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Selectivity of the separation of DNA fragments by capillary zone electrophoresis in low-melting-point agarose sol

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ABSTRACT

The properties of solutions of low-melting-point agarose for the separation of DNA fragments were investigated as a function of temperature and applied voltage. Giddings *et al.*'s definition of the selectivity of the separation according to molecular size, $S = |(\mathrm{d} \ln u)/(\mathrm{d} \ln p)|$, where u is the electrophoretic mobility and p the number of base pairs, was adopted as a suitable phenomenological description of the capability of the system to separate DNA fragments. It was found that the selectivity of separation in 1% agarose has its maximum at about 600 base pairs, decreases with increasing applied voltage and is a complex function of temperature. No decrease in selectivity was observed even at temperatures above the melting point. The high selectivity of agarose at these temperatures is probably evidence for the positive effect of conformational entropy of DNA molecules. A development of an apparatus for thermostating the capillary within the range 10–70°C that allowed the measurements to be performed is reported.

INTRODUCTION

Liquefied agarose, in the state of the sol, has been used successfully for separations of oligonucleotides by capillary zone electrophoresis [1–3]. The application of such a low-viscosity sieving medium brings some advantages. The capillary can be filled and refilled with the sol easily and thus a standard separation condition is established before each analysis. The concentration and the type of separation medium can be changed in the same capillary after successive runs. No polymerization or cross-linking of the medium is needed and so the lifetime of the capillary is limited only by that of the capillary wall coating. Even untreated capillaries could be used if the agarose sol was allowed to gel within a capillary below its gelling temperature [4]. The electroosmotic flow inside the untreated capillary filled with a hydroxyethylcellulose solution has been used as a non-selective transport for the separation of DNA fragments [5]. The hydrodynamic injection of the sample can be applied if a low-viscosity medium is used [6].

In addition to the separation efficiency, the selectivity is the other criterion of the quality of a separation, which evaluates the relative dis-

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placement of the zones of two components [7]. Giddings *et al.* [8] defined the selectivity of the separation of polymers according to their sizes. This definition of selectivity is more informative not only for the quality of separation, but also for the character of migration of a polymer solute through a separation medium.

Despite the fact that the efficiency of the separation of the DNA fragments decreases with increase in temperature, as shown for polyacrylamide gel [9] and for agarose solutions [6], the decrease has no direct connection with the sieving itself and can be avoided. As the efficiency of the separation of polyelectrolytes is intrinsically very high [7,10], the lowered separation efficiency is usually caused by other phenomena such as electromigrational dispersion, interactions with the capillary wall and inhomogeneities of the gel. The impact of all these factors can be decreased independently of the temperature. Hence the selectivity is of paramount importance for optimizing the separation conditions.

In this work, the selectivity of the separation of DNA fragments sized from 9 to 1353 base pairs (bp) in various low-melting-point agaroses was measured at various temperatures and electric field strengths. The relationship to possible migration mechanisms is discussed. Another objective was to prove the feasibility of separation at high temperature and consequently lowered viscosity of the sieving medium.

THEORY

Agarose transient structures

Gelation is one of the most interesting and complex phase transitions in polymer science and has been subject of considerable theoretical and experimental study. Agarose belongs to a class of polysaccharides that exhibit a marked thermal hysteresis in the sol–gel and gel–sol transitions. Key features of these transitions have been studied by both optical and reheological methods [11–16].

Fig. 1 shows a schematic diagram of the dependence on temperature of the absolute value of a rheological or optical property of an agarose, *e.g.*, viscosity, optical rotation, light scattering or turbidity. The pronounced thermal hysteresis between the gel–sol and sol–gel transition is caused by the existence of the metastable equilibrium. This is observed on cooling the substance (lower curve in Fig. 1), whereas stable equilibrium is attained by heating (upper curve in Fig. 1). The properties of agarose obtained on the cooling curve gradually change to the final equilibrium state on the heating curve. The rates of these relaxation phenomena depend on the temperature and concentration. At 20°C and an

