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Review

Comparison of properties of agarose for electrophoresis of DNA

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ABSTRACT

Agarose as a medium for separation of DNA was first introduced in 1962 and since the early 1970s agarose submarine gel electrophoresis has been synonymous with separations of DNA molecules larger than 1 kilobase pair (kb). The large pore size, low electroendosmosis and strength of the matrix have advantages over other media such as polyacrylamide for many applications. The variety of grades of agarose, developed by chemical manipulation of the substitutions on the agarose polymer, provides a range of matrices for separation of DNA molecules from a few base pairs (bp) to over 5 megabase pairs (Mb) in length. The introduction of low-melting-temperature agarose has revolutionised the extraction and manipulation of chromosome-sized molecules. On the other hand, the demand for analysis of very small quantities of DNA will most likely lead to the increasing importance of capillary electrophoresis. Many theories have been propounded to explain the electrophoretic migration of DNA in agarose. The most popular of these has been reptation theory but none can account for all of the reported anomalies in migration. However, anomalous migration has been exploited to study DNA structure, topology and catenation. An example of the use of two-dimensional electrophoresis to demonstrate the complexity of DNA migration through agarose is given. Generally, for molecules smaller than 50 kb, electrophoretic separation is a function of length. By alternately electrophoresing DNA in two different directions, molecules as large as 5.7 Mb have been effectively separated, although with such large molecules DNA structure as well as size may determine migration. In the case of separations of chromosomes from the intestinal protozoan, *Giardia duodenalis*, for example, a discrepancy of 1 Mb in the size of one chromosome, with an apparent size of 0.7–2.0 Mb, depended on the boundary conditions of separation. Major challenges for the molecular biologist are separation of larger chromosomal sized molecules, greater number of samples and smaller formats. Towards this challenge computer-aided technology is a key component in the control of electrophoresis parameters and analysis.

CONTENTS

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LIST OF ABBREVIATIONS

bp	Base pairs
kb	Kilobase pairs
Mb	Megabase pairs
CE	Capillary electrophoresis
CHEF	Contour-clamped homogeneous electric field electrophoresis
EEO	Electroendosmosis
FIGE	Field inversion gel electrophoresis
GTG	Genetic technology grade
OFAGE	Orthogonal field alternation gel electrophoresis
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RFLP	Restriction fragment length polymorphism
TAE	Tris–acetate–ethylenediaminetetraacetic acid
TBE	Tris–borate–ethylenediaminetetraacetic acid

1. BRIEF HISTORY OF DNA ELECTROPHORESIS IN AGAROSE

Electrophoresis as a method to separate single-stranded from double-stranded DNA was introduced by Matsubara and Takagi in 1962 [1], using starch as the medium on flat beds. Agar as a matrix was first used on flat glass sheets to separate cellular and polyoma viral DNA components in 1966 [2,3], and agarose, the agaropectin fraction of agar [4], was instituted for DNA separation in 1969 [5] after its successful use in RNA separation on a flat bed apparatus with a perspex mould for loading slots [6]. The subsequent development in running parameters led to Tris–acetate ethylenediaminetetraacetic acid (TAE) buffers which are still in use today [7,8]. Ethidium bromide was originally included in these buffers to improve separation of open and closed circular DNA forms by differential intercalation [7], but also allowed immediate visualisation under UV illumination [7,9]. These latter studies employed tube gels instead of the flat beds traditionally used for starch gel electrophoresis. To allow multiple sample loadings and improve lane-to-lane reproducibility, a vertical apparatus was introduced by Studier [10], although vertical agarose–acrylamide composite gels had been used for RNA since 1967 [11] and DNA [8]; the horizontal flat-bed approach was revitalised in 1977 [12]. The current use of horizontal gels which dispensed with wicks, either paper or agarose, and which totally immersed the gel in running buffer (submarine gels) has been attributed to Schaffner [13].

2. COMPARISON OF AGAROSE WITH OTHER METHODS OF DNA SEPARATIONS

2.1. Polyacrylamide

In the early 1970s, polyacrylamide and agarose became the media of choice for separating macromolecules but agarose has certain advantages over polyacrylamide for separating DNA molecules. Agarose of the same concentration as cross-linked polyacrylamide is more rigid and has larger pores (see Section 3). Agarose gels as dilute as 0.15% are strong enough to form slabs for electrophoresis [14]. Very firm 0.9% agarose gels permit entry of T4 bacteriophage during electrophoresis. In addition agarose gels unlike polyacrylamide gels do not require a cross-linking agent during gelation. Thus variability of polymerisation and the presence of potentially toxic catalysts do not affect data obtained in agarose gels in most applications. Recently introduced grades such as GTG (genetic technology grade, FMC Bioproducts, Rockland, ME, USA) have overcome some earlier difficulties with restriction endonuclease and ligase inhibitors. Polyacrylamide gel electrophoresis (PAGE) [15] is popular for separating DNA from 2–1000 base pairs (bp) and different concentrations of polyacrylamide are recommended for the separation of 2–25 bp in comparison with larger segments [16].

