage which are imposed upon slab gels as a result of Joule heating can be overcome if the slabs are extremely thin (25-100 μm). The use of ultra-thin polyacrylamide gels was pioneered by Radola [153, 154] for use in the isoelectric focusing of proteins. Ten years later, this idea was applied to polyacrylamide gels for DNA sequencing simultaneously by Stegemann *et al.* [155] and Brumley and Smith [156]. The apparatus of Brumley and Smith employed a horizontal polyacrylamide gel as thin as 25 μm, cast between two glass plates; a very thin comb was used to create many adjacent sample wells. On a single gel, 24 DNA samples (from 6 individual sets of sequencing reactions) were loaded manually. During electrophoresis, the bottom plate was cooled by circulating cold water underneath it. Using this apparatus, Joule heat could be dissipated more efficiently than for conventional slab gels, allowing electric fields as high as 215 V/cm to be applied (whereas conventional sequencing gels, which are usually about 250-500 μm thick, are run at approximately 40 V/cm). The thinness of the gel, in conjunction with autoradiographic detection, also resulted in greater sharpness of DNA bands, allowing more sequence to be read from a gel of a given length. Overall, Brumley and Smith found that the ultrathin horizontal slab gel provided a 77% increase in readable sequence per reaction in 1/6 the electrophoresis time [156]. Typically, conventional DNA sequencing gels allow the reading of about 300 bases per gel. Brumley and Smith [156] reported that using ultrathin slab gels, up to 560 bases could be read with single base resolution from a single, 25 cm-long gel. This limit was pushed to 1000 bases on a 50 cm-long ultrathin slab gel by Ansorge, *et al.* [6] and to 1040 bases on a 93 cm-long gel by Nishikawa and Kambara [157].

5. 5. Novel gels for DNA slab gel electrophoresis

Polyacrylamide and agarose gels are not the only separation matrices available for DNA electrophoresis, although they are by far the most common. Although there have been some dramatic departures, many novel gels are variations on the theme of polyacrylamide or agarose, molecularly engineered to have superior characteristics for certain types of separations. For example, in the polymerization of polyacrylamide gels, the use of

different crosslinking molecules beside Bis has been investigated [158], as well as the use of novel acrylamido monomers [159]. Many researchers have used various admixtures of polyacrylamide and agarose, in an attempt to combine the advantages of both types of gels. In 1968, Peacock and Dingman [160] suggested that adding some agarose to low-concentration polyacrylamide gels would endow them with greater mechanical strength. Accordingly, large pore-size agarose has been used as a support for relatively dilute polyacrylamide gels which alone would have unacceptably low mechanical strength [161-163]. Bode attempted to improve the ability of concentrated agarose to resolve small DNA fragments by adding linear polyacrylamide to the buffer solution [86, 87]. He found that covalent linkages between the polymers forming the separation matrix were not a prerequisite for DNA separation, and that in fact linear polymers in solution could also provide separation [85, 88]. Along the same lines, it was shown by Perlman *et al.* [164] that if DNA is separated in agarose gels (at low, non-restrictive concentrations) which were supplemented with solutions of linear polysaccharides (such as hydroxyethyl cellulose, methyl cellulose, or galactomannan), superior DNA resolution and optical clarity were obtained as compared to those obtainable in conventional high-concentration agarose gels. These composite gels are inexpensive and easily poured from the melt, like agarose. The authors noted, however, that this technique could not replace polyacrylamide gels for the fine resolution of DNA smaller than 75 bp [164].

Another approach has been to synthesize novel acrylic monomers to create gels which have similar but superior properties to those of polyacrylamide [165]. Low-concentration polyacrylamide gels (3%) are often used to separate DNA larger than 1000 bp, but these gels lack mechanical strength and usually contain a high proportion of unpolymerized monomer, which on one hand is a neurotoxin, and on the other hand can react irreversibly with the sample [103]. With the goal of improving the resolution of DNA > 1000 bp in polyacrylamide gels, Righetti *et al.* have employed novel polymerization techniques to create 'laterally-aggregated,' large-pore-size polyacrylamide gels [166]. Poly-*N*-acryloyl-tris (NAT) gels, introduced by Kozulic *et al.* [167, 168] were found to give substantially improved separation of DNA fragments larger than 200 bp, as compared to polyacrylamide. The authors suggested that NAT-based gels perfectly fill the gap between the optimal separation range of polyacrylamide and of agarose gels [167, 168]. Chiari, *et al.*, however, have pointed out that the NAT monomer is inherently unstable, and degrades with zero-order kinetics [169].

An undesirable trait of polyacrylamide gels is their susceptibility to hydrolytic cleavage at high pH (DNA electrophoresis is typically carried out at pH 8); thus their use cannot be prolonged, and they cannot be re-used, as might be desirable for a large-scale sequencing project [103, 169]. Chiari *et al.* have recently introduced another novel acrylic monomer to create more stable gels: *N*-acryloylaminoethoxyethanol (AAEE) [169]. In fact, the AAEE monomer has been shown to be 500 times more stable than acrylamide. Gels and physical networks of poly-AAEE have been shown to be useful for the separation of PCR products by slab gel electrophoresis [170, 171] and of larger DNA restriction fragments (up to 23 kbp) by CE [172]. In short, this novel polymer appears to be very promising for DNA electrophoresis applications.

Another novel gel is HydroLink™, invented several years ago by Righetti's group [173] and marketed by AT Biochem (Malvern, PA). This amphiphilic gel is composed of various crosslinkers, several of which are more hydrophobic than Bis, that are polymerized with acrylamide. HydroLink™ is reported to have properties intermediate between those of polyacrylamide and agarose. The polymers making up this mechanically strong gel matrix form "pillars" with a hydrophobic core and a hydrophilic coating [173]. The performance of HydroLink™ gels for slab gel electrophoresis of DNA was shown to be quite good [174, 175]. Linear resolution of DNA ranging from 100 to 5000 bp was obtained, and good (though non-linear) resolution from 6000 to 23000 bp [174]. Hydrolink™ gels have also been shown to provide the separation of single-stranded DNA sequencing products ranging in size from 35 to 230 bases in length with single base resolution [176].

6. Capillary electrophoresis (CE)

6. 1. The invention of CE

While electrophoretic DNA separations typically are carried out in slab gels, there are many practical advantages to performing these separations in microbore capillaries (inner diameter (i.d.) 25-100 μm). The technique of electrophoresis in tubes with sub-millimeter diameters was conceived in 1974 by the Finnish researcher Rauno Virtanen [177], and its potential further illustrated in 1979 by Mikkers, Everaerts, and Verheggen [178]. Virtanen separated alkali cations in 200-500 μm i.d. glass tubes using potentiometric detection [177], while Mikkers, Everaerts and Verheggen separated small charged molecules using 200 μm i.d. teflon tubes and UV absorbance detection [178]. In 1981, Jorgenson and Lukacs demonstrated that extremely high resolution and small plate heights could be obtained for

the electrophoretic separation of dansylated amino acids in a glass capillary with a 75 μm i.d. [179-181]. This work attracted the attention of other researchers in the area of biotechnology to the new technique of "capillary zone electrophoresis," as it was first named [182]; the word "zone" has since been dropped [183].

6. 2. Features and advantages of CE

It is interesting to note that in his seminal paper, Virtanen's first reference is to the classic chemical engineering text on transport phenomena by Bird, Stewart, and Lightfoot [184]; for it is the unique transport properties of microbore capillaries that make them eminently suitable for electrophoresis. As we have discussed, the most significant limitation in slab gel electrophoresis is the Joule heating which results from current flow through the system. Joule heating effects limit useful voltage gradients to approximately 5 V/cm for an agarose gel, 8 V/cm for a conventional polyacrylamide gel, and 40 V/cm for a thin polyacrylamide DNA sequencing gel [2].

The above-mentioned limits in applied voltage may be overcome if electrophoresis is carried out in a microbore capillary. Today, a typical capillary used for DNA electrophoresis has a 50-100 μm i.d., fused silica walls about 150 μm thick, and a thin exterior coating (10 μm) of polyimide to provide flexibility. During electrophoresis, Joule heat is produced in the tiny volume inside the capillary, but the thick fused silica wall acts as an efficient heat sink, absorbing heat which is then rapidly dissipated from the relatively large surface area of the outer walls. The efficient dissipation of Joule heat afforded by the high surface-area-to-volume ratio of a capillary virtually eliminates thermal and gravitational convection and allows electric fields of up to 700 V/cm to be used without deleterious Joule heating, if the capillary is effectively thermostatted during electrophoresis [185]. Field strengths of about 300 V/cm are more typically used, and can provide DNA separations which are up to 25 times faster than slab gel electrophoresis [186]. The smaller the capillary diameter, the higher the field which can be applied without substantial production of Joule heat; thermal effects become significant for capillaries with inner diameters of 100 μm or more [187]. For these rapid separations at high voltages, efficiencies of at least 100,000 theoretical plates/meter are routinely obtained [187], and with careful optimization, efficiencies as high as 30 million theoretical plates/meter have been achieved for oligonucleotide separation in a polyacrylamide gel-filled capillary [188]. Furthermore, DNA separations by CE are typically complete in under 20 minutes (as compared to analysis times of 2-20 hours which are required within a slab gel). Another

advantage of CE is its adaptability to automatic, on-column sample loading, as well as on-column detection.

Figure 9 shows a schematic illustration of a CE apparatus. Typically, CE is driven by a power supply with a 30,000-60,000 V capacity. The ends of the fused silica or quartz capillary are immersed in two electrolyte reservoirs, one of which is connected to the power supply by a platinum electrode, and the other of which is grounded. On a commercial CE instrument, such as the Beckman P/ACE system (Beckman Instruments, Palo Alto, CA, USA), one is able to program the apparatus to automatically inject a liquid sample slug by the timed application of a controlled driving force for injection -- a fixed pressure differential across the capillary for hydrodynamic injection, or a fixed voltage for electrokinetic injection (sample injection methods are discussed in Section 6. 5.). While injection usually occurs at the high-voltage end of the capillary, the on-column detection system (Section 6. 6.) is situated close to the grounded end. Commercial CE systems are usually equipped with an electronically-driven control system, and an ohmmeter to allow the monitoring of current. All of this is sealed in a chamber which serves two purposes: to allow precise thermal control of the environment around the capillary by air or water circulation, and to prevent the operator from sustaining electric shock (when the chamber is opened, the field is automatically cut off).

Although high-voltage electric fields are applied, the relatively high resistance of the fused silica capillary and of the buffers which are used results in the production of low currents (usually less than 20 μA), which essentially prevents electrolysis of the aqueous buffer from occurring. Buffer reservoirs at either end of the capillary are also large in volume (several mL) when compared to the capillary volume (~ 1 μL), once again acting to minimize electrolysis. Nonetheless, it is important to degas the electrophoresis buffer under a vacuum before use.

Optimum performance in CE is obtained when DNA samples are about three orders of magnitude smaller than those used in slab gel electrophoresis (nL of sample as opposed to μL). Thus the technique serves extremely well for analytical purposes. As an analytical technique, however, it demands stricter reproducibility and more precise quantification of peak areas and migration times than slab gel electrophoresis. By analogy to figures-of-merit in chromatography, the precision of the data should be about 1% and the standard deviation in the data for successive runs should be less than 5% [189]. Vespalec *et al.* [190]

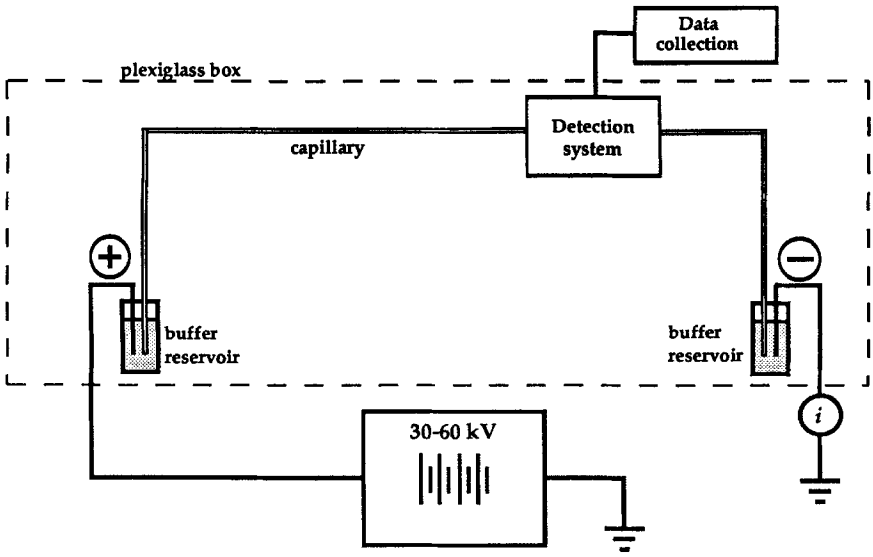


FIGURE 9
A schematic diagram of a typical capillary electrophoresis apparatus.

have shown that the injection of internal standards with known mobilities, along with the sample, allows the calculation of more reliable, reproducible electrophoretic mobility data; a similar approach has also been taken by Sirén *et al.* to reliably detect trace chemicals in body fluids using CE [191]. Lee and Yeung have proposed a method, using migration indices, to improve precision and to facilitate data transfer in CE [192]. With this method, data taken at different constant or gradient field potentials can be compared consistently.

6. 3. The effect of temperature on CE of DNA

Electrophoretic mobility is very sensitive to temperature (increasing about 2% for each 1°K increase in temperature, primarily due to a decrease in buffer viscosity [110]), hence good temperature control is essential for the reproducibility of analyte electrophoretic mobilities [193, 194]. If the capillary is not adequately cooled, there will be a parabolic temperature profile across the capillary cross-section due to the production of Joule heat. This will cause the formation of a parabolic electrophoretic velocity profile, leading to band broadening. Hjertén has pointed out that even if the temperature difference between the

capillary wall and its centerline is only 0.5°K, a band which has migrated 250 mm at the wall of an electrophoresis tube has accordingly migrated 3.5 mm further at the tube centerline [195]. This effect by itself has the potential to cause a significant amount of band broadening; and it also provides a driving force for Taylor dispersion, as shown in Figure 10 [195, 196]. Theory predicts that for a 50 μm i.d. capillary under a field of 300 V/cm and surrounded by air at 298°K, the difference in temperature between the capillary center and the wall is about 1.4°K (3.1°K for a 75 μm capillary and 5.6°K for a 100 μm capillary) [193]. In reality, then, it is highly desirable to use small-diameter capillaries (< 100 μm i.d.) in conjunction with efficient cooling systems, and to choose the field strength and current density to minimize temperature differences across the capillary diameter. To cool the capillary during electrophoresis, forced air convection at a precisely controlled temperature is typically employed; a flowing liquid bath surrounding the capillary provides more rapid heat transfer, but is somewhat more complex to implement [197]. Solid-state Peltier devices remove Joule heat from the capillary very efficiently [198], and have been incorporated into some commercial CE systems.

A considerable amount of theoretical work has been done to accurately predict the parabolic temperature profile which exists within the capillary during electrophoresis [110, 193, 196], as well as to estimate the average temperature at the center of the capillary during electrophoresis [199, 200] for various inner diameters, applied voltages, currents, and external temperatures. Under typical operating conditions, however, the peak variance due to thermal band broadening has been found to be negligibly small compared to other sources of variance, even if the temperature rises quite a bit [24, 193, 201-203]; more significant band broadening is likely to result from overloading of the capillary with sample, electrokinetic dispersion due to differences in the conductivity and/or pH of the sample and the surrounding buffer, and/or dynamic adsorption of the analyte molecules onto the capillary wall [187, 195, 204]. In a properly designed and optimized CE system, however, the effects of all of these factors can be minimized and extremely narrow bands can be obtained [188, 205, 206].