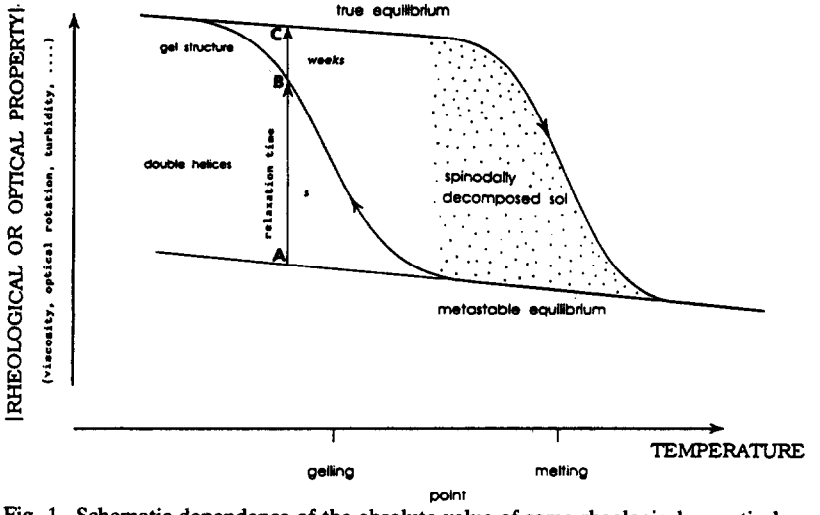

Fig. 1. Schematic dependence of the absolute value of some rheological or optical property (viscosity, optical rotation, turbidity, etc.) of an agarose solution on temperature. The arrows on the hysteresis loop indicate heating and cooling, respectively. The arrows A→B and B→C indicate the transitions to the metastable and true equilibrium, respectively. For explanation, see text.

agarose concentration of 1% the characteristic relaxation time of the sol–gel transition is a few weeks [14]. However, the first increase in viscosity can be observed after several hours. The higher the temperature, the longer is the relaxation time.

At temperatures close to the melting point, the relaxation time is very long and starts after a delay that is interpreted as the spinodal decomposition [17] of the sol, which occurs long before the beginning of gelation [13,15,16]. These sol–sol structural transitions are microscopic transport phenomena that lead to two sets of regions, one with higher and the other lower than average concentrations. The size of the high-concentration regions ranges from a fraction of a micrometre to a few micrometres [16]. In these regions the two random coils→double helix transitions occur, which give rise to the optical activity of the solution as indicated in Fig. 1 by the arrow A→B. The conformational ordering to a coaxial double helix is essentially complete within 100 ms [14].

The final gelation step, indicated by arrow B→C in Fig. 1, is the longest and takes several weeks, as stated above. From the molecular point of view this process is physical cross-linking based on the bundling of double helices. The gelation occurs in the high-concentration regions whereas the low-concentration regions act as early stages of the pores. If the initial concentration of the polymer is high enough, the physical cross-linking between the polymer-rich regions closes the pores, which are essential for the good sieving properties of agarose [16,18].

Selectivity

The objective of any separation technique is to obtain an adequate resolution of the bands of separated components. For a quantitative description of the mutual separation of two components the resolution R_s is used. The resolution is defined as the ratio of the distance between the concentration distribution centroids, Δx, to the mean width of the two peaks near the baseline, taken as 4σ, where σ is the mean standard deviation of their concentration distributions [19].

For the separation techniques where the concentration profiles are detected at a fixed point as a dependence of the concentration on time, the resolution should be defined with the help of time quantities as

$$R_s = \Delta t / 4\sigma_t \tag{1}$$

where Δt is the absolute value of the difference in the migration times of two separated components and σ_t is the standard deviation expressed in units of time. The numerator and denominator on the right-hand side of eqn. 1 describe the selective and the dispersive transport, respectively. The selective transport is the object of this study.

To make the selectivity of electrophoresis independent of applied voltage, the effective mobility difference Δu can replace the migration time difference in eqn. 1. This difference can be obtained from the definition of the electrophoretic mobility $u = L/(tE)$ differentiated by t, where L is the migration path length and E the electric field strength. Usually the dispersion is expressed by the dimensionless number of the theoretical plates N, which is independent on the total migration time and thus on the total length of the migration path. Hence σ_t can be replaced with N with the help of the equation $\sigma_t = \bar{t}/(\sqrt{N})$. After these transformations the resolution can be expressed as [7]

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{|\Delta u|}{u} \tag{2}$$

Now, the selectivity in eqn. 2 is expressed as a relative difference of the effective mobilities of two components. Even more universally, selectivity can be defined if related to a physicochemical parameter of separated components. As the separation of DNA fragments is according to their sizes, the number of base pairs p is accepted here as such a parameter. Consequently, the difference in mobilities can be expressed as $\Delta u = |\mathrm{d}u/\mathrm{d}p| \cdot \Delta p$. However, for the sake of universality it is more convenient to use the derivative according to a relative molecular size. Then

$$\Delta u = \left| \frac{\mathrm{d}u}{\mathrm{d}p/p} \right| \frac{\Delta p}{p} \tag{3}$$