2.2. Starch and agar

Starch gels, like polyacrylamide gels, have smaller pores and are weaker than agarose gels of the same concentration [17]. Agar has a lower gel strength than agarose because of the presence of the non-gelling ionic components, which serve as diluents and interfere with the overall structure of the gel network [18]. Agarose gels are thus preferred for the separation of nucleic acids.

2.3. Ultracentrifugation

A dye–buoyant density gradient method for the detection and isolation of closed circular duplex DNA was introduced by Radloff *et al.* [19], and has been used extensively for plasmids, closed circular mammalian DNA and viral genomes such as ΦX174, polyoma and SV40. Examples of the use of neutral and alkaline sucrose and cesium chloride–ethidium bromide isopycnic gradient centrifugation for SV40 are given in Sebring *et al.* [20] and Ganem *et al.* [21]. Today, commercially available, patented, affinity chromatography resins, often based on the technique whereby DNA is bound to glass in the presence of sodium iodide [22], are suitable substitutes for plasmid isolation.

2.4. Capillary electrophoresis

Recently, capillary electrophoresis (CE) is finding application in the field of macromolecule separation. The use of capillaries as the migration channel in electrophoresis, compared with open-bed electrophoresis, offers several advantages including minute sample requirements, reduced analysis time, increased sensitivity, the ability to use greater potential fields and, with the appropriate equipment, on-line sample detection [23]. Several investigators have replaced conventional gel-filled capillaries which are difficult to prepare and are destroyed at high current densities, with linear polymers such as hydroxypropylmethylcellulose and methylcellulose which generate a sieving effect [24]. The combination of hydroxyethylcellulose and ethidium bromide to separate DNA fragments in the size range of 20–2200 bp is claimed to be superior to the latter and to offer the same degree of resolution as gel-filled capillaries [25]. It appears that the advantages of CE may lead to wider application of this technique including the analysis of restriction fragment length polymorphism (RFLP) of restriction enzyme-cleaved polymerase chain reaction (PCR) samples [26].

3. STRUCTURE AND PROPERTIES OF AGAROSE

3.1. Structure of agarose

This topic is well covered in *The Agarose Monograph* [18] and several publications by Serwer [27,28] and is outlined briefly here. Agarose, a natural polysaccharide isolated from agar (agar–agar) of marine red algae, is a series of naturally occurring derivatives. The variable agarose polymers consist of a repeating unit of agarobiose, variously substituted with ester sulphate groups, pyruvic acid ketal and methyl esters. Because of the variable nature of agarose and fractionation procedures for different grades, consistency between grades and different lots cannot be assured [29].

The mechanism for gelation of agarose was first suggested by Rees [30] and later demonstrated by Arnott *et al.* [31]. It involves a shift from random coil in solution to a double helix in the initial stages of gelation and then to bundles of double helices in the final stage. The rigidity produced by the arrangement of the molecules in the gel is one of the most important characteristics of agarose. When gelled agarose is observed by freeze-fracture and electron microscopy, the aggregated double helices can be seen as a fibrous network. The diameters of the fibres tend to increase with rising concentration and the appearance of protuberances or cul-de-sacs in the network increases with falling concentration [32].

3.2. The chemical modification of agarose

Araki [33] first fractionated agarose from agar as an essentially electrically neutral gelling polysaccharide. These early separations involved acetylation of agar, separation of the resulting acetate esters in mixtures of chloroform and petroleum ether and saponification of the neutral product yielding the gelling polymer, agarose. Less complicated means of making the separation were not found until the 1960s when Blethen [34] added carrageenan to the agar in order to make a bulkier, but readily filterable precipitate. Other processes used to separate the agaropectin fraction from charged polysaccharides in agar included the adsorption of agaropectin with aluminium hydroxide followed by successive purification steps of precipitation with ammonium sulphate, polyethylene glycol and removal of ionic polysaccharides with DEAE cellulose.

Traditionally most agar came from various species of *Gelidium*. In the 1950s, in the face of a shortage of these species the Japanese developed a method of treating sea-weed of a different genus, *Gracilaria*, with hot sodium hydroxide. This converted the non-gelling polysaccharides into gelling agar by desulfating the polymer, producing agarose.