6.4. Electroosmotic flow within capillaries

Another important characteristic of the fused silica capillaries which are typically used for electrophoresis is the static negative charge which they carry on their inner surface. The surface silanol groups in fused silica have a pK_a near 2 [22]; since separations of native double-stranded DNA are typically carried out in buffers of pH 8, the fused silica surface is

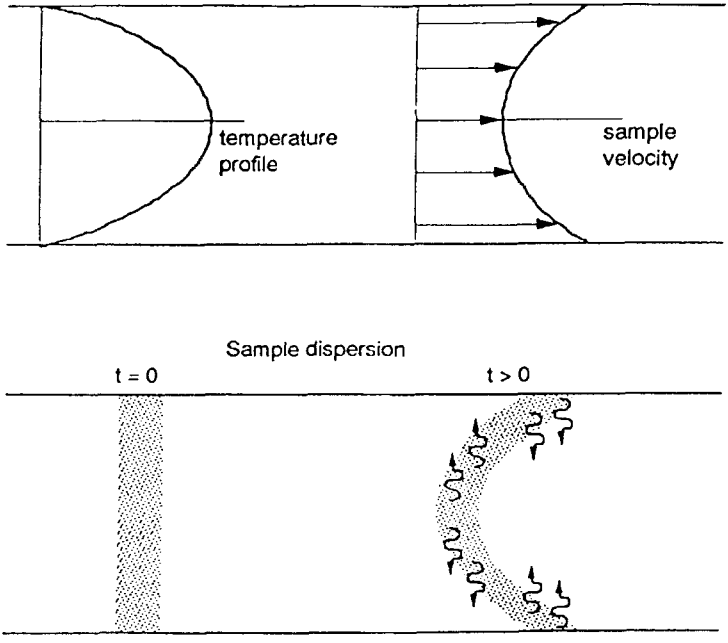


FIGURE 10
Taylor dispersion in a heated capillary. The temperature profile in the lumen of the capillary distorts the sample's electrophoretic velocity profile. Molecular diffusion ameliorates the effect by radially averaging the concentration. The sample is depicted migrating in a direction opposite to that of electroosmotic flow. t = time. [Reprinted, with permission, from Reference [193].]

virtually 100% ionized and highly negatively charged during DNA electrophoresis. Therefore strong electroosmosis occurs within the capillary when a high-voltage electric field is applied. Electroosmosis is the flow of a conducting liquid engendered by its contact with a charged surface, under the influence of a tangentially applied electric field [207]. We will provide a brief description of electroosmosis here; more detailed discussions of this electrokinetic phenomenon may be found in standard texts on the physical chemistry of surfaces (*e.g.*, [208, 209]).

Since the capillary surface carries a negative charge, positive counter-ions from the buffer solution are preferentially adsorbed there. As a result, an electrical double layer is formed

at the silica-buffer solution interface, consisting of an immobile layer of positive ions which are strongly adsorbed to the solid surface (called the *Stern layer*), and a diffuse double layer extending into the liquid (Figure 11). When a tangential electric field is applied, the mobile part of the diffuse double layer (referred to as the *Gouy-Chapman layer*) migrates toward the cathode in response to the electric force which acts on the excess positive charge within it. As a consequence, a shear plane is formed at some distance from the fused silica surface. A constant, steady-state flow velocity is reached when the force exerted on the positive counter-ions (and thus on the liquid as a whole, since the ions entrain solvent molecules as water of hydration, as well as by frictional drag) is balanced by the frictional forces which result from the viscosity of the solvent. The potential at this shear plane is termed the *zeta potential* (ζ). The zeta potential of an aqueous solution (a polar solvent) in contact with glass (a polar surface) can have a magnitude as high as 100 mV [207]. The double layer thickness, δ, is more or less arbitrarily defined as the distance from the immobile layer to a point at which the potential has dropped to 37% of the zeta potential at the interface between the immobile and diffuse layers. The linear velocity, u, of the solvent under the influence of the applied electric field, E, is given by the equation

$$u = \left(\frac{\varepsilon}{4 \pi \eta}\right) E \zeta \qquad (10)$$

where ε and η are the dielectric constant and the viscosity of the solution, respectively. The electroosmotic mobility, μ_{eo}, is then defined as

$$\mu_{eo} = \frac{u}{E} \qquad (11)$$

The velocity of electroosmotic flow can be determined by injecting a neutral, easily detectable molecule at the anodic end of the capillary [210-213], and recording the time it passes the detector (t_d), which should be situated near the cathode. An uncharged molecule will move at the velocity of electroosmotic flow, hence the average electroosmotic velocity u is equal to L_d/t_d, where L_d is the effective length of the capillary (the length to the detection window). Of course, for this purpose, the marker must be truly neutral and have negligible interactions with the capillary wall. Mesityl oxide is a molecule which fits this description [213]. More precise methods for measuring electroosmotic flow, some of which can detect minute variations in μ_{eo} occurring during a single run, have been proposed [191, 214-217].

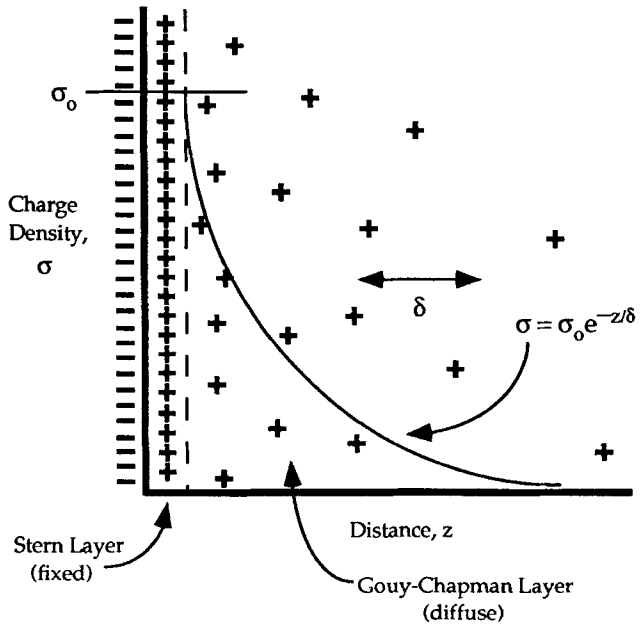


FIGURE 11
A schematic representation of the electrical double layer showing the negatively charged surface, fixed excess positive ions in the Stern layer and mobile excess positive ions in the diffuse Gouy-Chapman layer. The so-called "double-layer thickness" is shown as δ. [After a diagram in Reference [181], with permission.]

Unlike laminar flow, which has a parabolic velocity profile, electroosmotic flow is theoretically predicted to have an almost completely flat velocity profile, which varies only within the thickness of the double layer and reaches a constant velocity outside of it (see Figure 12). In a typical DNA electrophoresis buffer within a fused silica capillary, the double layer is around 3 nm (30 Å) thick [22]. Since the inner diameter of the capillary is typically 50 μm, the region of velocity variation is negligible and the flow profile should be essentially flat across the entire capillary. Tsuda *et al.* [202] studied the minimum plate height which is experimentally achievable in an open capillary in the presence of electroosmotic flow; it was not as small as theory predicted for a flat electroosmotic velocity profile. They concluded that in reality the electroosmotic velocity profile is not quite flat, but instead slightly parabolic, though much less parabolic than that of pressure-

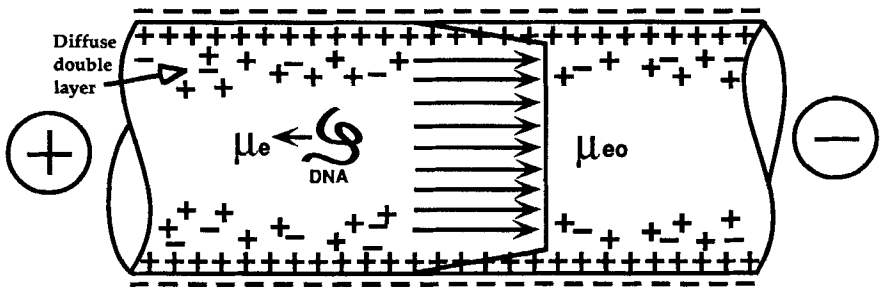


FIGURE 12
A schematic diagram of "counter-migration capillary electrophoresis" for the separation of DNA molecules. DNA migrates by electrophoresis in the anodic direction. Electroosmotic flow moves with a greater velocity in the cathodic direction, pulling the DNA toward a detector situated at the cathodic end of the capillary. Thus, in the presence of a polymer solution within the capillary (not shown) to separate the DNA based on its size, the largest, most slowly-migrating DNA molecules will be swept past the detecter, followed by the faster DNA in order of decreasing size.

driven flow. In later work, Tsuda and Ikedo, in collaboration with Zare's group, used a fluorescent solution and epifluorescence microscopy to visualize the flow profile of electroosmosis in rectangular capillaries (1 mm x 50 μm) [218]. They found that the electroosmotic velocity profile was close to, but not quite flat; instead, the profile had unexplained "leading edges." Based on their findings with the rectangular capillary, they predicted that in a cylindrical capillary, the electroosmotic flow profile would be an inverted, extremely shallow, parabola (*i.e.*, the velocity would be a bit lower at the center of the capillary than at the walls).

6. 4. 1. Electroosmosis as a "pump" for DNA electrophoresis

Although for some applications it is desirable to eliminate electroosmosis, as we shall discuss, for others it is useful to maximize its velocity. The use of electrophoresis buffers which are low in ionic strength and high in pH will produce the greatest electroosmotic flow velocities in fused silica capillaries [219, 220]. Under typical experimental conditions for DNA separations (300 V/cm), the electroosmotic mobility within a fused silica capillary is 1.25-3 times greater than the electrophoretic mobility of DNA molecules, and opposite in direction, since DNA is negatively-charged and hence migrates toward the anode (refer to Figure 12). This rapid flow of buffer with a relatively flat velocity profile makes

electroosmotic flow an excellent "pump" for electrophoresis, for it can be used to pull all analytes, regardless of their mobility, past the detector without contributing significantly to band broadening [181, 207]. Experimentally, it has been shown that the electroosmotic flow velocity is directly proportional to the value of the electric current per unit cross-sectional area (the current density); if the specific resistance remains constant during a run, it will also be proportional to the electric field [202]. However, even the introduction of a small bubble can change the specific resistance of the capillary/buffer system. Hence, if a constant electroosmotic flow velocity is desired it is best to run at constant current.

As we mentioned in Section 4. 4., DNA size separation can be carried out in dilute, low-viscosity polymer solutions, which will flow rapidly through the capillary by electroosmosis [90, 91, 195, 221]. This approach can be used for the separation of DNA restriction fragments and oligonucleotides. DNA will not adsorb ionically to the fused silica surface, but instead is repelled from the negatively-charged wall. This is important, as analyte adsorption causes significant band broadening. Thus, DNA can be injected into the anodic end of the capillary, and when the field is switched on it will be pulled by rapid electroosmosis in the *cathodic* direction, while it migrates electrophoretically in the *anodic* direction. In this case, the detector will be placed near the cathodic end of the capillary, and electroosmotic flow will serve to pull the DNA rapidly past the detector. This technique has been called *counter-migration CE* [222], because the DNA is "swimming upstream" against electroosmotic flow. Hence the slowest, largest-molecular weight DNA will be pulled past the detector first, followed by smaller DNA in order of decreasing size. This situation is illustrated schematically in Figure 12. If the DNA sample is mixed with a neutral marker, then for a given run the average electroosmotic velocity can be calculated with Equations (10) and (11). The *apparent* electrophoretic velocity of the DNA bands, μ_{app}, can be calculated with Equation (1). Since electroosmotic flow has a flat velocity profile, it displaces all of the DNA bands to the same extent. Thus, their actual electrophoretic mobilities can be calculated with the equation

$$\mu_e = \mu_{app} - \mu_{eo} \qquad (12)$$

Beckers, *et al.*, [213] studied the use of electroosmotic flow as a pump for the capillary electrophoretic separation of small charged molecules. Based on an extensive, quantitative study, they found this technique would yield accurate and reproducible values of electrophoretic mobilities, if a neutral marker (mesityl oxide) was used to calculate μ_{eo} and μ_e was calculated with Equation (12).

6. 4. 2. Methods of controlling electroosmotic flow in capillaries

For some applications there are deleterious effects which result from the charged silanoate groups on the capillary wall and the resultant electroosmotic flow. For example, when native proteins are separated by electrophoresis, basic proteins (*e.g.*, cytochrome c) will be attracted to the capillary wall if the buffer pH is greater than ~ 3. If adsorption interactions are significant, the proteins will elute as very broad bands; some adsorb so strongly that they do not elute at all. In general, the recovery of proteins will vary inversely with the protein isoelectric point (pI) [223]. Basic proteins will preferentially adsorb near the capillary inlet; and where they have adsorbed, the surface charge is altered and a non-uniform axial distribution of zeta potential within the capillary is set up, causing recirculation of buffer in this region with a resultant mixing of bands as they pass [223]. Clearly, local recirculation of buffer and a non-uniform electroosmotic profile can cause a huge amount of band broadening. This problem will usually occur only when basic analytes are included in the sample.

Even when exclusively negatively-charged, non-adsorbing moieties such as DNA are being separated, many researchers prefer to suppress electroosmotic flow completely. The perceived problem is that the flat velocity profile of electroosmotic flow can become distorted if there is a parabolic temperature profile in the capillary, or if there is some non-uniformity in the silica surface charge along the length of the capillary [195, 196, 202, 210, 224]. Electroosmotic flow is definitely undesirable when polyacrylamide gel-filled capillaries are employed for DNA separations; in this case, electroosmotic forces within the capillary may sometimes cause the gel to migrate out of the capillary during electrophoresis, clearly an undesirable occurrence [225]! Also, when DNA is detected using laser-induced fluorescence detection [226, 227] with intercalating fluorescent dyes such as ethidium bromide, the cationic dyes can adsorb onto the wall and progressively change the velocity of the electroosmotic flow.

For any one of the above reasons, it may be desirable to suppress electroosmotic flow within the capillary. Electroosmosis can be controlled by the application of radial electric fields [228-234], by chemical modification of the capillary wall [231, 235-248], by the adjustment of buffer pH [219, 249, 250] or buffer concentration [219, 251-255], and by the addition of surfactants [242, 256-259] or organic modifiers [260] to the electrophoresis buffer.

However, when DNA separations are to be carried out, it is generally preferable to perform them near pH 8 in 100-200 mM buffers, to keep DNA in its native double-stranded, helical conformation and to keep ionic strength low so that excessive currents are not generated during high-field CE. The addition of surfactants or organic compounds is to also be avoided if possible, since post-column purification of DNA would then become more difficult. When an additional, radial electric field is applied externally to a capillary, the polarity and magnitude of the zeta potential of the inner wall can be changed at will [228-234, 261]. Under certain conditions, this technique can be used to slow, stop, or even reverse the direction of the electroosmotic flow within fused silica capillaries. However, the control of electroosmotic flow by the application of radial electric fields is only effective when low pH buffers are used (pH < 6) [234] or when surface-modified capillaries having a very low surface concentration of charged silanol groups are used [245]. This pH limitation means they are not really applicable for DNA separations in fused silica capillaries, because DNA degrades rapidly at low pH. Furthermore, interactive control of the radial electric field is required to maintain a consistent electroosmotic velocity, and such a system is not yet available. In short, although this method of controlling electroosmotic flow in fused silica capillaries seems promising for electrophoretic separations which can be carried out at relatively low pH, but probably will not be very useful for DNA separations, which must carried out at pH 8 to maintain sample integrity.

At present, the only method of controlling electroosmosis which is easily compatible with DNA separations is the coating of the capillary walls [235-248]. This approach was first suggested by Hjertén in 1967 for electrophoresis in a rotating, 360 mm-i.d. quartz tube [235]. He based his idea upon an examination of the following general equation for linear velocity of electroosmosis, in which $\psi(x)$ is the potential at a distance x from the tube wall and ζ is the zeta potential,

$$u = \frac{\varepsilon E}{4\pi}\int_0^{\zeta} \frac{1}{\eta}\, d\,\psi(x) \qquad (13)$$

At constant viscosity η, this expression is equivalent to Equation (9). Hjertén realized that if the viscosity in the double layer could be made very large or infinite, then the electroosmotic flow velocity could be reduced essentially to zero [235]. He accomplished this by coating the wall of the 350 mm-i.d. electrophoresis tubes he was using with methyl

cellulose, crosslinked with formaldehyde at high temperature and low pH [235]. Later, after CE was invented in 1981 [179-182], it became clear that a more stable coating would be necessary if protein separations were to be carried out at reasonable pH by this new technique. The first practical, stable and effective coating for fused silica capillaries was also invented by Hjertén, who showed in 1985 that a covalently attached, hydrophilic coating of polyacrylamide practically eliminates electroosmosis in fused silica capillaries and also sterically prevents the adsorption of basic proteins [237]. In short, the procedure consists of two steps: (1) γ-methacryloxypropyl-trimethoxysilane is covalently bound to the fused silica wall, then (2) acrylamide is simultaneously polymerized and attached via the silane groups to the wall. The resultant coating consists of a thin layer of polyacrylamide covalently bound to the capillary wall, which extends beyond the thickness of the double layer, greatly increasing the viscosity in this region and effectively preventing electroosmotic flow. Hjertén's polyacrylamide coating has become widely used, for it is relatively simple to synthesize and remains stable over a wide range of pH (pH 2-pH 11). Several other coating procedures have since been proposed [238-242, 244-248, 262], but none has yet supplanted Hjertén's procedure in popularity. (Recent advances in capillary coatings have been reviewed by Wehr [263].) Recently, Hjertén and Kubo [243] reported the invention of a new type of coating which uses hydrophilic polymers such as dextran or methyl cellulose which are less susceptible to hydrolytic cleavage than polyacrylamide, and is stable between pH 0.3 and pH 12.7. Hence, the coating will withstand short rinses of 0.05 M NaOH or 1 M HCl, to remove adsorbed proteins, etc. The protocol for synthesizing this ultra-stable coating is somewhat more complicated than that for Hjertén's polyacrylamide coating [237], and this wider range of pH stability is presently only necessary for certain applications (*e.g.*, some protein separations), and not for DNA electrophoresis. In summary, electroosmotic flow, with its relatively flat velocity profile, is useful for some applications of DNA electrophoresis and must be completely suppressed for others.