Actually, the molecules are "sized" during the migration through the separation medium on the basis of their absolute lengths and, accordingly, the resolution is a result of relative differences in interactions of molecules with the separation medium. After substituting eqn. 3 into eqn. 2 the final relationship for resolution is [8]

$$R_s = \frac{\sqrt{N}}{4}\left|\frac{du}{dp}\cdot\frac{p}{u}\right|\frac{\Delta p}{p} = \frac{\sqrt{N}}{4}\left|\frac{d\ln u}{d\ln p}\right|\frac{\Delta p}{p} \quad (4)$$

Where the selectivity S is [8]

$$S = \left|\frac{d\ln u}{d\ln p}\right| \quad (5)$$

The ratio $p/\Delta p$ is called the separation power, f [8]:

$$f = p/\Delta p \quad (6)$$

where Δp is the difference in base pairs of two polynucleotide molecules of mean size p which are separated with a resolution $R_s = 1$. Then it follows from eqn. 4 for the separation power [8]

$$f = \frac{\sqrt{N}}{4}\cdot S \quad (7)$$

Assuming the difference $\Delta p = 1$, the separation power is the maximum size of the molecules that differ in one base pair and are separated completely.

The selectivity defined by eqn. 5 reflects not only the separating ability of a system but also the character of migration through a separation medium. Now it is interesting to compare the selectivities derived from the two most commonly used models of migration of macromolecules through a gel, the Ogston model [20] in the form extended by Rodbard and Chrambach [21];

$$u \approx \exp(K\, p^{1.2}) \quad (8)$$

where K is a constant and the radius of the equivalent sphere of DNA was taken as proportional to $p^{0.6}$ [22], and the reptation model [23–25]:

$$u \approx 1/p \quad (9)$$

Now after the substitutions of u in eqn. 5 by eqn. 8 for the sieving and by eqn. 9 for the reptation, respectively, the selectivities are proportional as follows:

$$S_{\text{sieving}} \approx p^{1.2} \quad (10)$$

$$S_{\text{reptation}} \approx p^{0} \quad (11)$$

While the selectivity of the sieving is proportional to the size of a molecule, the selectivity of the reptation mechanism is constant. Hence the sieving mode is similar to partition chromatographic techniques and the reptation mode to gel permeation chromatography or field-flow fractionation techniques.

EXPERIMENTAL

Chemicals

A 123 bp ladder of double-stranded DNA in the size range 123–4182 bp (Gibco BRL No. 5613 SA), a ϕX-174 DNA Hae III digest in the range 72–1353 bp and a pBR-322 DNA Msp I digest in the range 9–622 bp (New England Biolabs, Beverly, MA, USA) were used. The solutions were stored overnight at 4°C.

The agaroses used were SeaPrep (Cat. No. 50302; FMC Bioproducts, Rockland, ME, USA) and AcrylAide (Cat. No. 51013, a 2% solution; FMC). Solution of 1% were prepared gravimetrically in 89 m*M* Tris base–89 m*M* boric acid–2.5 m*M* Na_2EDTA (1 × TBE) at boiling temperature and stored at 60°C as described [2].

Capillary

The fused-silica capillary (75 μm I.D., 367 μm O.D.) was obtained from Polymicro Technologies (Phoenix, AZ, USA). The capillary was internally coated with linear polyacrylamide [2,26]. The total length of the capillary was 0.41 m and the length from the end to the window was 0.3 m. The window was cut off by a blade under a microscope.

Apparatus

The laboratory-made apparatus [27] was constructed in a similar way to a cartridge of a Varian (Walnut Creek, CA, USA) 2550 variable-wavelength detector and connected to a thermostated water-bath (Multitemp 2209; LKB, Bromma, Sweden) circulating doubly distilled water.