3.3. Sieving properties and pore size

It has been estimated that the average pore size of agarose at a concentration of 1.5% is 0.1 μm [35–37], while Slater *et al.* [38] have estimated

that a 1% gel has a pore size of 0.14 μm using the data of Righetti *et al.* [39]. SeaPlaque agarose has a pore size which is only slightly smaller than that of SeaKem LE agarose, as judged by the sieving of spherical particles [40]. However, agarose structure is far from the proposed simple pores simulating a tube [27,41,42] and the average pore sizes are greater than twice the persistence length of DNA [38] such that DNA could bend sufficiently to enter a pore [43]. Furthermore, agaroses of different grades have slightly different structures, *e.g.*, SeaPlaque is thought to have fewer double-helical agarose segments in the suprafibre which constitutes the matrix between the "pores", and is hydroxyethylated [27]. These minor modifications have a profound effect on the migration of DNA which is not expected from a simple reptation model (see Section 5.1).

3.4. Electroendosmosis

Electroendosmosis (EEO) is a phenomenon of electrophoresis whereby neutral molecules which would not normally migrate in an electric field are carried toward an electrode by the hydrated counter ions of the ionic groups on the gel. One of the advantages of agarose is its low EEO but the ionic ester sulphates and pyruvates do contribute to residual EEO in most commercially available agaroses. Adherence to the charged groups in these may cause a problem with some samples [27].

3.5. Other properties of agarose

Other factors which affect DNA migration are voltage gradient and the buffer type and concentration. These effects can be conveniently expressed by plotting log (mobility) *versus* agarose concentration, referred to as a Ferguson plot [15]. Interpretation of the plots can yield information on particle size and free mobility (related to surface net charge density) as well as radius, volume and length of the gel fibre. In application to most proteins, this plot is linear but those of DNA in agarose are concave [44]. Ferguson plots may also reflect the conformation of the particle since rod-shaped virus particles generate concave plots [45] as does DNA at elevated polymer concentrations capable of stretching the molecule [44].

4. COMPARISON OF DIFFERENT GRADES OF AGAROSE

4.1. Low-melting-temperature agarose

The various substitutions on the agarose polymer are responsible for many agarose properties and by careful selection of raw materials these properties can be controlled. The exent of natural methylation was found by Guiseley [46] to affect the gelling temperature of agarose. Synthetically methylated, and more conveniently hydroxyethylated, agaroses and a number of simple derivatives have lower melting and gelling temperatures and are suitable for supporting living cells and for melting (at 65°C) without disrupting DNA double helices therein. The recovery of DNA fragments following electrophoretic separation is an integral part of the technology required for the characterisation and manipulation of nucleic acids. A variety of methods to perform this task include electrophoresis onto either DEAE cellulose membranes [47] or dialysis membranes [48], absorption onto glass beads [22], electroelution [49] and, most conveniently, extraction from low-melting-temperature agarose [50,51]. A recent variation on the low-melting-temperature agarose extraction involves freezing at −70°C of the molten agarose followed by a high-speed spin to separate the DNA from the agarose pellet [52]. Recovered DNA in all of the above methods can be successfully ligated, restriction enzyme cleaved and cloned. However, it does appear that impurities from the gel are inevitably associated with the recovered DNA [53], but are inconsequential for many procedures using appropriate grades of agarose.

4.2. Separation of small DNA fragments

Until recently, separation of DNA fragments smaller than 1 kilobase pair (kb) were carried out

in polyacrylamide gels. Many of these separations can now be effected in agarose such as Nu-Sieve GTG agarose (FMC). This agarose is low-melting and has a low viscosity, which allows concentrated (8–10%) agarose gels to be cast. Segments smaller than 100 bp can be separated in these gels [54] with a limiting resolution of about 8 bp between resolvable bands.

The Agarose Monograph [18] gives details of the choice of FMC agarose grades for separation of different-size DNA molecules. Other manufacturers including Pharmacia, Beckman, Bio-Rad and Sigma offer proprietary grades of agarose.

4.3. Separation of chromosomal sized DNA

The choice of agarose for separation of large DNA molecules in pulsed-field gel electrophoresis (PFGE) applications is still undergoing revision based upon the perceived parameters influencing separation and resolution. The choice of agarose for high-molecular-mass DNA separation is largely undefined, except perhaps for the recently defined increase in speed of separation afforded by low endosmosis agarose described below [55,56]. Early work utilised standard grades such as SeaKem (FMC) with great success, and has progressed to more refined grades such as SeaKem GTG, as they became available. Manufacturers have introduced specialised products such as Chromosomal Grade (Bio-Rad), Megarose (Clontech), Agarose MP (Boehringer), Pulsed Field Grade (Stratagene) and FastLane (FMC) with varying assertions for low sulphate, low endosmosis and/or high gel strength being important. Under the conditions that we have examined in the last three years for separation of chromosomes from the intestinal protozoan parasite, *Giardia duodenalis*, for example, it is clear that each application, apparatus and the boundary parameters have considerable influence on the outcome of the separation. Good separation and resolution, although influenced by the type of agarose, is still very empirical and subject to "fine tuning".