In the following sections, we will discuss the methods which have been developed for DNA sample introduction into capillaries, on-column detection of DNA, and methods for collecting fractions after electrophoretic separation is complete.

6. 5. Sample injection methods for CE of DNA

In slab gel electrophoresis, DNA samples are manually pipetted into pre-formed sample wells in a submarine gel, and care must be taken to load the samples quickly and

accurately in order to minimize diffusion. One of the great advantages of performing electrophoresis in microbore capillaries is that samples can be loaded automatically, and the amount of sample injected can be calculated and controlled precisely. The potential of CE for efficient, high resolution separations can only be realized if the sample injection method does not contribute significantly to band broadening. The best performance is obtained when extremely small sample volumes are injected (2 - 100 nL). Overloading of the capillary can cause significant band broadening, so it is also imperative that injection methods be able to deliver precise sample volumes. Two types of on-line injection satisfy these criteria: electrokinetic injection, and hydrodynamic injection.

6. 5. 1. Electrokinetic injection

When electrokinetic injection is performed, one end of the capillary is removed from the buffer reservoir and placed in the sample vial (which may contain as little as 2 μL of sample) along with a platinum wire electrode. An electric field is then applied for a short period of time, to force the charged analytes to migrate into the capillary both by electrophoretic migration and/or by electroosmotic flow of the sample solution. Usually, however, for DNA electrophoresis, electrokinetic injection is used in conjunction with polyacrylamide-coated capillaries in which electroosmotic flow has been eliminated[187]. In this case, the DNA sample is injected electrokinetically at the cathodic end of the capillary, and the detector is located near the anodic end. If *uncoated* capillaries are used, electrokinetic injection of DNA is more difficult, due to the strong electroosmotic flow which prevails in the cathodic direction. In general, when uncoated capillaries are used for DNA separation it is simpler and more efficient to use hydrodynamic injection, as we shall discuss. If gel-filled capillaries are used there is no electroosmotic flow and electrokinetic injection at the anodic end of the capillary is appropriate. The following equations apply generally to the cases of electrokinetic injection in both the presence and absence of electroosmotic flow.

For both electrokinetic and hydrodynamic injection, the quantity of sample, Q, injected into the capillary by on-column injection is given by the product of the sample volume introduced and the sample concentration, C, such that [264]

$$Q = \pi r^2 L_s C \qquad (14)$$

where r is the inner radius of the capillary and L_S is the length of the sample zone. When a

sample is introduced electrokinetically, the length of the sample zone is given by the equation

$$L_s = t_{inj}(v + u) \tag{15}$$

where t_{inj} is the injection time, v is the electrophoretic velocity of the analytes within the sample, and u is the electroosmotic velocity. The values of v and u are given by the following equations:

$$v = \mu_e E = \mu_e \frac{V}{L_t} \tag{16}$$

$$u = \mu_{eo} E = \mu_{eo} \frac{V}{L_t} \tag{17}$$

where μ_e is the electrophoretic mobility of the sample, μ_{eo} is the electroosmotic mobility of the buffer, E is the field strength in V/cm, V is the total applied voltage, and L_t is the total capillary length. Substituting Equations (16) and (17) into Equation (15), the expression for the sample length becomes

$$L_s = t_{inj} \frac{V}{L_t} (\mu_e + \mu_{eo}) \tag{18}$$

Combining Equations (14) and (18), the amount of sample introduced to the capillary can be expressed as

$$Q = \frac{\pi r^2 (\mu_e + \mu_{eo}) t_{inj} V}{L_t} C \tag{19}$$

Thus, for a given sample concentration, the quantity of sample introduced can be directly controlled through the applied voltage, V, or the injection time, t_{inj} [264]. In order to inject equal amounts of sample into capillaries with different diameters, the injection time (or injection voltage, for electrokinetic injection) must be carefully adjusted [187]. It is also important to note that because of the contribution of analyte electrophoretic mobility to the quantity injected, there is some bias in the amounts of various analytes introduced during electrokinetic injection, with the more mobile analytes injected in greater quantities than

less mobile analytes [264, 265]. The actual amount of each analyte injected can be calculated using Equation (19), once the electrophoretic mobilities are known; however, this sampling bias is a general disadvantage of electrokinetic injection. In principle, however, all DNA molecules > 8 bp in length have the same steady-state electrophoretic mobility in free solution (and they will usually be injected from free solution, into a gel or polymer solution), so sampling bias between DNA molecules of different lengths should result only from differences in the ability of the DNA molecules of various sizes to migrate into the separation matrix within the capillary. This sampling bias may or may not be significant, depending on the DNA size and the density and pore size of the separation matrix which is used.

Equation (18) is only strictly correct when the sample conductivity is equal to the conductivity of the electrophoresis buffer. The efficiency of electrokinetic injection is also affected by the conductivity of the sample solution; the amount of sample injected increases almost linearly with increasing resistance of the sample solution [265]. This phenomenon can be exploited to increase the amount of sample injected in a given time (*field amplified sample injection* [266, 267]). However, this effect may not always be beneficial, and must be considered whenever electrokinetic injection is performed using a sample buffer which differs in composition from the electrophoresis buffer [268, 269].

6. 5. 2. Hydrodynamic injection

Another important method for sample introduction into capillaries is hydrodynamic injection (also called hydrostatic injection, siphoning, or gravity flow injection). In hydrodynamic injection, the capillary is removed from the buffer reservoir and placed in the sample vial. Then, a pressure differential is applied across the capillary for a specific amount of time, drawing a sample slug into the capillary. Alternately, the sample vial may be vertically raised to a certain specified height for an interval of time, creating a hydrostatic head between the sample vial and the other end of the capillary. Hydrodynamic injection has the decided advantage over electrokinetic injection that no sampling bias is introduced during injection [264, 265]. The quantity of sample introduced during hydrodynamic injection can be calculated in a manner analogous to that for electrokinetic injection. The length of the sample zone injected is given by [264]

$$L_s = t_{inj} v_{hf} \tag{20}$$

where t_{inj} in the injection time and v_{hf} is the average velocity of the hydrodynamic flow. Using the Poiseuille equation for flow in a cylindrical tube [270], v_{hf} can be expressed as

$$v_{hf} = \frac{\Delta p\, r^2}{8\, \eta\, L_t} \tag{21}$$

where Δp is the applied pressure differential (or the hydrostatic head, $\Delta p = \rho g \Delta h$, if injection is accomplished by raising one end of the capillary), r is the capillary inner radius, η is the sample solution viscosity, and L_t is the capillary length. Substituting Equation (21) into Equation (20) gives the length of the sample zone as

$$L_s = \frac{\Delta p\, r^2\, t_{inj}}{8\, \eta\, L_t} \tag{22}$$

Using Equation (14), the quantity of sample introduced by hydrodyamic injection can be written as

$$Q = \frac{\Delta p\, \pi\, r^4\, t_{inj}}{8\, \eta\, L_t} C \tag{23}$$

For a given capillary, the amount of sample injected can be controlled through the injection time and the applied pressure differential (or introduction height, Δh). Furthermore, the dependence of Q on capillary diameter is very strong; the practical effect of this dependence is that for very small capillaries (i.d. < 20 μm), it is often difficult to hydrodynamically inject a sufficient volume of sample during a reasonable injection time (for 50-100 μm i.d. capillaries, no such problem is encountered). There is no dependence of the amount injected on any specific properties of the sample analytes, such as electrophoretic mobility, so all analytes are injected to the same extent from a homogeneous sample solution. If the quantity injected is to be known very precisely, the viscosity of the buffer should be measured, and Δp and t_{inj} should be mechanically controlled. This is difficult to do if the "raise the sample vial" method of hydrodynamic injection is used; we therefore recommend the use of an automatically timed system which creates a specific, small pressure differential at one end of the capillary. A disadvantage of hydrodynamic injection, theoretically, is that the flow profile of the sample as it enters the capillary is parabolic, whereas in electrokinetic injection it is flat. Electrokinetic injection is a better method to use for coated, gel-filled capillaries, since the application of a pressure gradient across a dense gel matrix is not an effective way to introduce a sample plug.

Rose and Jorgenson [264] performed a quantitative comparison of electrokinetic and hydrodynamic injection methods in uncoated capillaries, and found that the reproducibility (when both were automated) was at best 4.1% relative standard deviation for electrokinetic injection, and 2.9% for hydrodynamic injection. Thus, hydrodynamic injection is somewhat superior to electrokinetic injection for analytical purposes. Liu *et al.* have noted this result as well [187]. Rose and Jorgenson also reported that hydrodynamic injection can be used for samples as small as 250 nL, while electrokinetic injection requires more sample (~ 2 μL) since a platinum wire electrode has to be in continuous contact with the sample solution during injection. Lastly, they found that neither injection technique created a significant amount of band broadening [264].

6. 5. 3. Sample injection by spontaneous fluid displacement

Fishman, *et al.* [271], have recently introduced a new sample injection technique for CE: *spontaneous fluid displacement*. This method is based on the fact that when a capillary is withdrawn from a sample solution, a droplet is formed at the end of the capillary. Furthermore, the interfacial pressure difference across the curved surface of the droplet will drive the droplet into the capillary entrance. This method allows the injection of the smallest sample which is physically possible. If a very sensitive detection technique is used, use of this sample injection method could theoretically maximize the peak sharpness and resolution. Furthermore, the injection volume can be varied by changing the shape of the capillary's outer diameter. Fishman *et al.* found that 3.5 nL samples could be injected by hand using this method with a reproducibility of 6.0% R.S.D; they suggest that automation (as well as some modifications to the capillary) would likely result in significantly improved reproducibility [271]. Certainly, this is the easiest injection method which one could possibly use, but if analytical reproducibility is required, it will probably require automated dipping of the capillary tip, as well as special treatment of the capillary's outer surface at the injection tip.

6. 5. 4. Methods to significantly increase the sample loading capacity of capillary electrophoresis

One of the disadvantages of CE for some applications is its limited sample capacity (< 100 nL), making it primarily an analytical and at best, a micro-preparative technique. Tsuda *et al.* [272] have shown that the sample capacity of CE can be increased through the use of rectangular capillaries (ranging in size from 195 μm x 16 μm to 1000 μm x 50 μm). These rectangular capillaries have a sufficiently high surface area-to-volume ratio, despite a

relatively large cross-section, to allow large samples to be loaded without loss of separation efficiency. Additionally, Fujimoto *et al.* [273] have recently demonstrated the use of a microcentric capillary column, constructed by winding a plastic line helically on a fused-silica capillary and then inserting it into a larger-diameter fused silica capillary (Figure 13). The microcentric column allows the separation of samples an order of magnitude larger than those normally used for CE (about 17 times larger; the authors managed to separate and collect a 16 μL sample), while maintaining peak integrity because the separation takes place in a thin annular chamber. With the application of these novel geometric approaches, CE has the potential to become a preparative separation technique, which will make it more competitive with slab gel electrophoresis.

6. 6. DNA detection methods for CE

A major advantage of CE is that separated zones can be conveniently detected on-line [235, 274]. This is accomplished simply by removing a short stretch (1-2 mm) of the amber-colored polyimide coating from the outside of the capillary (by burning it with a direct flame, a red-hot filament, or a strong acid, followed by cleaning with methanol, or by scraping it off with a razor blade or diamond-edged knife), and placing this fused silica "window" inside the detector. On-line detection prevents contributions to band broadening from flow through the joints, fittings, and connectors that are necessary for off-column detection (as in high-performance liquid chromatography (HPLC)). Furthermore, the ease of on-line detection has made CE much more amenable to automation than slab gel electrophoresis; several different commercial, automated apparatuses are available. It is crucial that a CE apparatus be equipped with a very sensitive detector, because due to the tiny inner diameter of the capillary, the volume of a typical zone is extremely small. For example, in a 75 μm i.d. capillary 100 cm long, a zone exhibiting 500,000 theoretical plates has a zone length of 5.8 mm and a zone volume of 26 nL [275]. If a 25 μm-i.d. capillary 100 cm long is used, a zone exhibiting 500,000 theoretical plates has a volume of only 3 nL [275]. Strategies for sensitivity enhancement in CE were recently reviewed by Albin *et al.* [276]. Important on-line detection methods for CE of DNA include UV absorbance detection, first used for CE in 1979 [178, 182], and laser-induced fluorescence detection, introduced in 1985 [226, 227].

6. 6. 1. UV absorbance detection of nucleic acids

UV absorbance is a simple, inexpensive, and universal on-line detection method for CE, and as such is offered with virtually all commercial CE systems; hence it has been the most

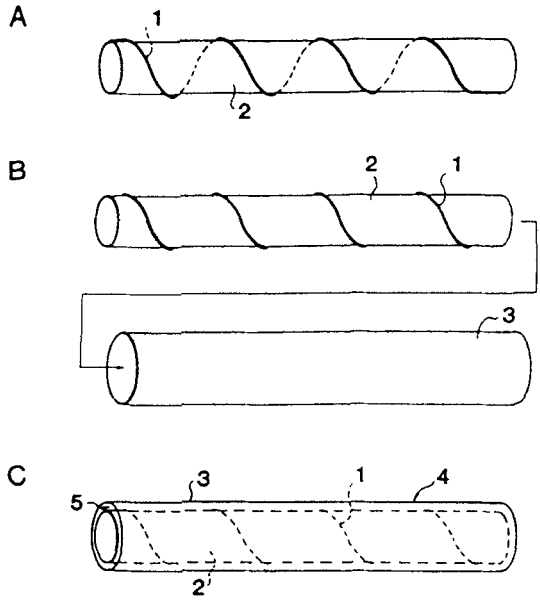


FIGURE 13

Steps illustrating the construction of the concentric column. (A) A naked fused silica tube (o.d. 340 μm) is obtained by removing the polyimide coating with strong acid and washing. Then a synthetic resin thread with nominal diameter 90 μm is wound around the capillary helically, and glued at each end; (B) this inner tube is inserted into a 530 μm i.d. x 630 μm o.d. fused silica capillary with a polyimide coating. (C) the completed concentric capillaries. 1 = sythetic resin thread; 2 = inner tube; 3 = outer tube; 4 = polyimide coating of outer tube; 5 = separation chamber. [Reprinted, with permission, from Reference [273].]

widely used detection method for DNA separations [33, 34, 90, 91, 170-172, 185, 188, 205, 206, 222, 277-321]. Glass capillaries are not transparent to UV light at wavelengths below 300 nm, making them useless for UV detection in the most important wavelength range (*e.g.*, DNA absorbs maximally at 260 nm and proteins at 280 nm). Instead, high-quality fused silica capillaries are used, which have a UV cut-off of 170 nm [275]. Proper design of the UV absorbance detector is important, so that the signal due to DNA bands can to be distinguished from absorbance changes which may result from differences in refractive index between the sample and the electrophoresis buffer [322]. Typically, UV absorbance detectors for CE are HPLC detectors, modified to allow the detection window in a capillary to be held in the light beam. The capillary geometry places conflicting demands on the

detection cell for UV absorbance: reducing the cell volume will provide high performance with respect to theoretical plates, but a certain minimum cell volume is required for sufficient passage of light, and to provide a favorable signal-to-noise ratio [322]. In typical capillaries, the sensitivity of UV absorbance detection is limited by the short pathlength which is available across the capillary tube. Because of this limitation, conventional UV absorbance detection systems are not useful for DNA sequencing, in which a typical band contains only 1-10 attomoles of material.

One of the primary advantages of using UV absorbance detection for the CE of nucleic acids is that detection is based on the intrinsic UV absorbance of DNA; no intercalating dyes or fluorophores need be attached to DNA molecules, as in laser-induced fluorescence detection methods, and thus the DNA helix and its electrophoretic mobility are not perturbed. Since all four DNA bases (A, G, T, and C) absorb roughly the same amount of 260-nm UV radiation [1], the UV absorbance signal increases linearly with the number of DNA base pairs in the band, which helps in peak identification and sample quantitation.