Another factor which has allowed analyses of high-molecular-mass DNA molecules is the immobilisation of the chromosomes in an agarose matrix to prevent shearing of the chromosomal sized DNA. Lysis of microorganisms (and mammalian cells) requires agarose with low-melting-point characteristics, no nucleases and no inhibitors of restriction endonucleases. Specifically, low-melting-temperature GTG agaroses are suitable and products such as InCert Agarose from FMC and others from Bio-Rad, Beckman and the Imbed kit from New England Biolabs are specifically designed for this purpose.

4.4. New types of agarose

A new type of agarose reported recently, will allow up to 30% higher mobility of all size ranges of DNA [56]. The mobility increase was predominantly due to the low EEO of the agarose and was most pronounced in PFGE. A modified galactomannan-agarose binary gel has been described and is claimed to offer improvements over conventional agaroses especially in optical clarity of the gel, increased gel strength, improved nucleic acid sieving and resolution and greater economy since lower agarose concentrations can be used as a consequence of the improved sieving qualities [57].

5. ELECTROPHORETIC SEPARATION OF DNA MOLECULES

5.1. Theoretical considerations of electrophoretic migration

The hydrodynamic behaviour of DNA has been thoroughly investigated in relation to viscosity and sedimentation [58,59]. Theoretical treatments of the DNA molecule as a "stiff worm-like coil" are adequate to describe these hydrodynamic parameters (*e.g.*, refs. 58 and 60). The interaction of these stiff DNA polyions with a fixed matrix and in an electric field, as in the case of agarose gel electrophoresis, has not been treated as successfully at a theoretical level, notwithstanding thorough empirical description [7,12,61–66] and theoretical considerations of polymers in gels [67–78]. The behaviour of DNA

molecules during gel electrophoresis shows numerous anomalies in all but the simplest conditions [12,61,79–82]. More recently, reptation theory has been modified to allow for many internal degrees of freedom and to incorporate observed anomalies [83–85].

In a recent review, Noolandi [86] discussed modelling efforts to understand the process of continuous-field DNA gel electrophoresis as a prerequisite to some understanding of two-dimensional and pulsed-field gel electrophoresis. The models have progressed from primitive and biased reptation [77] to more elaborate models to explain anomalies as they have been discovered. These included anharmonic bead-spring reptation [84], Monte Carlo simulation [87,88] and the elastic bag model [86]. His conclusion was "no theoretical model is expected to account for all experimental details; often the most useful output of the theory is to determine which aspects of a model do not agree with experiment, in this way those features which are important for obtaining high resolution separations can sometimes be inferred".

Alternative approaches to the theoretical understanding of electrophoresis have been raised by Calladine and co-workers [89,90]. The path of migration in these statistical models is more direct rather than "wormlike and tortuous" as in reptation models [72,73]. Light microscopy studies of the migration of long DNA molecules are more consistent with these conclusions also [91,92]. Calladine *et al.* [90] have argued that the popularity of reptation models lies in "the ease of programming a lattice of rigid gel 'points' for studies of migration of a totally flexible DNA molecule on a computer". However, Smith *et al.* [93] argue that fluorescence microscopy studies of single molecules "clearly show the molecules moving principally by reptation" but that retardation is caused by extended U-shapes slipping through the gel. Clearly, theoretical studies are in need of considerable refinement to bridge the gap between them and practical electrophoresis.

5.2. Electrophoretic techniques

5.2.1. Slab gels

The most commonly used technique for DNA separation is submerged horizontal electrophoresis in 0.5–2.0% agarose, usually in one of two buffers, Tris–borate–ethylenediaminetetraacetic acid (TBE) [11,94] or TAE [7,8]. By variation in the agarose concentration and buffer, it is possible to reliably separate double-stranded DNA in the approximate size-range of 200–50 000 bp. The type of agarose used will depend to some extent on the size of the fragments to be separated [18]. Discontinuous buffer systems have also been used successfully to separate DNA fragments up to 50 kb in size [95]. Apart from separating DNA molecules agarose slab gel electrophoresis has been useful for analysing structural elements of DNA such as the determination of DNA interstrand crosslinks [96] and specific interactions between proteins and nucleic acids [97].

5.2.2. Transverse gels

Other techniques include transverse agarose pore-gradient gel electrophoresis whereby electrophoresis of DNA is carried out at 90°C to a gradient usually of the order of 0.2–1.5% agarose. This technique and associated computer technology, including video acquisition, digitisation and densitometry, extends to agarose gel electrophoresis the benefits of semiautomated analysis of Ferguson curves [98].

5.2.3. Capillary electrophoresis

Boček and Chrambach [99,100] tested the possibility of using liquefied agarose similarly to the previously investigated linear polymer polyacrylamide [101] in CE. Mitochondrial DNA (16 kb) and plasmid DNA were retarded in their electrophoretic migration in proportion to their size. Using various grades of low-melting-temperature agarose these workers demonstrated that CE of DNA fragments in agarose solutions at 40°C were equivalent to those of CE of DNA in polyacrylamide gels [101] but without the associated problems. The application of liquefied agarose CE to the separation of the entire range of bi-

ological macromolecules could render agarose electrophoresis universal.

5.2.4. Two-dimensional agarose electrophoresis

Two-dimensional electrophoresis of DNA has been used to improve the resolution of complex mixtures of restriction endonuclease cleavage products by enriching in the first dimension on a size basis [102]. This approach has also utilized an *in situ* cleavage prior to electrophoresis in the second dimension for mapping purposes [103–106] and has been used to examine DNA methylation patterns [107]. Alternatively, treatment of DNA prior to electrophoresis in the second dimension with UV irradiation allowed identification and separation of closed circular from linear DNA molecules [108].

The introduction of alkaline denaturing gel electrophoresis in horizontal beds [12] also allowed the combination of two dimensions under different denaturing conditions to analyse DNA strand breakage [109].

Complex conformers, such as catenated intertwined dimers, have been analysed with two-dimensional electrophoresis [110]. Branched molecules, including replication forks and replication intermediates, have also been separated from linear DNA molecules by manipulation of the boundary conditions in two-dimensional electrophoresis [111]. Both unidirectional and bidirectional replication have been subsequently analysed by two-dimensional agarose gel electrophoresis (ref. 112 and references therein). One of the most useful applications of two-dimensional electrophoresis has been the resolution of topological isomers of supercoiled DNA and other unusual structures in which treatment with topoisomerases followed by relaxation prior to separation in the second dimension with agents such as chloroquine demonstrates the relief of torsional stress [113–116]. This approach is an extension of similar analyses in a single dimension [117–119].

An example of the use of two-dimensional agarose gel electrophoresis to demonstrate the complexity of DNA migration through agarose is shown in Fig. 1. Linear DNA in the range 1–46 kb has migrated through a thin strip of different-grade agarose and has been retarded to the extent that some DNA in the size range 5–12 kb still remains in the strip while DNA outside this range shows less or no retardation. It is clear that the short passage through SeaPlaque has dramatically affected migration characteristics of some size classes of DNA molecules under standard electrophoretic conditions. Under these conditions migration does not appear to be a function of simplistic models.

5.2.5. Affinity agarose electrophoresis

Although agarose as a medium for affinity binding studies has been used extensively in column chromatography it has not been exploited in electrophoresis. An example of this approach using various DNA intercalating dyes has shown the potential of this approach for separating a range of DNA segments based on GC content and related structures [120].

5.3. DNA separations

5.3.1. DNA less than 50 kb

50 kb is an often quoted upper limit for reasonable resolution and separation of DNA molecules although higher-molecular- mass DNA can be separated by extending conventional techniques. To separate DNA fragments of 100–2000 bp, agarose gel concentrations of 2–3% are recommended and a lower limit of 0.25% is usually recommended for fragments in the 5–50 kb range. In general it is thought that separation is achieved by a sieving mechanism, *i.e.*, the chance that the migrating particle intersects a gel fibre (see Section 3.3), where the size or effective radius of the DNA molecules are smaller than the average pore size of the gel [75]. Thus linear DNA molecules in this size range are generally regarded to migrate as a monotonic function of their length.

5.3.2. DNA greater than 50 kb

The limit of resolution for very large DNA molecules (> 50 kb) is reached when the size or radius of gyration of the DNA molecule exceeds the maximum pore size obtainable with an aga-

Fig. 1. Two-dimensional electrophoretic separation of DNA through a thin strip of SeaPlague agarose. A 15-cm square perspex plate was poured with 100 ml of 0.8% Pharmacia NA (nucleic acid) grade agarose in 40 m*M* Tris acetate, 2 m*M* EDTA, pH 7.8. Both lanes contain 0.8 μg of bacteriophage T4dC DNA (Amersham) cleaved with KpnI and 0.3 μg of the 1-kb "ladder" from BRL. The first dimension of electrophoresis was from top to bottom, at 30 V (22 mA) for 15 h in a Bio-Rad Sub-Cell at constant voltage and ambient temperature (22°C), after which a 3-mm-wide slot was excised from the gel between the two running tracks. The resulting trough was filled with 2.5% SeaPlaque agarose in the same buffer and was allowed to set for 30 min. The gel was rotated 90° and the second dimension was electrophoresed (after replacement of the same buffer) at 60 V (47 mA) for 5.5 h, such that the right hand lane passed through the SeaPlaque strip and the left lane did not. The gel was stained with ethidium bromide and photographed after UV transillumination. DNA in the bottom band is 1 kb in length and the top 46.1 kb. Retardation of DNA between 5 and 12 kb is such that some is still retained in the SeaPlaque strip; with longer running times, the retained DNA eventually leaves the strip.

rose gel. At this point, it is thought that the DNA molecules are no longer resolved by sieving, and begin to migrate at the same rate via "reptation" [72,73]. Separation of yeast chromosomes by Schwartz and Cantor [121] and Carle and Olson [122] led the field in significantly extending the range of large DNA molecules which can be separated by gel electrophoresis. By alternately electrophoresing the DNA in two different directions within the gel, the phenomena which normally render large DNAs unresolvable are bypassed. The subject of PFGE has been covered in detail elsewhere [55,123]. In practice, separation of chromosomes up to 5.7 Megabase pairs (Mb) are achievable [124], and separation of larger eukaryotic chromosomes is being actively sought.