6. 6. 2. Laser-induced fluorescence detection of DNA

If highly efficient fluorophores are used, the sensitivity of laser-induced fluorescence (LIF) detection can be up to six orders of magnitude greater than UV absorbance [323]. Clark *et al.* [324] have shown that a band containing only ~ 1 pg of DNA can be detected using ethidium bromide (EtBr) as a fluorescent label; the use of more efficient dyes, such as thiazole orange (TO) and oxazole yellow (YO) with enhanced spectroscopic properties and enhanced DNA binding affinities, is predicted to lower this detection limit by another factor of 50 [121-126]. Both glass and fused silica capillaries may be used for fluorescence detection, but fused silica is to be preferred because its luminescence levels are intrinsically lower [275]. Although an LIF detection system is much more expensive and complicated to build than a UV absorbance detector, its phenomenal sensitivity as well as other positive features which we will discuss have made it an increasingly popular method of DNA detection [23, 113, 186, 187, 194, 225, 278, 286, 324-357]. Most of the recent flurry of research activity with LIF detection has been in the area of DNA sequencing by CE [23, 113, 176, 186, 194, 225, 325-328, 330-338, 341, 342, 345, 351, 353, 358], the detection of DNA restriction fragments [278, 324, 329, 339, 346-350, 354-356, 359] and for the analysis of polymerase-chain reaction-amplified DNA fragments for genetic typing and forensic applications [347, 348, 350, 357, 359].

To perform DNA sequencing by CE, single-base resolution of single-stranded DNA fragments is required, so the capillaries are filled either with crosslinked polyacrylamide gels [277, 360] or with very concentrated solutions of linear polyacrylamide [194]). (We will discuss intra-capillary sieving matrices in Section 6. 8.) The resolution and peak sharpness obtained by capillary gel electrophoresis is significantly superior to those obtained in commercial, automated slab gel DNA sequencers [186, 326]. Furthermore, the classic Sanger method of sequencing by dideoxynucleotide triphosphate (ddNTP) chain termination employs radioactive labels and autoradiographic detection [4]. Radioactive detection has the disadvantages of long exposure times for autoradiography, complex protocols, and significant health risks. In 1986-87, several groups reported the use of fluorescent labels and LIF detection, rather than radiolabels and autoradiography, for the products of the chain-termination method of sequencing, separated both in capillaries and on slab gels [361-363].

A disadvantage of LIF detection is the necessity for fluorescence labeling of DNA molecules. The intercalation of fluorescent dyes into a double stranded DNA helix both lengthens and stiffens the molecule, increasing its persistance length and changing its higher-order structure [364], and almost certainly affecting its conformation during electrophoresis. The intercalation of the cationic dyes also reduces the net charge on a DNA molecule, which proportionally decreases the electrophoretic mobility. Thus, it is impossible to quantitatively compare electrophoretic mobilities between experiments in which the DNA samples were stained with different dyes or with different dye:DNA ratios. The presence of different covalently-attached fluorophores also causes dye-specific mobility shifts [334]. The use of fluorescent probes is also disadvantageous because their shelf life is brief, complex manipulations are required to attach them to DNA molecules, and their presence has deleterious effects on DNA separation efficiency [365].

6. 6. 3. DNA sequencing by CE with LIF detection

Since the introduction of LIF detection for DNA molecules, one of the major areas of research has been labeling and detection strategies, *i.e.*, the best method for distinguishing the products of four sequencing reactions (for A, G, C and T), within a single capillary. It is better to separate and detect the products of all of sequencing reactions in one capillary, rather than in four parallel capillaries, because there is generally a 5% capillary-to-capillary variation in the migration velocity of the DNA fragments [366]. In a slab gel, detection is more straightforward since the products of the four sequencing reactions can be separated

in adjacent lanes without such lane-to-lane variation, if the slab gel is properly cooled. For example, Ansorge *et al.* [6, 362] introduced a single-spectral-channel, four-lane sequencer, in which a different fluorescent label is attached to each of the four ddNTP chain-terminators, and the products are separated in adjacent lanes of a slab gel.

There are several ways to accomplish this goal by CE, making use of fluorescent labels which upon excitation with laser light at a certain wavelength will emit fluorescent light at another wavelength. Several different labels, with distinct spectral properties, are available (*e.g.*, FAM, JOE, TAMRA, and ROX are four fluorophores marketed by Applied Biosystems (Foster City, CA, USA) to be used for DNA sequencing, which have relatively widely spaced absorbance and emission spectra). In the method of Smith *et al.* [361], as for that of Ansorge *et al.*, the oligonucleotide primers are labeled with four different fluorophores and each labeled primer is uniquely associated with one of the four terminating ddNTPs through use of separate chain-terminating reactions for each. The products of the four sequencing reactions are separated in a single gel-filled capillary. At the detection window, the fluorescence of the reaction products is excited by two laser lines and collected in four different spectral channels (*i.e.*, through four different spectral filters, one for each fluorophore). In a somewhat different approach, Prober *et al.* [363] used a two-spectral channel, single-lane sequencer, in which four fluorophores are each associated with the one of the DNA bases, by applying each fluorophore to a different ddNTP chain terminator (rather than to the primers). The fluorescence of the four different labels is excited by a single laser line and detected in two spectral channels. Various permutations of this labeling and detection strategy (*i.e.*, the use of four uniquely labeled primers, and separation in a single capillary), have also been employed by Lee *et al.* [367], Tomisaki *et al.* [353] and Carson *et al.* [345].

Mathies *et al.* [334] introduced a "binary" coding scheme for DNA sequencing fragments, using primers which are labeled with two different dyes only (FAM and JOE). One of the dyes emits in the green spectral channel, and the other in the red spectral channel. The strategy is to label the A-specific reaction products with both red- and green-emitting dyes, so that A fragments are detected in both spectral channels; G fragments are detected in the red channel only, T fragments in the green channel only, and C fragments are omitted from the reaction/labeling scheme and appear only as "gaps" in the progression of DNA peaks. Thus, only three sets of chain-termination reactions need be carried out, a labor-saving strategy first conceived by Tabor and Richardson [368]. Mathies *et al.* [334] point out that it

is advantageous to use only two different dyes, because they can be selected to cause similar electrophoretic mobility shifts for the DNA sequencing fragments, facilitating the accurate reading of sequence data.

Another method is that of Pentoney *et al.* [331], which requires only a single fluorophore and reportedly results in significant reduction in the time required to read a DNA sequence, while employing simpler instrumentation than the preceding methods. Their strategy, another modification of that of Tabor and Richardson [368], is to employ two sets of sequencing reactions, each containing complementary mixtures of only three ddNTPs in the concentration ratio 4 : 2 : 1. The DNA sequence can be determined by relative peak heights and by assigning the missing ddNTP to "gaps" between the peaks. Both of the complementary mixtures contain complete sequence information, and the results from each can be compared, allowing the sequence to be called out more confidently. (In most sequencing experiments, the same four sequencing reactions are generally performed at least twice so that such a comparison may be performed, as shown in Figure 1.) One disadvantage of this method is that it requires the use of a modified T7 DNA polymerase with Mn^{2+}, which is less processive than the native enzyme (*i.e.*, polymerizes shorter contiguous DNA strands before dissociating from the template) and hence reduces the usable length of the sequencing run [368]. Another disadvantage of using this method is that it may be difficult to confidently compare peak heights for larger DNA fragments, which are broader than peaks for small DNA fragments and tend to be misshapen. An advantage is that with only a single fluorophore, the problem of different mobility shifts being associated with different dyes is no longer existent, as it is with multi-color systems. In addition, multi-color systems require more complicated and expensive instrumentation for excitation and signal collection. This basic strategy, *i.e.*, the use of modified T7 polymerase and various combinations of peak heights and different numbers of dyes labeling the primers, have also been used by Ansorge *et al.* [369], Chen *et al.* [333], Ruiz-Martinez *et al.* [194], and Lu *et al.* [370].

The lower detection limit (*i.e.*, the amount of analyte that produces a signal which is 3 times greater than the standard deviation of the background signal) of a laser-induced fluorescence detection system for DNA sequencing is more stringent for CE than for slab gel electrophoresis, due to the low sample capacity of the capillary. When LIF detection is used for slab gel electrophoresis, the detector is required to register a lower limit of 10-100 attomoles per band; if larger samples are loaded on a slab gel, the band resolution

decreases. When the method of DNA separation is CE, however, Drossman *et al.* have estimated that only 1-10 attomol per band can be applied without overloading the capillary gel [186].

6. 6. 4. Capillary array electrophoresis with laser-induced fluorescence detection

In section 5.4 we discussed the use of ultra-thin slab gels for more rapid parallel separations of DNA fragments than is possible by conventional slab gel electrophoresis. Another important new technique, which is designed to accomplish the same goal, is capillary array electrophoresis [356, 366]. In capillary array electrophoresis, multiple capillaries (so far, as many as 100 [356]) are set up in parallel; an array of 100 multiplexed capillaries is about 3 cm across [356]. This approach was pioneered by the Mathies group [324, 334, 366], and is also being developed by Yeung's group [343, 356] and by Kambara, Takahashi, and co-workers [341, 351]. A schematic representation of the capillary array electrophoresis system developed by Mathies and co-workers, which utilizes laser-excited confocal fluoresence detection, is shown in Figure 14.

Capillary array electrophoresis was first developed for rapid, high-throughput DNA sequencing, funded by the Human Genome Project [371]. Using the fluorescence-labeling strategies we have discussed in Section 6. 6. 3., a complete set of DNA sequencing reaction products can be separated in each capillary. Accordingly, capillary array electrophoresis is at present a source of hope to those involved in the Human Genome Project, as a method which has the potential to greatly increase the rate of DNA sequencing. A capillary array electrophoresis system has the advantages of CE which we have discussed, most importantly the ability to apply high field strengths for rapid, high-performance DNA separations, and the ease of system automation (especially sample injection). However, large capillary arrays also present a significant challenge for the design of LIF detection systems; the best approach to accurately and simultaneously gather and process of the fluorescence signal from a large number of capillaries in parallel is still a matter of discussion in the literature [356]. For example, there is a problem with the elimination of signal "cross-talk" between adjacent capillaries (one capillary may present its own signal to the detection system plus 10% of the signal from the neighboring capillary; this makes it difficult to obtain an electropherogram without spurious "phantom" peaks) [356]. The primary goal of the Human Genome Project is to sequence all 3 billion base pairs of human chromosomal DNA, as well as the genomes of other organisms (*e.g.*, mouse, Drosophila fly) in a reasonable amount of time and at a reasonable cost. Employing the current

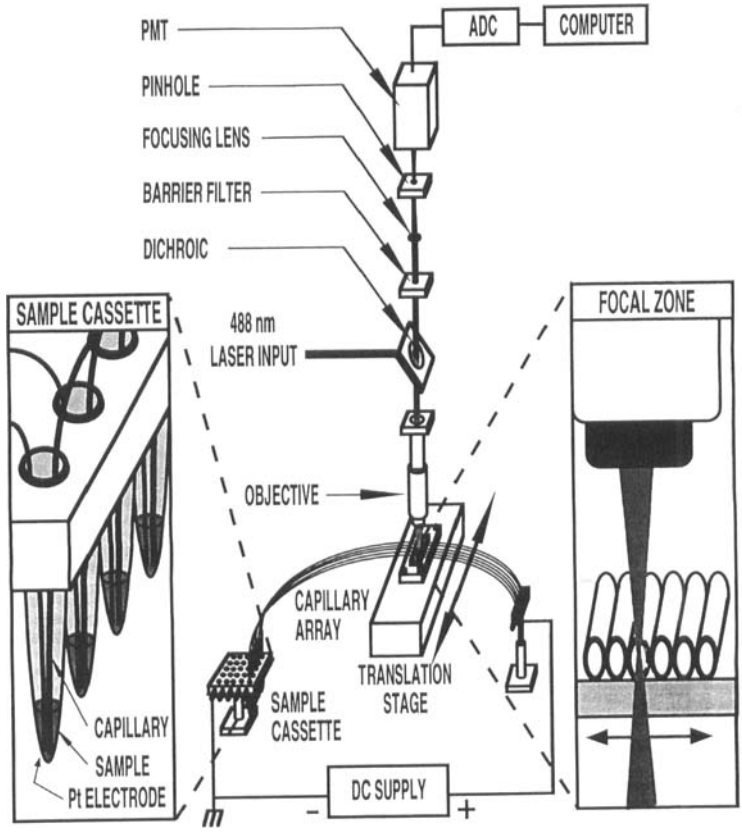


FIGURE 14
Schematic of the laser-excited, confocal-fluorescence capillary array scanner built by the Mathies group. [Reprinted, with permission, from Reference [325].]

technologies, *i.e.*, conventional sequencing gels, a single competent sequencing laboratory would require 1000-10,000 years to sequence the human genome; and as Ueno and Yeung point out, the patience of the taxpayer would most likely run out before the project's completion [356]. An interesting review of cutting-edge DNA sequencing technologies, including capillary array electrophoresis and other novel approaches which are being developed for the Human Genome Project, has recently been published [372].

In addition to DNA sequencing applications, running multiple capillaries in parallel would ideally allow electrophoresis patterns in many different "lanes" to be compared as easily as they are in slab gels [2]. However, due to irreproducibilities in casting intra-capillary gels, the electrophoretic velocity of DNA often differs up to 5% from one capillary to another [366], which complicates the comparison of mobility patterns between lanes. This problem, however, may be conquered in the near future, as there is a great deal of promising research currently being conducted in the area of reproducible casting of intra-capillary sieving matrices for DNA electrophoresis, as we will discuss in the Section 6. 8.

6. 6. 5. Other detection methods for CE of DNA

Other detection systems which will be applicable for DNA molecules and which are currently under investigation, but not yet in use by the wider community, include native fluorescence detection [365], and radioisotope detection [373]. The primary advantage of native fluorescence detection is that it obviates the need for fluorescent labeling with DNA structure- and charge-perturbing dyes, shortening and simplifying experimental protocols, and allowing easier quantitation of results. Milofsky and Yeung [365] were the first to show that it is possible to detect the laser-induced *native* fluorescence of DNA molecules separated in a polyacrylamide gel-filled capillary. Although they predicted that this technique has the potential to be significantly more sensitive than UV absorbance detection, the performance of the system was poor with polyacrylamide-filled capillaries because of their high background fluorescence and tendency to quench the fluorescence emission of the DNA analytes [365]. When a solution of uncrosslinked methyl cellulose was employed as the DNA separation matrix, however [374] McGregor and Yeung found that this background fluorescence problem was overcome. With a methyl cellulose separation matrix, an unusually low buffer pH of 2.8 (to maximize the native fluorescence), and the employment of a sheath flow cuvette, the performance of the native fluorescence detection system was an order of magnitude more sensitive than UV absorbance detection [374]. However, degradation of the DNA sample occurred in this low pH buffer.

Radioisotope detection in small-diameter tubes (300 μm) was first reported by Kaniansky *et al.* [375, 376], and applied to capillaries by Altria *et al.* [377] for the detection of radiolabeled pharmaceuticals. Pentoney *et al.* [373, 378] have recently described the design of two on-line radioactivity detectors for CE. One type of detector employs a commercially available semi-conducter device, placed outside the detection cell to count impinging γ or high-energy β rays. The second type of device exploits a commercially-available plastic

scintillator material which is formed to completely surround the capillary's detection window, preventing any signal from escaping detection. To date, these detection devices have only been tested on mixtures of 5'-[α-^{32}P]-labeled nucleotide triphospates [373], but will be applicable to detection of any radiolabeled nucleic acids. Pentoney *et al.* mention an impressive list of advantages gained by the use of this detection method [373]. One obvious advantage of radioisotope detection is its extremely high sensitivity. A second advantage is its selectivity, which is superior to that of all other methods since there is no background signal. Third, radioisotope detectors can be precisely calibrated to provide quantitative measurement of analyte concentration. Fourth, a radioactive molecule possesses the same structural properties as an unlabeled molecule, so there would be no effect on DNA electrophoretic mobility. Finally, radioisotope detection, when implemented on-line in conjunction with automated injection of the minute samples typically using in CE, presents a lower health hazard to researchers than autoradiographic detection in a slab gel format.