While separation of a wide range of chromosomes from different species is achievable, the relation between DNA size and mobility during PFGE is complex. We have shown that chromosome mobility varies with switch interval and between the type of agarose used [125]. *Giardia duodenalis* has chromosomes which can be resolved by PFGE and other systems [126,127]. *Giardia* chromosomes separated by field inversion gel electrophoresis (FIGE) [79] appear to be smaller when compared with yeast chromosomes than when separated in contour-clamped homogeneous electric field electrophoresis (CHEF) [128] and compared with the same markers [127,129]. The type of agarose is also important. When *Giardia* chromosomes were separated in three different grades of agarose simultaneously, SeaKem GTG, SeaKem ME and SeaPlaque, compared with a lambda ladder as marker, the apparent sizes of the chromosomes varied markedly [125]. This indicates different interactions between the molecules being separated and the gel matrices.

The human genome project has depended greatly on separation of human DNA for long-range genetics and mapping coupled with cosmid and yeast artificial chromosome construction [130].

6. SEPARATION OF STRUCTURALLY DIFFERENT DNA MOLECULES

6.1. Circular DNA molecules

Circular and linear DNA, circular and nicked-circular DNA, and the various topoisomers of

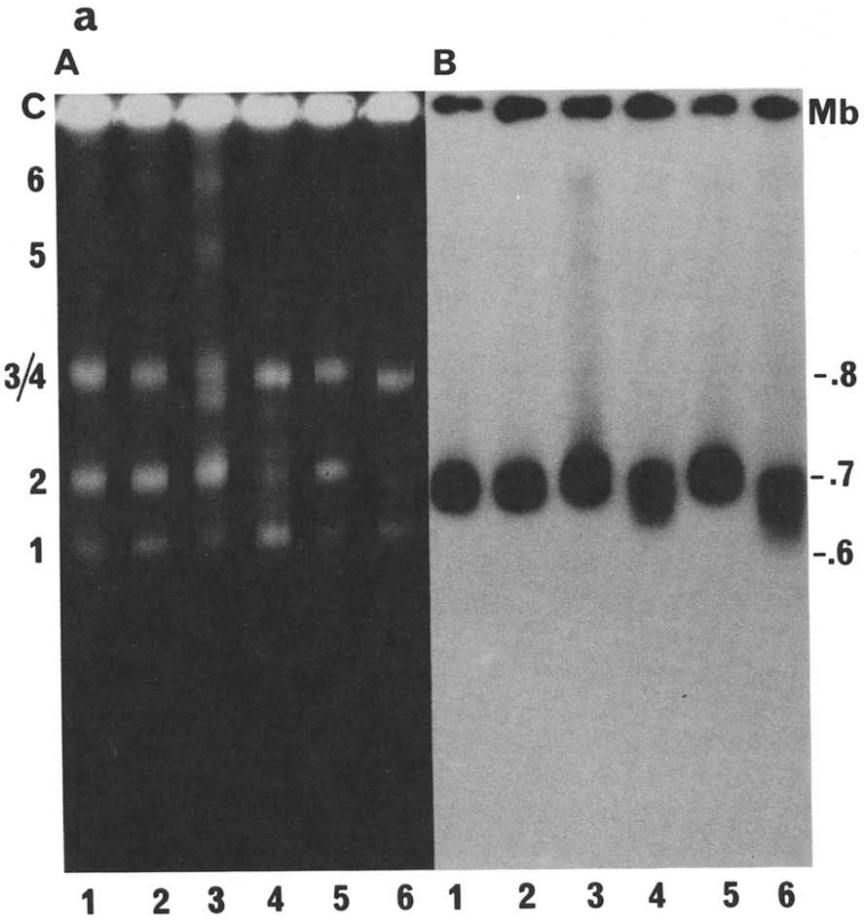

Fig. 2.

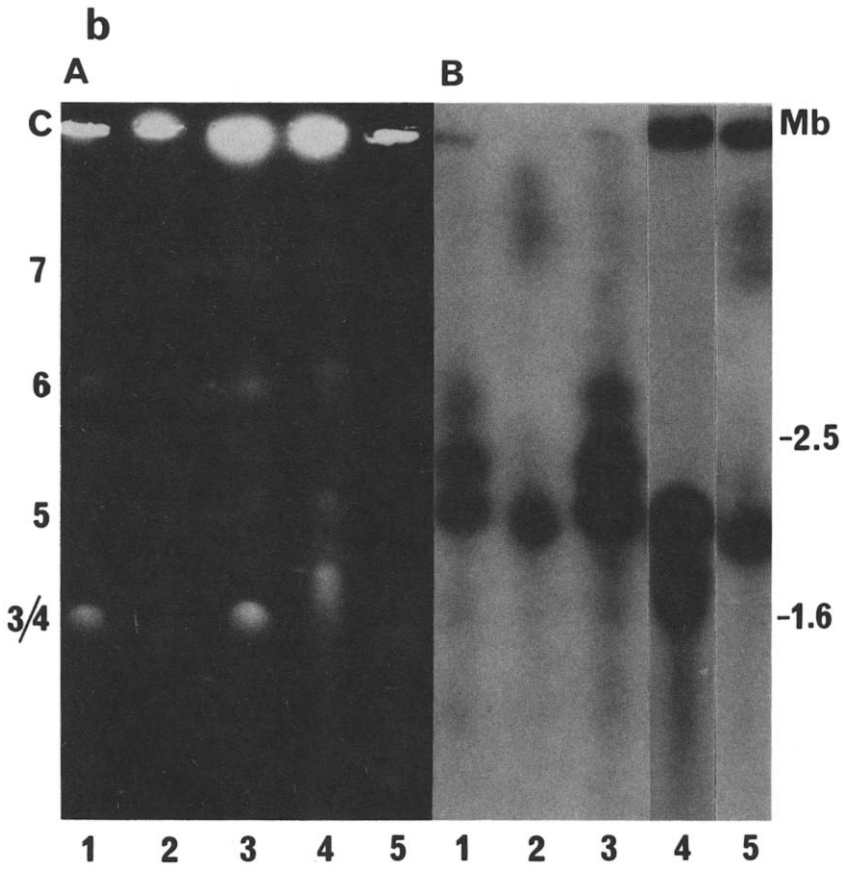

Fig. 2. Anomalous migration of chromosome 2 from *Giardia duodenalis*. *Giardia* chromosomes separated in FIGE have been identified by chromosome-specific markers cloned from separated chromosome bands in a gel [129]. When the chromosomes were separated in CHEF, chromosome 2, identified by several FIGE chromosome 2 markers, migrated more slowly than the chromosome 3 and 4 bands and in some strains resolved as multiple chromosomes. The chromosome 3/4 bands were identified by chromosome 3/4 specific probes [129]. When compared with yeast chromosomes in FIGE separations, chromosome 2 of *Giardia* is approximately 0.7 Mb and chromosomes 3 and 4 are of the order of 0.8 Mb. In CHEF separations of *Giardia* chromosomes, chromosome 2-specific probes predominantly identify a chromosome of approximately 2 Mb (designated chromosome 5 in b). In the same gels chromosomes 3 and 4 are estimated to be 1.6 Mb. (a) FIGE of *G. duodenalis* chromosomes. *Giardia* chromosomes were prepared in agarose blocks, separated and ethidium bromide-stained (A) [127]. Chromosomes were Southern transferred and hybridised with a chromosome 2-specific probe (G21/1/9) (B). Chromosomes 1, 2, 3 and 4 are in the size range of 600–800 kb when compared with yeast chromosomes in the same system [127]. Lanes 1–6 are the same in each panel and each lane shows the separation of chromosomes from a different strain of *G. duodenalis* isolated from humans (lanes 1, 2, 3 and 6), a cat (lane 4) and a sheep (lane 5). Lanes: 1 = WB1B; 2 = H8; 3 = Bris/83/HEPU/106; 4 = BAC1; 5 = OAS1; 6 = BRIS/88/HEPU/862. (b) CHEF of *G. duodenalis* chromosomes. *Giardia* chromosomes were separated and stained (A), Southern transferred and hybridised with the same chromosome 2-specific probe as shown in (a) (B) [129]. Chromosomes 3 and 4 were identified with chromosome 3-specific probes [129] and were shown to be the same as chromosomes 3 and 4 in (a). Each lane shows the separation of a different human strain; lanes 1–5 are the same in each panel. Lanes: 1 = Ad6; 2 = Ad1; 3 = Ad10; 4 = BRIS/83/HEPU/106; 5 = WB1B. C refers to chromosome designation. Sizes are shown in Mb.

closed circular DNA, of the same size, can be distinguished on the basis of their migration distance in agarose gel electrophoresis [131–133]. Several methods have been published to distinguish or separate linear and various forms of circular DNA molecules and rely on altering the agarose concentration or quality. Upcroft [134] has used the method of entrapment in a Sea-Plaque plug to enrich for open-circular recombinant DNA molecules. Similarly, entrapment in

SeaPlaque was used for the production of a SfiI linking library for *Theileria parva* [135]. Wheeler *et al.* [136] have used a gradient agarose gel, transverse agarose pore-gradient gel electrophoresis, to detect supercoiled plasmid-sized DNA in mixtures with linear DNA.