6. 7. Sample collection methods for DNA separated by CE

When it was first developed, CE was considered to be an analytical technique only. However, it has become desirable to run CE in a micropreparative mode as well, so that separated peaks can be subsequently collected and identified. It has been shown that analytes which have been separated by CE can be collected in sufficient quantities for subsequent analyses [188, 264, 279, 379, 380]. For example, Cohen *et al.* [279] separated an oligonucleotide primer by capillary gel electrophoresis and then collected it, by placing the anodic end of the capillary in a microcentrifuge tube containing 2-5 µL of water along with the Pt electrode. When the electric field was applied, the sample (and ions from the electrophoresis buffer) eluted into the tube, and the purified oligonucleotide primer was then used it as a probe in a standard dot-blot assay. The capillary can be moved from one collection tube to another, breaking the circuit and halting the flow of current in between tubes. Reportedly, the sample bands can be stopped and started in gel-filled columns with little resultant band broadening [279]. Guttman *et al.* [381] manually collected picomoles of small peptides separated by CE in SDS-polyacrylamide gel-filled columns, and successfully microsequenced the fragments. Guttman *et al.* [188] have also manually collected oligonucleotide fractions, using a decreased field strength during sample delivery to make the precise collection of the very narrow bands feasible. Using a similar strategy, *i.e.*, temporarily breaking the circuit in order to collect samples, Rose and Jorgenson [264] designed an automated fraction collector to collect purified DNA and proteins. Sample

collection performed by temporary interruption of the electric field while the capillary is placed in a collection vessel has also been demonstrated for peptides and proteins by Albin *et al.* [379] and by Nashebeh, *et al.* [382]; Altria and Dave demonstrated the collection and analysis of pharmaceutical compounds separated by CE [380]. In many of these cases, the collected samples were reinjected and recovery was found to be very high. The collection of analytes by these methods has the disadvantage that the field is broken when the capillary is moved between sample collection vials, and that 2-5 μL of water must be present in the vial to complete the circuit and allow the sample be collected by electromigration. This results in a dilution of the collected fractions, which have volumes on the order of a few nL. However, the sample can be reconcentrated by evaporation.

Huang and Zare [383, 384] designed a more advanced type of sample collection device for CE, which employs an on-column frit (constructed by sintering a mixture of glass powders) to the side of the capillary 1.5 cm prior to its outlet. For this purpose, a 40-μm diameter hole is burned in the side of the capillary with a focused laser beam, and then a slurry of glass powder is pasted over the hole and set by heating at 1000°C for 30 seconds. A detector is placed right before the frit, and the detector signal is programmed to trigger fraction collection. Electrophoretic separation takes place in the first part of the capillary (inlet to frit); the second, 1.5-cm part of the capillary (frit to outlet) is used for field-free eluent collection (electroosmotic flow in the first segment pumps the sample out of the capillary outlet). Through the porous glass frit, the capillary is grounded at all times, while the separated bands are eluted from the end of the capillary onto a moving surface (filter paper placed on the periphery of a rotating disc) tracing a pattern of bands on the surface which can be probed later [383]. Sample collection requires about 30 minutes. The collected samples may be cut out of the filter paper and eluted with methanol. By this method, Huang and Zare accomplished 90% recovery of the original material [384]. It is also possible to collect the purified fractions in sample vials [384]. A satisfactory on-column glass frit is somewhat difficult to make (the authors report a 50% success rate). However, a single fritted capillary can be used for six months or more.

Commercial CE instruments are now available with on-column fraction collection by the first method, in which the field is interrupted. In comparison to slab gel electrophoresis, micropreparative CE provides shortened analysis times as well as greater resolution, recovery, and purity. Fraction collection can also be automated more easily in CE (although automated electrokinetic fraction collection is also available in some advanced

commercial slab gel electrophoresis systems). Standard slab gel purification of oligonucleotides requires several manual steps and about 2 days of work; in CE purer (though much smaller) samples can be obtained within 30 minutes [383]. Depending on the resolution between the peaks, between 500 ng and 1 μg of sample can be collected from one capillary.

6. 8. DNA separation matrices for CE

As we have discussed in Section 3, DNA fragments cannot be separated by size when they are electrophoresed in free solution. Hence, in order for CE to be used for DNA separations, the capillary must contain a DNA separation matrix. CE in both *chemical gels* (such as crosslinked polyacrylamide) and *physical gels* (concentrated solutions of entangled, but uncrosslinked, polymers) provides high-resolution, high-efficiency DNA separations. Furthermore, dilute and ultra-dilute polymer solutions (which are most certainly *not* gels) also provide size separation of DNA, by a mechanism of separation which is altogether different from those which we have discussed in Section 4. We will discuss each of these types of DNA separation matrices for CE.

6. 8. 1. The use of gel-filled capillaries for DNA electrophoresis

Soon after the advantages of performing electrophoresis within a capillary rather than on a slab gel had been demonstrated, *gel-filled* capillaries were applied to the separation of nucleic acids. Polyacrylamide gel-filled capillaries, which would become important for DNA electrophoresis, were first introduced for protein separations in 1987 by Cohen and Karger [277] and Hjertén *et al.* [360]. Since then, many groups have investigated the use of capillaries filled with crosslinked polyacrylamide gels for DNA separations [23, 113, 186, 205, 206, 225, 278, 279, 282, 284, 289, 290, 293, 298, 304, 312, 319, 320, 325-331, 333-338, 341, 342, 346, 351, 353, 357, 358, 385-388], as well as for the separation of SDS-complexed-proteins, which have electrophoresis properties quite similar to those of DNA molecules [277, 296, 305].

DNA electrophoresis in crosslinked polyacrylamide gels can provide excellent separations, with single-base resolution for DNA ranging from 15 to more than 500 bases [358], and with an astoundingly high number of theoretical plates (*e.g.*, 15-30 million plates/m, for the separations shown in Figures 15(a) and 15(b) [205, 279, 386]). Furthermore, for gel-filled capillaries made in their laboratory, Baba *et al.* have reported that average relative standard deviations in migration times for polynucleotides in the size range of 50 to 250 bases were 1.1% (run to run), 1.5% (day to day), and 2.1% (batch to batch).

However, in general the investigation of DNA separations in polyacrylamide gel-filled capillaries has been hindered by the difficulty of polymerizing gels of adequate quality, and by the persistent occurrence of gel breakdown during prolonged runs at high voltage [186, 225, 297, 325-327, 385, 389]. At present, this remains the primary stumbling block to the widespread adoption of CE techniques for DNA sequencing [332]. These problems have been examined by several groups, and it appears that capillary gel polymerization can be accomplished reproducibly with the aid of various strategies, including the use of novel polymerization methods [390], the application of high pressure during polymerization [391, 392], the use polymeric additives [389], and the consistent observation of good laboratory practice [186, 188, 205, 206, 326, 385, 386, 389].

The other problem, gel instability under high fields, has proved less tractable. Gel breakdown is manifest by a decrease in current (at constant voltage [186]) and by loss of resolution [188, 225, 325, 326, 328]. These conditions are generally accompanied by bubble formation near the injection (cathodic) end of the capillary gel, as detected by microscopic examination of capillaries [225, 325, 327]. Attempted solutions to gel instability problems have included chemical crosslinking of gels to the capillary walls [154, 186, 188, 328, 385, 389], running electrophoresis at relatively low fields (< 300 V/cm) to reduce stress on the gels [186, 188, 205, 326-328, 385], trimming the injection end of the capillary after injection to remove the bubbled region of the gel [326, 328, 385], or trimming *both* ends of the capillary after the gel polymerization, but before injection [205]. It has also been reported that the gel instability problem can be conquered with the use of well-designed injection equipment [205]. If gel-filled capillaries are to be used for a large-scale DNA sequencing project, it is imperative that the high-field instability problem be solved; for if the cathodic end of the capillary must be examined under a microscope and then trimmed after each injection, automation will be impossible and much of the speed advantage of CE is lost [225]. In particular, for an array of 100 capillaries such as that built by Ueno and Yeung [356] (who envision an automated DNA sequencer running up to 4000 capillaries in parallel), the time required for capillary inspection and trimming would be prohibitive.

When the separation of single-stranded DNA sequencing products is performed in capillaries filled with *denaturing* polyacrylamide gels, additional problems are encountered. As Smith [332] points out, instability problems exist even for denaturing polyacrylamide gels cast in a slab format. The presence of a strong chemical denaturant (such as 8 M urea) in the gel is the cause; urea is unstable to hydrolysis at pH 8, and at a concentration of 8 M

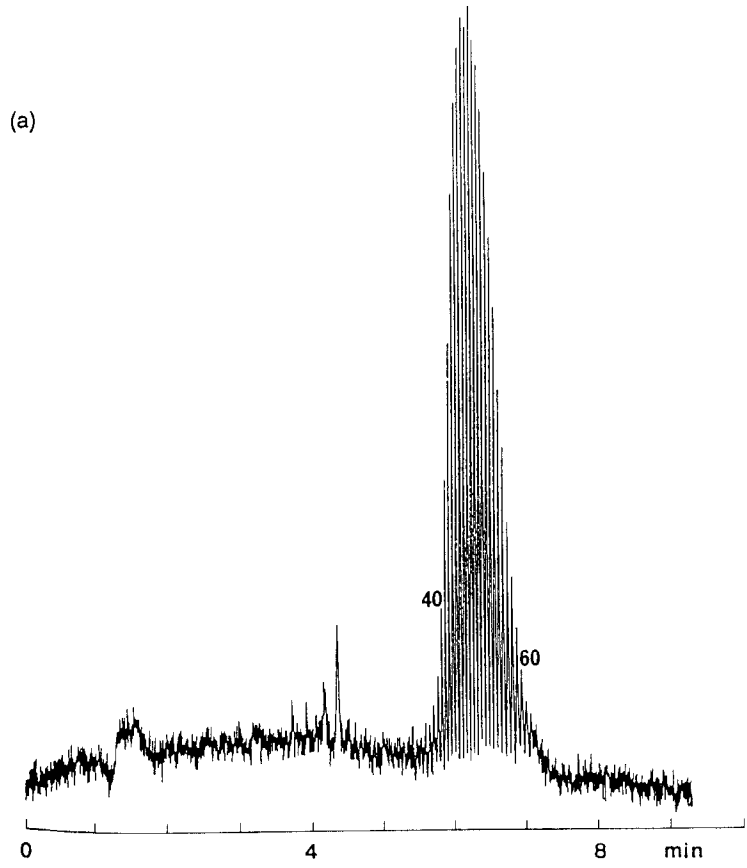


FIGURE 15

(a) Separation of a polydeoxyadenylic acid mixture, $(dA)_{40\text{-}60}$, by capillary gel electrophoresis. Capillary was 27 cm long (to detector), with a 75 μm i.d.; running buffer was 0.1 M Tris/0.25 M borate/7 M urea, pH 8.3, and the gel contained 7.5% acrylamide and 3.3 % Bis crosslinker. Detection by UV absorbance at 260 nm. The applied field was 400 V/cm. [Reprinted with permission from Reference [279].] (b) Separation of a mixture of polydeoxyadenylic acids digested by nuclease P1. Capillary was 35 cm long (to detector), with a 100 μm i.d.; running buffer was 0.1 M Tris-borate and 7 M urea, pH 8.6; and the gel contained 5% acrylamide and 1.5% Bis crosslinker. Detection by UV absorbance at 260 nm. The applied field was 200 V/cm. [Reprinted with permission from Reference [203].]

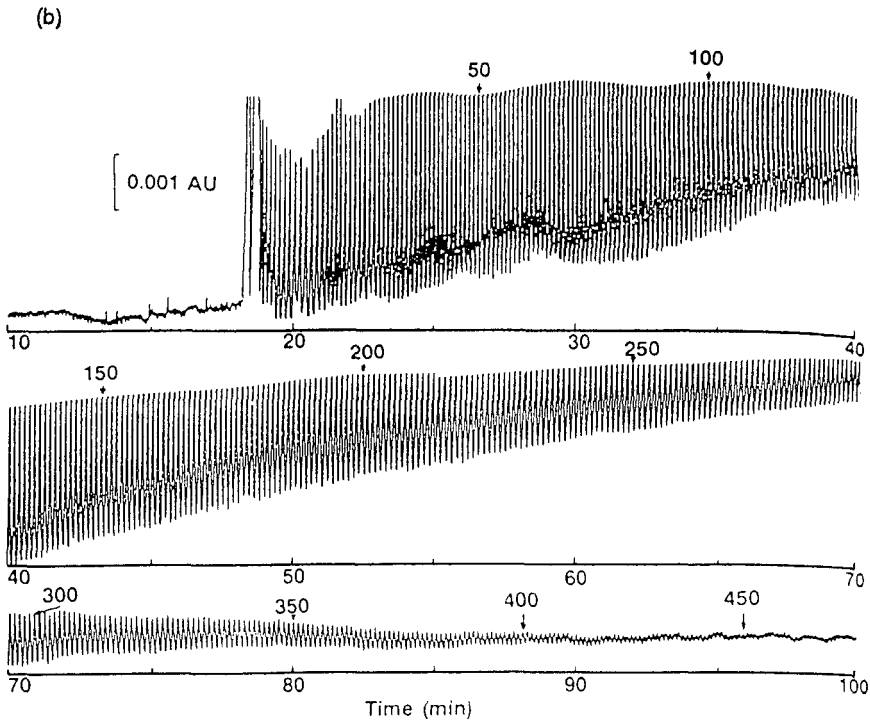


FIGURE 15 Continued

also tends to crystallize out of solution [332]. Both native and denaturing gel-filled capillaries will also, on occasion, irreversibly bind high molecular weight DNA [295]; therefore, with each successive run there is an increasing chance that the presence of impurities will produce anomalous peaks.

For all of these reasons, the performance of gel-filled capillaries necessarily degrades over time, and in practice their lifetimes are severely limited. This is a serious problem, since they are time-consuming to produce in the laboratory and expensive to buy from a commercial supplier. The prevailing attitude of researchers toward the preparation of gel-filled capillaries can best be summed up by two quotes from recent publications. Paulus and Hüsken, of Ciba-Geigy Ltd. (Basel, Switzerland) wrote, "Although we were successful

in producing and running crosslinked gel capillaries, it was difficult to communicate proper handling to novice user [297]." And Lloyd Smith, of the University of Wisconsin, Madison, noted drily that, "...indeed, the procedures for preparing such gels even in research laboratories remains something of a black art [332]."

In addition to the DNA sequencing applications which we have discussed in Section 6. 6. 2., high-voltage CE clearly has the potential to be much faster than slab gels for DNA restriction mapping separations. However, until recently this technique could not be used for the separation of DNA molecules larger than 1000 bp. This is because, although polyacrylamide gel-filled capillaries have been successful for the separation of oligonucleotides [188, 279, 290, 393], the network formed by crosslinked polyacrylamide is too constrictive to separate restriction fragments greater than 1000 bp in length [100]. Agarose gels, which have useful pore sizes for restriction mapping, have proven to be a poor medium for CE. They are even more unstable than polyacrylamide at high temperatures and field strengths [278, 394], and furthermore are not optically transparent. Therefore, no known crosslinked gel matrix is effective for the CE separations of DNA restriction fragments longer than 2 kbp. Instead, electrophoretic separations of larger DNA are now often performed in capillaries filled with polymer solutions, having low enough viscosities that they can be pumped in and out of the capillary (earning them the misnomers, "replaceable gels," "sieving buffers," and "gel buffer solutions"). Polymer solution-filled capillaries are easy to prepare, inexpensive, stable at high electric fields, and can separate DNA ranging in size from 20 bp to current maximum of about 2 million bp, depending on the polymer type, concentration, and the applied field.

6. 8. 2. The potential of uncrosslinked polymer solutions as DNA separation matrices for CE

Early predictions were that separations of DNA fragments longer than 8 bp would be impossible without the "sieving effect of a high density gel [395]." The use of a non-gel separation matrix for electrophoresis was first investigated by Bode on cellulose acetate strips [85-87]. Tietz *et al.* [396] later demonstrated the separating ability of viscous solutions of linear polyacrylamide, by electrophoresis in a tube with a 6 mm inner diameter. Convection of bands in the 6 mm i.d.-tube was problematic, and the authors predicted that the potential applications of uncrosslinked separation matrices must await their combination with a suitable anti-convective device. The putative anti-convective device turned out to be a microbore capillary. The use of uncrosslinked polymer solutions

to separate DNA restriction fragments by CE was first reported by Zhu *et al.* [397] and Chin and Colburn [222]; in both of these cases, the dissolved polymers were hydrophilic cellulose derivatives. Heiger, *et al.* [283] soon thereafter reported the utility of concentrated solutions (6, 9, and 12 %T) of linear polyacrylamide which lacked crosslinking, for high resolution CE separations of DNA smaller than 1000 bp in length. Since the publication of these pioneering papers, there has been a great deal of active research on the subject of DNA and SDS-protein electrophoresis in uncrosslinked polymer solutions [39, 54, 55, 61, 63, 90, 170-172, 187, 194, 206, 280, 285-288, 291, 292, 294, 295, 297, 300, 302, 303, 310, 311, 313-318, 324, 339, 345, 348-350, 354-356, 359, 398-412].