6.2. Structural variants of linear DNA

The migration of circular DNA molecules in comparison with linear molecules can be compared with other anomalies in migration caused by local polymorphisms of DNA. Sequence-directed bending of the DNA helix is the origin of anomalously slow electrophoretic mobility of DNA fragments isolated from the kinetoplast body of trypanosomes. The striking feature of the bending locus is a regular repeat of the sequence element $CA_{5-6}T$ with 10 bp periodicity around the centre bend. Each A_n tract produces a small bend in the DNA helix axis and repetition of these elements in phase with the helix screw results in their coherent addition to form a large overall bend [137]. A recent study by Calladine *et al.* [90] has examined the migration of curved DNA in terms of two theories which they have proposed. A second example is the GC-rich rDNA repeat array [138] in *Giardia*. Cleavage of *Giardia* chromosomes with restriction endonucleases which leave the array intact show that the rDNA array migrates quite differently from the remaining segments of the chromosome which carried the array (unplublished data); boundary parameters are critical in determining the extent of the anomalous migration.

6.3. Chromosomal sized DNA

The anomalous migration of chromosome molecules in pulsed-field gels results not only from the structure of the molecules but from the conditions of electrophoresis. Yeast chromosome XII, which contains the rRNA gene array, migrates anomalously under orthogonal field alternation gel electrophoresis (OFAGE) and CHEF conditions [55,122,128]. Similarly, chromosome 2 of *Giardia duodenalis* alters in relative position to the other *Giardia* chromosomes when separated in different pulsed-field systems (Fig. 2). There is a size discrepancy of more than 1 Mb in chromosome 2 between separations in FIGE and CHEF systems and this almost certainly indicates unusual structures modifying chromosomal migration. Lambda concatamers migrate more slowly than the same-sized yeast chromosomes in FIGE gels [127] and changes in relative migration positions of *Giardia* chromosomes have also been described in different agaroses [125]. It has been suggested that this latter phenomenon is related to the EEO value of the agarose [56].

Although this interpretation may be consistent with a generalised change in chromosome mobility, *i.e.*, all chromosomes observed, a complete description must encompass the relative changes in migration depending on the agarose grade and boundary conditions. A more subtle interaction between the agarose and DNA structure is implied in this case [125]. Under complex migration conditions imposed during PFGE, arrays such as repeated rDNA units migrate differently from other sequences and other repeat arrays in the same chromosome. A delicate balance ensues in order to display each chromosome at reasonable resolution and separation without sacrificing some to their competing chromosomal regions. Compounding the problem are compression zones which cause chromosomes of different sizes to comigrate [139–141].

7. COMPUTER AIDS TO AGAROSE GEL ELECTROPHORESIS

Computer technology has made advances possible both in running DNA separation programs (*e.g.*, in PFGE, the Bio-Rad CHEF systems, and in CE, the ABI systems) and in analysing data from Ferguson plots [132,133,142,143], CE [26], RFLPs [144] and DNA sequencing gels [145]. The miniaturisation of DNA separations described by Heller and Tullis [146] were achieved with the aid of a high-resolution electronic imaging system. DNA fragments of 72–1353 bp were separated into compact, microscopic banding patterns in 1-cm gels or capillaries in less than 2 min. This complements fluorescent studies of single DNA molecules [91,93].

8. CONCLUSION

Progression from agar to agarose as a matrix for sieving DNA molecules has allowed separation of very-high-molecular-mass molecules which encompass chromosomes of parasitic protozoa and lower eukaryotes and enabled the separation of conformers (including supercoils, open circles, linears, topological and structural variants). Demand for these techniques has been paralleled by commercial manufacturing of many varieties of agarose to improve quality, separation and resolution, and reflects the importance of the technique during the past twenty years. The challenge in the future is in separation of even larger chromosomal sized molecules, greater numbers of samples and smaller formats.

There is no other known medium which has comparable properties with agarose and it will probably maintain its position as foremost medium for separating DNA until a synthetic medium with controlled and fixed fibre widths and pore sizes is developed. Separation of mixtures of DNA molecules in the range of 200 bp to 5.7 Mb, under the appropriate conditions, can almost be guaranteed. However, estimation of the sizes of the separated molecules depends on electrophoretic migration in comparison with standard molecules of known size, and this in turn depends on the structure of the molecules. The structure as well as size of the molecule dictates electrophoretic mobility and no all encompassing theory has adequately explained migration of all DNA molecules in agarose.

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