To date, researchers have used solutions of thirteen different polymers as DNA separation media for CE, with varying degrees of success: hydroxyethyl cellulose (HEC) [90, 285, 348, 350, 355, 359, 399, 406], hydroxypropyl cellulose [206], hydroxypropylmethyl cellulose [206, 286, 311, 313, 314, 349, 354, 404], methyl cellulose [287, 295, 300, 339, 356, 374], polyethylene glycol [286, 288], polyethylene oxide [315, 316, 318, 407], galactomannan [288], pullulan [317], dextran [291], and polyvinyl alcohol [288, 310, 404], as well as liquefied agarose [54, 62, 401, 404, 405], uncrosslinked polyacryloylamino-ethoxyethanol (pAAEE) [171, 172], and uncrosslinked linear polyacrylamide [61, 63, 170, 187, 194, 283, 291, 292, 297, 302, 303, 310, 345, 354, 396, 398, 402-404, 412]. This great variety of polymers, most of which can be obtained or synthesized with several different molecular masses and which can be dissolved in aqueous buffers over a wide range of concentration, present a rather bewildering choice to potential users of CE with uncrosslinked polymer solutions. As we shall discuss, solutions of uncrosslinked polymers have been found to have DNA separation potential over a wide range of concentrations, depending on the specific polymer, from ultra-dilute polymer solutions well below the polymer entanglement threshold [91], to semi-dilute, low-viscosity solutions [90, 285, 399], to extremely concentrated, 'syrupy' solutions so viscous that they cannot be injected into a capillary and must be polymerized *in situ* [413]. The advantage of syrupy polymer solutions is that they provide single-base resolution of small DNA fragments [413]. The problem with *in situ* polymerization using acrylamide monomers, however, is that inside the capillary the polymerization reaction cannot be driven to better than 85%, leaving "a huge amount of potentially harmful reacting species [413]," which must then be removed from the capillary in a multi-step chemical scavenging process.

It has been recently shown by Karger's group that capillaries filled with concentrated solutions of polyacrylamide (9-10% T) have the single-base resolving power to be used for

DNA sequencing, providing more than 350 readable bases per 30-minute run at 200 V/cm, with none of the instability problems associated with the gel-filled capillaries which are usually used for DNA sequencing by CE [194, 345]. The linear polyacrylamide solutions developed in this study were polymerized outside of the capillary and had sufficiently low viscosities to be manually pumped in with a Hamilton syringe. The final viscosity of these polyacrylamide solutions was decreased by controlling the polymerization conditions to favor the production of shorter polyacrylamide chains. This was done by adding unusually high concentrations of catalyst to the polymerization reaction. The excess of catalyst causes the initiation of a greater number of polymer chains, having shorter chain lengths when all of the monomer has been consumed [194]. Using this method, the molecular mass of the linear polyacrylamide in the separation matrix was optimized to give the maximum resolution of DNA at minimum solution viscosity (the viscosity of the most effective solution was 2500 cP) [194]. The success of this approach, in which high concentrations of relatively short polyacrylamide chains are used to achieve fine resolution of small DNA fragments while minimizing solution viscosity for ease of handling, has since also been demonstrated by Heller and Viovy [412] and Gelfi *et al.* [414]. It is likely that these "replaceable" polyacrylamide solutions will soon become the separation matrix of choice for automatic DNA sequencing and the separation of short DNA fragments by CE, replacing polyacrylamide gels.

6. 8. 3. Biological applications of CE in uncrosslinked polymer solutions

During the last year it has become apparent that the advantages of CE in uncrosslinked polymer solutions are beginning to be realized outside of the analytical chemistry community, in which it is typical for researchers to test the separation efficiency of a system using off-the-shelf restriction digests of DNA such as ΦX174-*HaeIII* or λ–*HindIII*. Encouragingly, molecular biologists around the world have begun to apply this rapid, efficient technique to perform DNA mapping and sizing separations of medical and genetic interest. Kuypers *et al.* [303] have used CE in 4% linear polyacrylamide to detect point mutations (which cause single-strand conformational polymorphisms) linked to cancer, and concluded that CE was a powerful technique for this application. Del Principe *et al.* [311] used a capillary filled with 0.5% hydroxypropyl methyl cellulose (HPMC) to detect a restriction fragment length polymorphism associated with the dystrophin gene on the X-chromosome (associated with muscular dystrophy and other diseases). Also using 0.5% HPMC, Cheng *et al.* [313] performed a heteroduplex polymorphism analysis of PCR products, and concluded that CE in polymer solutions would provide a rapid and highly

efficient way to screen for rare mutations among a large population. Bianchi *et al.* [314, 415] reported the use of CE with 0.5% HPMC to study the hybridization of oligonucleotide primers to PCR-amplified portions of HIV-1 genomic DNA; they suggest that CE "could be an integral part of automated diagnostic systems." Nesi *et al.* [171] detected and sized a restriction fragment length polymorphism associated with Kennedy's bulbospinal amyotrophy disease by CE in 8% pAAEE, noting that with this viscous polymer solution matrix, the capillary could be repeatedly used for 80-100 runs (pAAEE is a novel polymer with increased hydrolytic stability as compared to polyacrylamide [169]). Similarly, 6% linear polyacrylamide was used by Gelfi *et al.* to detect the GATT microsatellites associated with cystic fibrosis. Finally, Butler *et al.* [359] used CE in 1% HEC solutions to rapidly analyze the short tandem repeat HUMTH01, useful in identifying individuals for forensic purposes.

6. 8. 4. The choice of polymer type, molecular mass, and concentration for the separation of double-stranded DNA

In separating DNA restriction fragments, cellulosic polymer solutions typically are employed at concentrations of 0.20 - 1.0 %, while linear polyacrylamide is used at much higher concentrations (3.0 - 10.0 %T) to produce comparable resolution of DNA restriction fragments. For example, Strege and Lagu [287] depict the separation in 18 minutes of a 1-kbp DNA ladder (a size standard useful for the restriction mapping of plasmids) in a 0.5% methyl cellulose solution, while Chiari *et al.* [307] chose a concentration of 4.5%T linear polyacrylamide to provide the same separation, with similar results but a much longer analysis time (120 minutes). It is not yet clear why much greater percentages of polyacrylamide are required for comparable resolution of DNA restriction fragments. Most likely, it results from differences in the properties of polyacrylamide, an extremely flexible polymer, and hydrophilic cellulose derivatives, which are typically quite stiff in comparison [90, 91]. If indeed similar levels of resolution for DNA restriction mapping separations may be obtained either with relatively dilute, low-viscosity cellulosic polymer solutions and with concentrated, syrupy polyacrylamide solutions, then clearly the cellulosic polymers should be preferred, for three reasons. One, DNA moves through the less viscous cellulosic solution much more rapidly, providing faster, more efficient separations. Two, it is much easier and faster to pump dilute polymer solutions in and out of a microbore capillary than concentrated ones. Three, the acrylamide monomer is a potent neurotoxin and its routine use should be avoided if possible. Cellulosic polymers are completely harmless, as evidenced by the fact that they are used as industrial

thickeners for a myriad of personal products such as shampoo and conditioner (this also means that they are inexpensive: *e.g.*, HEC presently costs about $5 per 50 lb. bag, if it is purchased from an industrial manufacturer). The advantage of polyacrylamide and related acrylamido-based polymers like pAAEE is that their polymerization can be accurately controlled to give a desired range of molecular mass. Both Ruiz-Martinez *et al.* [194] and Heller and Viovy [412] have shown that the molecular mass of linear polyacrylamide may be optimized to provide good resolution at a reasonably low viscosity, so that the solution may be pushed in and out the capillary easily with a syringe. Although cellulose derivatives are also available in a wide range of molecular masses, they are not synthetically polymerized, but instead are derived from various natural sources (*e.g.*, cotton); they therefore tend to be quite polydisperse. For a systematic study of the dependence of DNA separating ability on molecular weight, it might be desirable to use a monodisperse polymer sample; but there has been no indication that polydispersity has any deleterious effects on DNA resolution.

While it seems clear that concentrated solutions of linear polyacrylamide are the most suitable choice for single-base resolution of DNA sequencing products by CE [194], it remains unclear which is the best polymer, and what is the optimum concentration range, to use for the CE separation of larger DNA (*e.g.*, for restriction mapping). An increasing number of systematic studies of these variables are appearing in the literature, such as those undertaken by Chrambach's group [63, 398], Righetti's group [172, 307], Barron *et al.*, [90, 91], Karger's group [194] and Baba's group [206]. A few guidelines for the use of polymer solutions for DNA separations in capillaries can be gleaned from these studies. HEC greater than 100,000 g/mol in number-average molecular mass (M_n) is easy to dissolve in an aqueous buffer over a wide concentration range and provides excellent, reproducible separations for DNA up to 23 kbp in size [90, 91]. In our experience, HEC seems to be a preferable DNA separation matrix as compared to hydroxypropyl cellulose (HPC), which is more surface-active. Although the two closely related polymers provide roughly the same level of DNA separation, relatively concentrated solutions of HPC have the tendency, even after thorough degassing, to exhibit high-frequency noise in the vicinity of the DNA peaks, probably due to the formation of bubbles when the sample is hydrodynamically injected into the viscous polymer solution (it is possible that this problem could be avoided if electrokinetic injection is used). We have also found that, in general, low-molecular mass hydrophilic polymers provide significantly poorer resolution, particularly of DNA larger than 600 bp [90, 91]. Baba *et al.* [206] found that there was very

little difference in DNA separation efficiency between solutions of hydroxypropyl cellulose, methyl cellulose, and hydroxypropyl methyl cellulose (HPMC); although it appears from the electropherograms presented in the paper that the peak shapes for DNA separations in HPMC were not quite as good as for the other two cellulose derivatives. These workers also reported that special procedures were required to dissolve these polymers, which are all less hydrophilic than HEC [206].

In our studies [90, 91], as well as those of Baba *et al.* [206], Guszcynski and Chrambach [61], a consistent theme emerges: the longer the polymers which are dissolved in the electrophoresis buffer, the better the DNA separation performance and the wider the range of DNA sizes which can be separated (this is true for both cellulosic polymers and polyacrylamide).

For large DNA (0.6-23 kbp), relatively dilute solutions of long cellulosic polymers provide the best resolution [90, 91], although very viscous, concentrated solutions of polyacrylamide [307] and pAAEE [172] can also provide excellent, if much slower, separations of DNA in this size range. To achieve the high-resolution CE separation of DNA smaller than 600 bp, higher-concentration and hence higher-viscosity polymer solutions must be used. More concentrated cellulosic polymer solutions can deliver the resolution required for restriction mapping of small DNA, but polyacrylamide seems to be the most useful polymer to achieve the single-base resolution of DNA sequencing fragments smaller than 500 bases. An advantage of using low-viscosity polymer solutions for the separation of intermediate-sized DNA is that for a given capillary length, one can then exploit the rapid electroosmotic flow in fused silica capillaries to pump DNA past the detection window (see Figure 12 and discussion in Section 6. 4. 1.). CE run in this mode can yield significantly better resolution of DNA fragments than CE in coated capillaries without electroosmotic flow, because the electroomosis serves to increase the DNA residence time in the capillary without significantly contributing to band-broadening [416].)

At present, the many research groups which are researching CE in polymer solutions are racing to explore parameter space for the DNA separation efficiency achievable in solutions of virtually every hydrophilic polymer available; some are even inventing new hydrophilic polymers for this purpose [169]. Eventually this should lead to a consensus on the optimum polymer type, length, and concentration for the separation of DNA in various size ranges.

6. 8. 5. The effect of fluorescent dyes on DNA electrophoresis in uncrosslinked polymer solutions

It has been found by several groups applying laser-induced fluorescence detection to CE in polymer solutions that the addition of ethidium bromide to the electrophoresis buffer can improve DNA resolution and peak shape [294, 339, 347]. There are complicating issues which arise when positively-charged, intercalating dyes are added to the system. First, intercalators lengthen and stiffen the DNA double helix; the ethidium cation can intercalate at up to one per four base pairs [2]. Second, the intercalation of these cationic dyes reduces the net charge on the DNA molecule. A lower net charge decreases electrophoretic mobility, increasing the DNA residence time in the capillary, hence increasing band separation. This effect will complicate quantitative comparisons of DNA electrophoretic mobility between experiments. Third, when cationic dyes (especially *dimeric* dyes, such as TOTO-1, YOYO-1, or ethidium dimer [355]) are used, each dye molecule has the potential to form an effective charge link between the ionized silanols on the silica surface of the capillary and the anionic DNA fragments [339]. If this occurs, DNA adsorption on the wall becomes significant and severe band broadening and tailing may occur [324, 339, 349, 355]. For monomeric dyes, this disastrous occurrence can be avoided if the capillaries are thoroughly and thickly coated with an uncharged polymer. So far, for dimeric dyes, which can intercalate into DNA with one linked monomer and charge-link to the silica surface with the other, no capillary coating appears to completely eliminate this ruinous interaction[349, 355]. Dimeric fluorescent dyes, which were designed to have improved spectroscopic properties and DNA-binding affinities as compared to EtBr [28, 29, 122-124], may be more useful for separations by slab gel electrophoresis in which DNA adsorption and dye charge-linking is not a problem.

6. 8. 6. The mechanism of DNA separation in uncrosslinked polymer solutions

The mechanism of DNA separation in uncrosslinked polymer solutions has been a matter of controversy. Some researchers have asserted that the mechanism is essentially the same as that of traditional slab gel electrophoresis [61, 83, 172, 206, 285, 286, 297], while others have attributed separation to the attraction and interaction of DNA fragments with the cellulose derivatives in the buffer, without further specification of what those interactions might be [222, 287]. Still others have suggested that the separation involves a mechanism of exclusion from the polymer fiber network similar to that occurring in gel permeation chromatography [63, 404]. One of the reasons that researchers have tried 13 different types of polymers (and counting) to separate DNA is that the relative significance of various

polymer properties (such as molecular mass, concentration, stiffness, polydispersity, and hydrophilicity) for DNA separation is not well understood. With a lack of understanding of how and why DNA separation occurs in uncrosslinked polymer solutions, it is difficult to optimize the system.

Furthermore, it has not been definitively established whether the mechanism of DNA separation in extremely dilute polymer solutions is the same as that which occurs in concentrated, syrupy solutions, although we will discuss the results of recent research which have shed considerable light on this question [90, 91, 411]. While it is certain that a striking difference in polymer-polymer interactions exists between a dilute, uncrosslinked polymer solution and a semi-rigid crosslinked gel [417], there is much less difference between an extremely concentrated, syrupy polymer solution and the aforementioned gel. When the polymer concentration is sufficiently high, an entangled polymer network is formed which is quite stiff and confining for the migrating DNA molecules, causing DNA electrophoretic motion to resemble that which occurs in crosslinked gels [411].

Viovy and Duke [93, 94] have, for the case of low electric fields and completely entangled polymer solutions, attempted to account for the dynamic nature of the physical network formed by uncrosslinked polymers, by modifying a version of the biased reptation model which includes tube length fluctuations to also include constraint release. Such constraint release occurs when the polymers which form the tube are allowed to reptate away, changing the tube dimensions even while the DNA remains within it. Nonetheless, for uncrosslinked polymer solutions in general, dilute or concentrated, the relationship between electrophoretic resolving power and the molecular mass and concentration of a given polymer is not yet fully understood, although as we have discussed, certain qualitative guidelines are beginning to emerge. At present, no unified theory exists to predict the appropriate polymer size and concentration for a desired DNA separation, or to model the electrophoretic mobility of DNA restriction fragments as a function of field strength, polymer concentration, DNA size, and polymer properties such as average molecular mass and persistance length.

At present, most researchers interpret their electrophoresis data, regardless of polymer type and concentration, with the assumption that the mechanism of DNA separation in uncrosslinked polymer solutions is basically the same as that postulated for DNA separation in polyacrylamide or agarose gels [61, 172, 206, 285, 297]. This assumption may be appropriate when the polymer solution in question is highly concentrated and forms an

entangled network [172], but is probably incorrect for the semi-dilute and dilute polymer solutions which are often used for the CE separation of DNA restriction fragments. It is usually assumed that DNA separation occurs because the polymers have formed an entangled network in solution; such a network may be termed a "physical" gel, as opposed to a crosslinked, or "chemical" gel [94]. This entangled polymer solution is further assumed to contain "transient pores," similar to the pores of an agarose or polyacrylamide gel [285]. The average size of the transient pores within a polymer solution is thought to determine the range of DNA lengths which can be separated, this average transient pore size having the same implications as the "pore size" of a true gel [100, 104, 166, 285, 418].

An influential publication in this area was that of Grossman and Soane [285] in which the proposal was made that in order for polymers in semi-dilute solution to be effective for DNA separation, they must be *entangled*; and that it was the physical similarity of this entangled network to the pore network of a gel which allowed size-dependent separation of DNA. They thus proposed that the Ogston-Rodbard-Chrambach and reptation models of DNA gel electrophoresis were appropriate to high-field DNA separations in uncrosslinked polymer solutions as well. A polymer solution is entangled if it is well above the *entanglement threshold concentration* (Φ^*), *i.e.*, the concentration at which the polymer chains begin to interact strongly in solution [419]. Experimentally, this concentration can be estimated from a log-log plot of solution viscosity vs. polymer concentration. In dilute solution, when there is no strong interaction between solvated polymer molecules, viscosity increases in direct proportion to polymer concentration, and the slope of this plot is constant at about 1.0. The formation of an incipient entangled polymer network in solution [420] is evidenced by a large increase in viscosity, with a corresponding increase in the slope of the viscosity vs. concentration curve (for example, Figure 16 shows this type of plot for a sample of hydroxypropyl cellulose dissolved in Tris-Borate-EDTA, a common DNA electrophoresis buffer). The entanglement threshold concentration of a given polymer sample in aqueous solution is strongly dependent on its average molecular mass, with a length dependence ranging between $N^{-0.8}$ for flexible polymers to $N^{-1.2}$ for stiff cellulose derivatives (where N is the number of monomers in the polymer chain) [90, 419].

The theory that an "entangled polymer solution" is necessary for DNA separation [285] has been proven to be incorrect. We have shown in a recent paper [91] that large DNA (2 - 23 kbp) can be separated in *ultra-dilute* hydroxyethyl cellulose solutions, up to two orders in

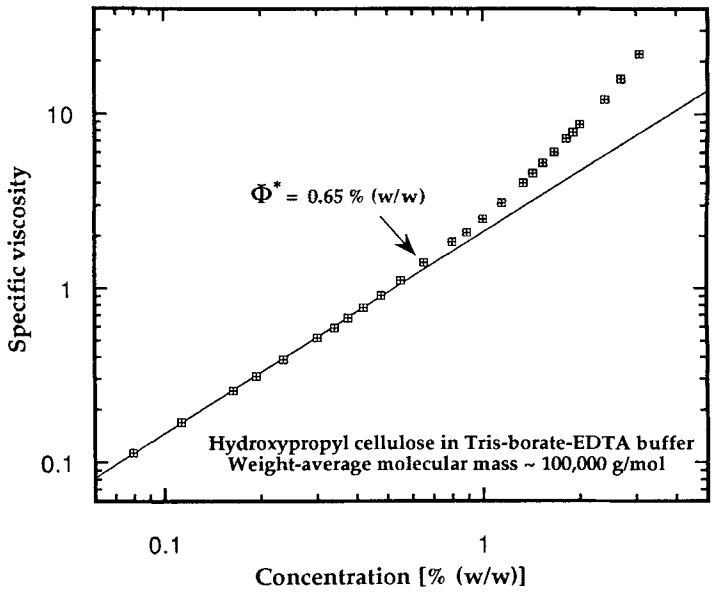


FIGURE 16
A log-log plot of the specific viscosity of a polymer solution as a function of polymer concentration. This plot can be used to determine the approximate concentration at which an entangled polymer network is formed within the solution. In this case, the polymer is hydroxypropyl cellulose, with a weight-average molecular mass of about 100,000 g/mol, dissolved in a 89 mM Tris-89 mM borate-5 mM EDTA buffer, pH 8.15. Viscosity measurements were taken with an automated Ubbelohde viscometer.

magnitude lower in concentration than the measured entanglement threshold. These findings confirmed the theoretical prediction of Viovy and Duke that duplex DNA up to 1 kbp or more could be separated in dilute, nonentangled solutions of high molecular weight polymers [93]. Figure 17 shows the separation of DNA ranging in size from 72 bp to 23130 bp in solutions of HEC (M_n ~ 105,000 g/mol) at concentrations of 0.15%, 0.0125%, and 0.00125% (w/w) [91]. This HEC sample forms an entangled polymer network at a concentration of 0.37% (w/w) [90]; it is apparent from these results that the formation of an entangled polymer network is not necessary for DNA separation in uncrosslinked polymer solutions [91]. Moreover, we determined that the *minimum* HEC concentration for the separation of large DNA is about 0.0006% (w/w) HEC (6 ppm). Figure 18 shows a plot of DNA electrophoretic mobility vs. HEC concentration, for very dilute solutions [91]. This

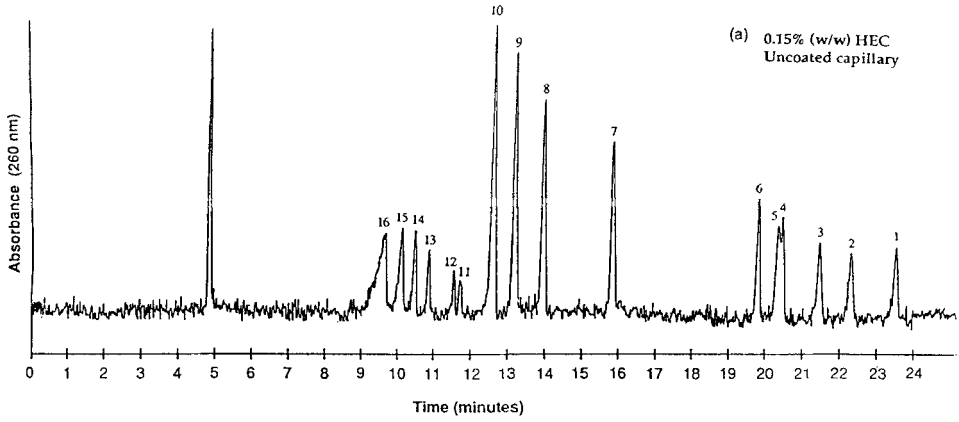


FIGURE 17

Separation by capillary electrophoresis of λ–*HindIII* and ΦX174-*HaeIII* restriction fragments (in non-stoichiometric mixture) **(a)** in 0.15 % (w/w) HEC (M_n 105,000). The far left peak corresponds to a neutral marker (mesityl oxide), used to determine the velocity of electroosmotic flow in the capillary. Peak identification: **1** = 23130 bp, **2** = 9416 bp, **3** = 6557 bp, **4** = 4361 bp, **5** = 2322 bp, **6** = 2027 bp, **7** = 1353 bp, **8** = 1078 bp, **9** = 872 bp, **10** = 603 bp, **11** = 310 bp, **12** = 281 bp, **13** = 271 bp, **14** = 234 bp, **15** = 194 bp, **16** = 118 + 72 bp. λ–*HindIII* restriction fragments of 125 bp and 564 bp are present in such small concentrations that they are too faint to be seen. (b) in 0.025 % (w/w) HEC (M_n 105,000). The far left peak corresponds to a neutral marker (mesityl oxide), while the second peak at left is an impurity present in the DNA sample. Peak identification: same as 3 (a). (c) in 0.00125 % (w/w) HEC (M_n 105,000). The far left peak corresponds to a neutral marker (mesityl oxide). Peak identification: same as 3 (a). Buffer: 89 mM Tris(hydroxyethyl-aminomethane), 89 mM boric acid, 5 mM Ethylenediamine-tetraacetic acid (EDTA), pH 8.15. Capillary: 51 μm i.d., 50 cm total length (35 cm to detector); temperature, 30 ± 0.1°C. Detection was by UV absorbance at 260 nm. Injection was hydrodynamic. Electrophoresis conditions: field strength 265 V/cm, current was (a) 8.5 μA, (b) 7.2 μA, (c) 7.2 μA. RSD of absolute electrophoretic mobilities: (a) 0.32 %, n=3; (b) 0.41 %, n=5; (c) 0.28 %, n=3. The entanglement threshold of this polymer sample in the electrophoresis buffer is ~ 0.37% (w/w). [Reprinted, with permission, from Reference [91].]

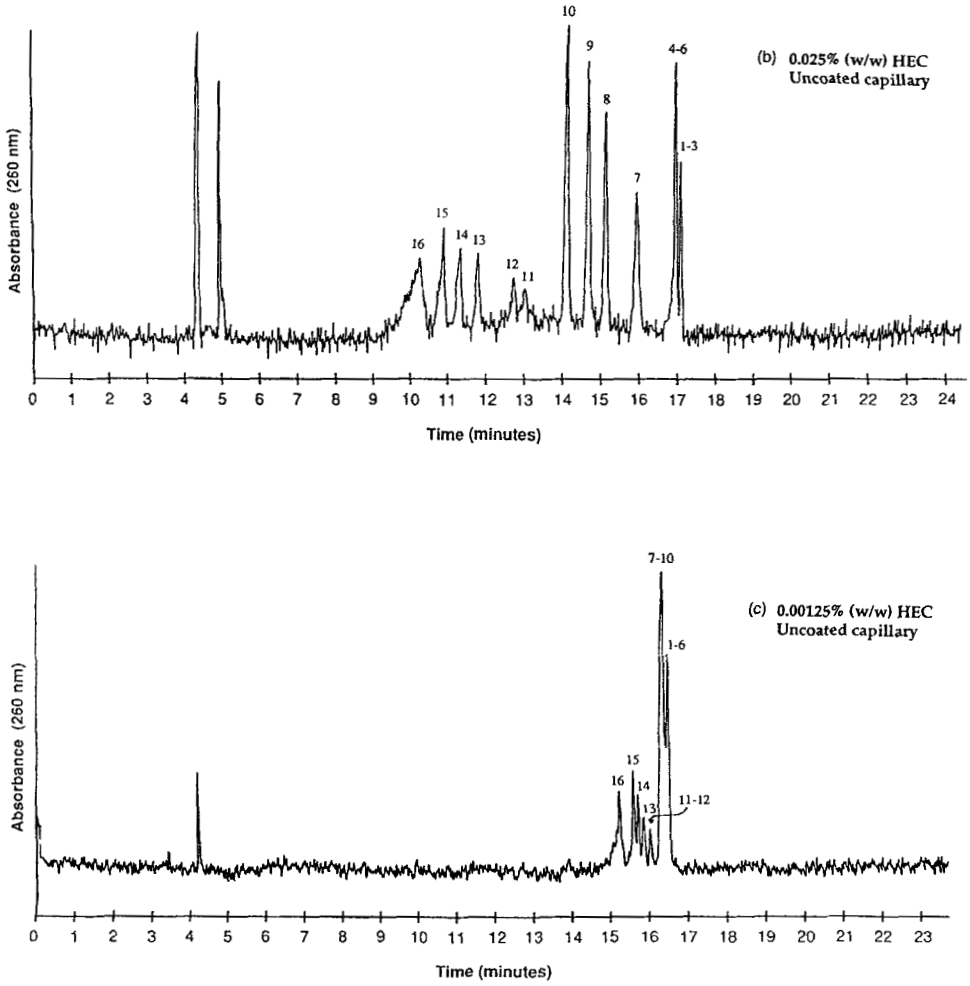


FIGURE 17 Continued

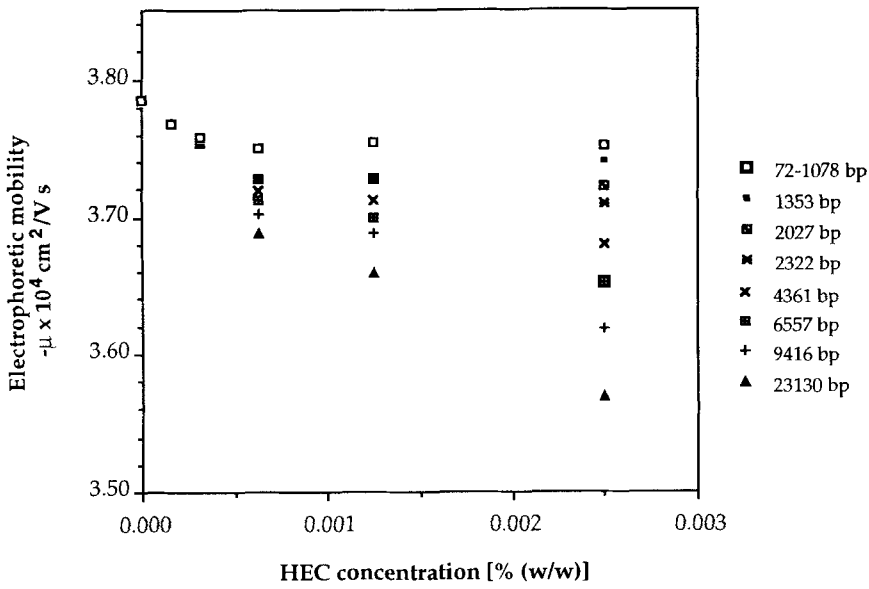


FIGURE 18

A plot of DNA electrophoretic mobility vs. HEC concentration (HEC M_n 105,000 g/mol) for DNA restriction fragments ranging from 72 bp to 23130 bp in length, plotted on an expanded scale to show detail at extremely low HEC concentrations. Data points at each HEC concentration are the average of 3 - 5 individual determinations. Average run-to-run variation in calculated electrophoretic mobilities: ± 0.46 %. DNA electrophoretic mobility was calculated by subtracting the electroosmotic mobility, calculated from the elution time of a neutral marker, from the *apparent* electrophoretic mobility of the DNA fragments, as DNA electrophoretic motion was opposite in direction to the electroosmotic flow which was used to drive it past the UV absorbance detector. The electrophoresis buffer, all conditions, and DNA sample, same as in Figure 17. [Reprinted, with permission, from Reference [91].]

finding suggests that the Ogston-Rodbard-Chrambach and reptation theories for the mechanism of DNA separation in gels are not sufficient to explain DNA separation in dilute polymer solutions. When a polymer solution is far below the entanglement threshold concentration, the polymers do not form a physical network and are relatively isolated in solution; they cannot be envisioned to form "pores," transient or otherwise.

According to pore-based models of DNA electrophoresis, no DNA separation should be possible in dilute polymer solutions. To explain our experimental results, we must assume

that another mechanism of separation is operative, at the very least in dilute polymer solutions, and possibly in more concentrated polymer solutions as well. The proposed mechanism of separation is based on a consideration of the physical properties of HEC and DNA, as well as those of polymers in general. HEC is a linear (*i.e.*, non-branched), uncharged cellulose derivative, having bulky ethylene oxide side chains terminating in hydroxyl groups (Figure 19). In aqueous solution, these hydrophilic side groups force the polymer into a stiff, extended conformation. This stiffness is evidenced by a Porod-Kratky persistence length of 8.3 nm, roughly 10 times that of a typical flexible, random-coil polymer [421]. Double-stranded DNA is even more stiff and extended in solution than HEC, with a Porod-Kratky persistence length of 45 nm in 0.2 M buffer [30]. At comparable concentrations, stiff, extended polymers exhibit the effects of entanglement coupling more strongly than flexible, random-coil polymers [421]. Given this, it is likely that when DNA molecules encounter isolated HEC polymers, they can become entangled with them. These transient entanglement interactions would be augmented by the stiffness of the two participants in the interaction. In this way, DNA molecules are forced to drag the uncharged HEC polymers along with them as they move by electrophoresis, resulting in a decrease of DNA electrophoretic mobility. It has been demonstrated theoretically by Bueche that the molecular friction factor of a polymer in solution is much increased by entanglement coupling with other polymers [421]. Therefore, this type of DNA/polymer entanglement coupling interaction could alter the frictional characteristics of the DNA molecules moving under the influence of the electric field in a size-dependent manner. Larger DNA molecules would have a higher probability of encountering and entangling with one or more HEC molecules. Figure 20 is a schematic illustration of the proposed DNA motion in dilute HEC solution, showing the relative sizes of large DNA (9461 bp) and small DNA (118 bp) compared to HEC (M_n 105,000). In this figure, both DNA and HEC polymers are drawn with the correct number of properly-scaled persistence lengths. And, as shown in the figure, large DNA is likely to adopt an open, free-draining conformation in an ultra-dilute polymer solution, as it does in free solution, allowing HEC chains to penetrate the DNA coil and increasing the probability of transient entanglement coupling. One of the advantages of this model for the mechanism of DNA separation is that it requires no theoretical constructs such as "pores" or "tubes."

Furthermore, we have shown that unlike DNA, charged microspheres having roughly the same radius of gyration as DNA molecules of interest exhibit no improvement in their size-separation as HEC concentration is increased [91]. As the Ogston-Rodbard-Chrambach

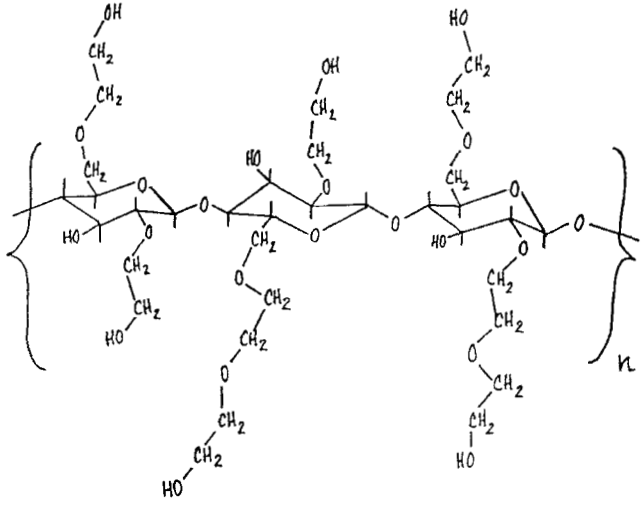


FIGURE 19
A typical structural element of a hydroxyethyl cellulose chain (three monomers shown). [Reprinted, with permission, from Reference [90].]

model would predict, Ferguson plots are completely straight for the separation of charged spheres (Figure 21); however, they are deeply curved for large DNA fragments [91]. Based on the proposed model, we have suggested that this is because they are incapable of participating in transient entanglement coupling with the HEC polymers, and because the Ogston-Rodbard-Chrambach model is simply not appropriate for the electrophoresis of relatively large DNA (> 600 bp) in dilute polymer solutions. The reptation model would not be expected to be appropriate either, since in a dilute polymer solution no obstacles would be long-lived enough to form the reptation tube.

Using a transient entanglement coupling model of DNA/polymer interactions during electrophoresis, we can explain the experimental finding that low-molecular mass HEC (M_n ~ 27,000 g/mol) does not separate DNA as efficiently as high-molecular mass HEC (105,000 g/mol) [91]. We found that unlike high-molecular mass HEC polymers, the smaller HEC chains do not have the ability to separate DNA larger than 600 bp very well at *any* concentration. These results point to the fact that the relative sizes of the cellulosic polymers and DNA molecules are very important in size-separation. The (average) total

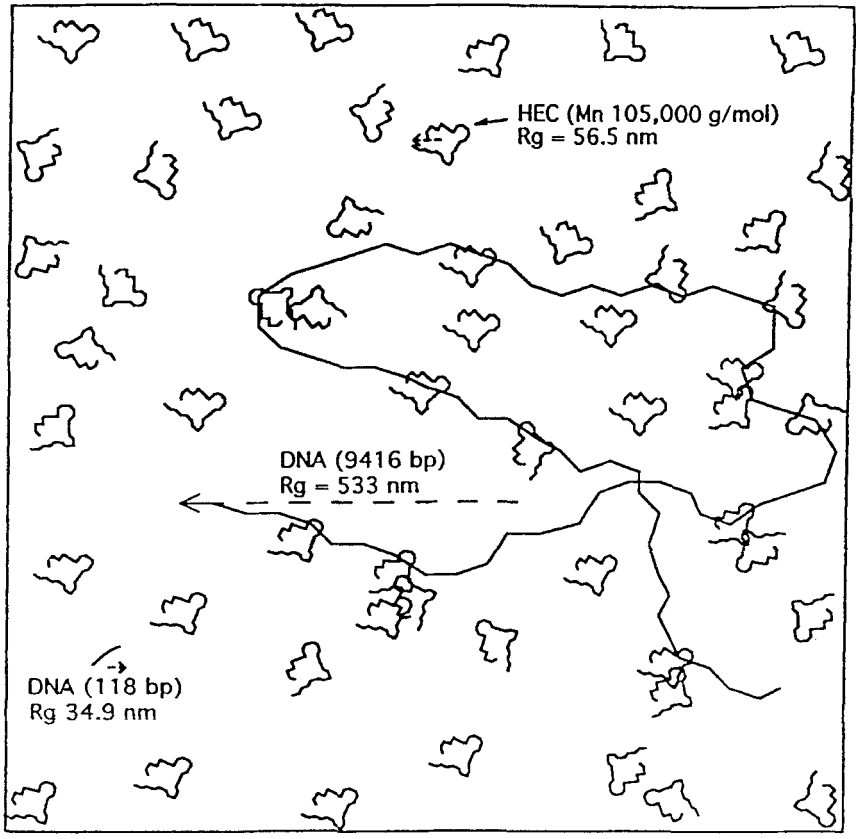


FIGURE 20

A schematic illustration of DNA motion in a dilute HEC solution. In the figure, the approximate relative sizes of HEC and small and large DNA are shown. Using the Porod-Kratky model for a stiff, worm-like coil, the calculated radius of gyration (R_g) of HEC (105,000 g/mol) is 56.5 nm. For a small, 118-bp DNA restriction fragment (from ΦX174-*HaeIII*), the Porod-Kratky R_g is 35 nm, while the contour length is 40 nm. Since the Porod-Kratky persistence length of double-stranded DNA is 45 nm, this represents less than one persistence length and the 118-bp fragment is small and rod-like, and unlikely to entangle strongly with HEC in dilute solution. A larger DNA restriction fragment, consisting of 9416 bp (from λ-*HindIII*) has a Porod-Kratky R_g of 533 nm; thus, it is ~ 10 times larger in radius than the HEC molecules. With a contour length of 3201 nm, this 9416-bp DNA fragment contains ~ 71 persistence lengths, as shown schematically in the figure, and has a high probability of undergoing transient entanglement coupling with many HEC molecules. When transient entanglement coupling occurs, the larger DNA molecule must drag the uncharged HEC molecules along with it during electrophoresis, decreasing its electrophoretic mobility in a size-dependent manner (the average number of HEC chains coupled to a DNA molecule must increase with DNA size). [Reprinted, with permission, from Reference [91].]

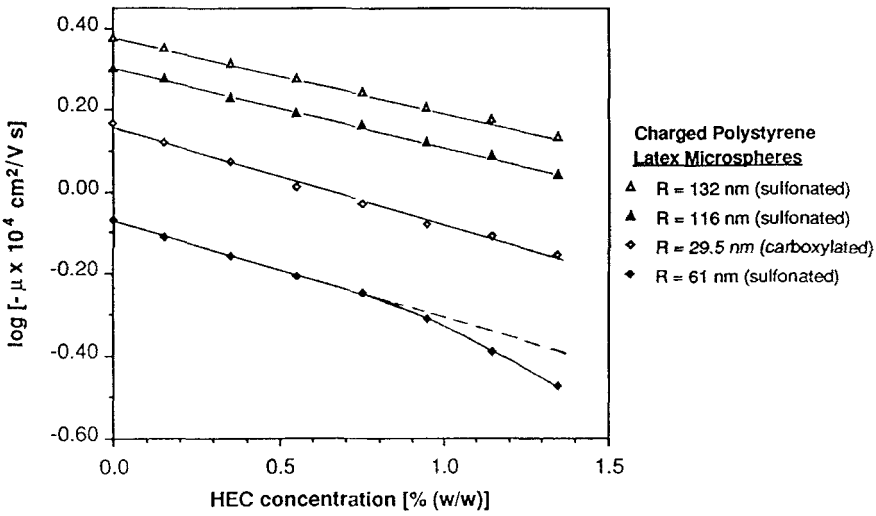


FIGURE 21

A Ferguson plot for the electrophoresis of charged polystyrene latex microspheres in solutions of HEC (M_n 105,000 g/mol). Note that the carboxylated spheres have a higher surface charge density than the three sulfonated sphere samples (which have similar surface charge densities). Thus, although the carboxylated spheres have the smallest radius, their electrophoretic mobility is intermediate between that of the two smallest sulfonated spheres. Each data point on the graph is the average of 3-6 individual determinations. Average run-to-run % RSD of electrophoretic mobilities was 1.18 %. Buffer: 0.05 M sodium chloride, 0.05 M boric acid, 6 mM sodium hydroxide, pH 8.2 , with 0.5 % (v/v) Triton X-100® non-ionic surfactant. Capillary: 51 μm i.d., 50 cm total length (35 cm to detector); temperature, 30 ± 0.1°C. Detection was by UV absorbance at 260 nm. Injection was hydrodynamic. Electrophoresis conditions: field strength 310 V/cm, current ~ 55 μA. [Reprinted, with permission, from Reference [91].]

contour length of the of the small and large HEC polymers can be calculated using their number-average molecular weights, the average monomer molecular weight (272 g/mol) and the contour length per monomer (0.519 nm [421]). Using these quantities, the M_n 27,000-HEC has a 51.5 nm contour length, while the M_n 105,000-HEC has a contour length of 200.3 nm. Using the Porod-Kratky persistence length of HEC (8.3 nm) [421], we calculate that the shorter cellulose has only 6.2 persistence lengths on average; the larger cellulose has about 24 persistence lengths, and can thus entangle more strongly with DNA chains, as well as with other HEC polymers. Furthermore, when a larger cellulosic polymer is entangled with a DNA molecule, it engenders more frictional drag than a

smaller one. It seems likely that if the dissolved polymers are too small, they may be too easily displaced by the larger DNA restriction fragments, because they form weak points of entanglement and are also too small to significantly hinder DNA electrophoretic motion. In this case, small HEC polymers would be less efficient in introducing size-dependence to the molecular friction factor of larger DNA, as is observed experimentally.

Recent experimental results support the idea that the interaction of DNA with HEC during electrophoresis is likely to involve transient entanglement coupling. Shi, Hammond and Morris [411] used epifluorescence videomicroscopy with a CCD camera to study the conformational dynamics of yeast chromosomal DNA (225 kbp - 1.9 Mbp) during electrophoresis in solutions of high-molecular mass HEC (M_n 438,800 g/mol), both above and below the HEC entanglement threshold. They found that in concentrated HEC solutions, DNA electrophoretic behavior is very similar to that which has been observed in agarose gels, *i.e.,* DNA cycles between compact conformations and extended U- or V-shapes which appear to be caught on some obstacle. The only difference between concentrated HEC solutions and agarose gels appeared to be that even when the DNA is caught on an obstacle and stretched into a U- or V-shapes, the apex of the U or V is able to move slowly in the direction of electrophoresis. In other words, the obstacles in concentrated polymer solutions are not immovable, as they are in agarose. However, in *dilute* HEC solutions (at one-third the entanglement threshold concentration), in which no entangled polymer network exists, the DNA molecules are observed to travel for most of the time in globular conformations, which occasionally catch on a glob of HEC polymers and cycle through a short-lived, less extended U shape (as they drag the uncharged HEC glob through the solution) and then return to a globular conformation (see Figure 22).

It is clear that the mechanism of DNA electrophoretic separation in uncrosslinked polymer solutions involves additional complexities in comparison to agarose gel electrophoresis, due to the deformability of the polymer network and due to the fact that at very dilute concentrations, no polymer network exists at all -- just isolated polymer chains. Shi *et al.* [411] noted that their microscopic observations show a continuum of DNA electrophoretic behavior from quite concentrated down to very dilute polymer solutions; this suggests that it should be possible to formulate a single, unified model of DNA electrophoresis in polymer solutions. This interesting and complex new problem will present a significant challenge to polymer physicists.

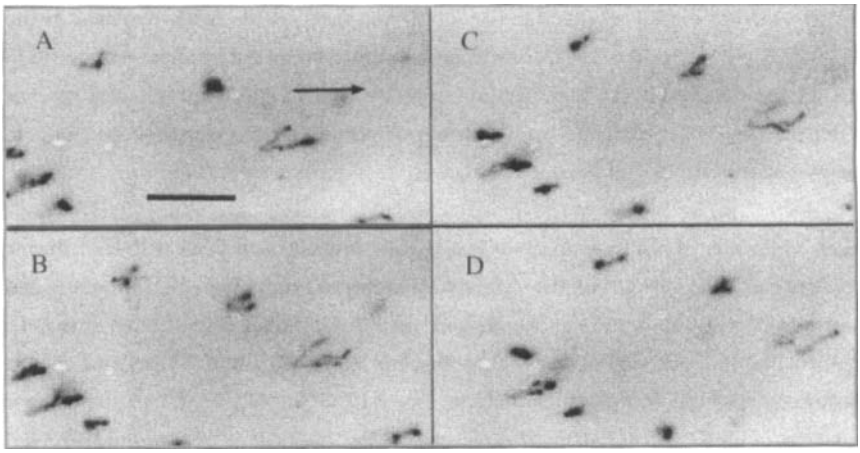


FIGURE 22

Time sequence (A-D) showing the existence of both small U-shaped DNA molecules and deformed globule conformations for yeast chromosomal DNA molecules (stained with ethidium homodimer I) undergoing electrophoresis in 0.032% HEC (M_n 438,800 g/mol), 60% sucrose and Tris-borate-EDTA buffer. For this HEC sample, the entanglement threshold concentration was measured to be 0.09% (w/w) [90]. At higher HEC concentrations, near and above the entanglement threshold concentration, DNA molecules were observed to assume longer-lived and significantly more stretched U-shaped conformations. Field strength: 25 V/cm. Frames at 4.0 second intervals; contrast is inverted (fluorescent regions black) for clarity. The migration direction is indicated by an arrow and the scale bar represent 10 μm. DNA was contained between two microscope coverslips which had been coated with polyacrylamide to eliminate electroosmotic flow. Illumination was with 10mW 532 nm light from a frequency-doubled CW Nd-YAG laser, whose output was passed through a coil of optical fiber. The fiber was mechanically vibrated to break up laser coherence. Images were taken with a scientific CCD camera (Photometrics Star-I). Sucrose was added to the buffer to increase its viscosity, thus slowing the DNA electrophoretic mobility. [Reprinted with permission from Ref. [411].]

Finally, we would like to point out that researchers who have attempted to separate very large DNA restriction fragments by steady-field CE in polymer solutions have not yet shown the separation of DNA fragments larger than 23.1 kbp (the largest fragment in the commonly used λ*HindIII* restriction digest). In fact, it has consistently been observed that DNA resolution degrades appreciably for fragments larger than a few thousand base pairs. It is likely that this limitation results from the orientation and stretching of DNA larger than 25 kbp which occurs under the high, steady fields typically used for CE. It is known that large DNA molecules are more easily oriented in the direction of the electric field

during electrophoresis than small ones [422]. At present it seems likely that steady-field CE in uncrosslinked polymer solutions is limited to the separation of DNA smaller than 25 kbp in size. Even so, this technique has the potential to be a rapid, efficient technique for plasmid mapping [302]. Furthermore, this is not a serious limitation, as Kim and Morris [410] and Sudor and Novotny [408] have shown that the CE separation of DNA fragments larger than 23.1 kbp can be achieved if lower-voltage, pulsed electric fields are applied. For the physical and genetic mapping of larger DNA molecules, or for the study of chromosomal DNA, pulsed field CE has great potential, as we discuss in the next section.

6. 8. 7. Pulsed-field CE in dilute, uncrosslinked polymer solutions for the rapid separation of large DNA

The potential of pulsed-field CE for DNA separations was first studied in 1992 by Heiger *et al.* [283, 423]. In 1993, Sudor and Novotny studied its use for the resolution of polysaccharides [423, 424]. Then, in 1994, Sudor and Novotny [39, 408] and Kim and Morris [409, 410] simultaneously reported that very large DNA could be separated in dilute polymer solutions upon the application of pulsed fields of a precise frequency. Sudor and Novotny reported the separation of DNA as large as 1 Mbp in 180 minutes in 0.4% (semi-dilute) linear polyacrylamide solution [408]. Kim and Morris, on the other hand, have reported the separation of DNA as large as 1.6 Mbp in ultra-dilute (0.002%-0.004%) mixed HEC/polyethylene oxide solutions, in only 12 minutes [409]. However, to date neither of these groups have been able to directly confirm the peak assignments in their electropherograms (e.g.., by spiking with a DNA fragment isolated from a slab gel). If indeed, as it appears, these researchers have achieved the separation of megabase DNA in just minutes, these are truly stunning and potentially revolutionary experimental results, given that slab gel separations of megabase DNA often require from 1 to 3 *days* of pulsed field electrophoresis. Coupled with a good injection and sample collection system, pulsed-field CE has the potential to become an important and rapid technique for the study of chromosomal DNA and for long-range DNA restriction mapping.

7. 0 Summary and Conclusions

The electrophoretic separation of DNA is an indispensible technique in a modern molecular biology laboratory. Although slab gel electrophoresis is a powerful method with many important applications, it is also time consuming, not readily amenable to complete automation or quantitation, and potentially hazardous due to the common use of radioactive labeling, neurotoxic acrylamide monomers, and the common requirement for

large amounts of buffer containing mutagenic intercalating dyes. CE represents a new frontier for electrophoretic DNA separations. CE is at least an order of magnitude faster than slab gel electrophoresis, may be completely automated, allows easier and more precise quantitation of results, and due to the small scale and self-contained nature of the apparatus, reduces health risks from dangerous chemicals. Moreover, the initial disadvantages of CE (*e.g.*, the instability of gel-filled capillaries, the difficulty of sample collection, the inability to run more than one "lane" in a single capillary, or to load large sample volumes) are rapidly being overcome. Although CE will most likely never completely replace preparative slab gel electrophoresis, it is an important complementary technique which will serve molecular biologists well in the coming years.

Acknowledgements

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