The capillary was coiled inside the cartridge and thermostated by direct contact with the flowing water within the range 10–70°C. The cartridge temperature in that range had no marked effect on the noise and the sensitivity of the detector. A pair of silica lenses, which focused the light beam as a condenser and objective, respectively, were fixed in movable holders that allow for focusing the light prior to any change of the wavelength. Separations were monitored at a single wavelength of 260 nm. After each run of the analysis the capillary was rinsed with fresh agarose solution using an injection syringe. Both the cathodic and anodic chambers, with a volume of 2 ml, were filled with agarose solution. Samples were injected hydrodynamically for 20 s with a level difference of 10 cm. A high-voltage supply (Glassman, Series EH; High Voltage, Whitehouse Station, NJ, USA) was used. The detector signal was monitored with a C-R5A Chromatopac integrator (Shimadzu, Kyoto, Japan).

RESULTS AND DISCUSSION

Fig. 2 shows an electropherogram of a 123 bp ladder of DNA molecules differing in size by a constant 123 bp. The recorded peaks represent a separation in 1% SeaPrep agarose at 40°C and an electric field strength of 4.9 kV/m. As stated above, the differences in migration times do not indicate much about the real separation properties and are incomparable with other systems. Hence the consecutively decreasing migration time differences of the adjoining fragments do not indicate that the separation ability of the medium is the best for the smallest molecules.

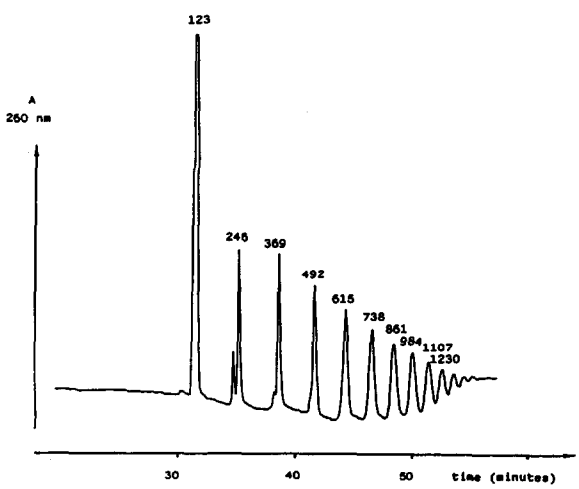

Fig. 2. Electropherogram of the separation of a 123 bp ladder of DNA fragments in 1% SeaPrep agarose. The number of base pairs is given on each peak. Conditions: temperature, 40°C; electric field strength, $E = 4.9$ kV/m.

Fig. 3 shows the electrophoretic mobilities of molecules of the 123 bp ladder analysed in 1% SeaPrep agarose at an electric field strength of 4.9 kV/m and within the temperature range 20–60°C. The dependences can be compared with other systems where different lengths of capillaries and different voltages were used. However, it still does not reveal the nature of the selective transport. The higher gradients of the curves at higher temperatures cannot simply be regarded as a symptom of high selectivity but as the non-selective effect of temperature on the mobility.

Fig. 4 shows a third-order least-squares polynomial fit of the selectivity (eqn. 5) on the number of base pairs of DNA molecules at 30, 40 and 50°C. The selectivity values were calculated from the raw experimental results of separations of restriction fragments (pBR-322 DNA

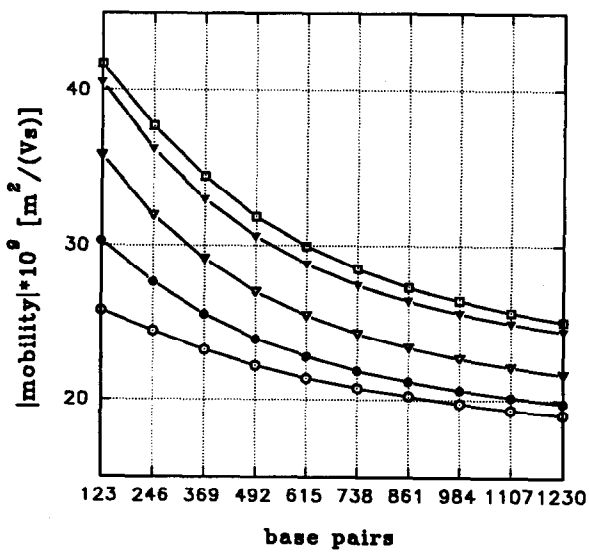

Fig. 3. Dependence of the electrophoretic mobilities of a 123 bp ladder of DNA fragments in 1% SeaPrep agarose at $E = 4.9$ kV/m and temperature of (○) 20, (●) 30, (▽) 40, (▼) 50 and (□) 60°C.

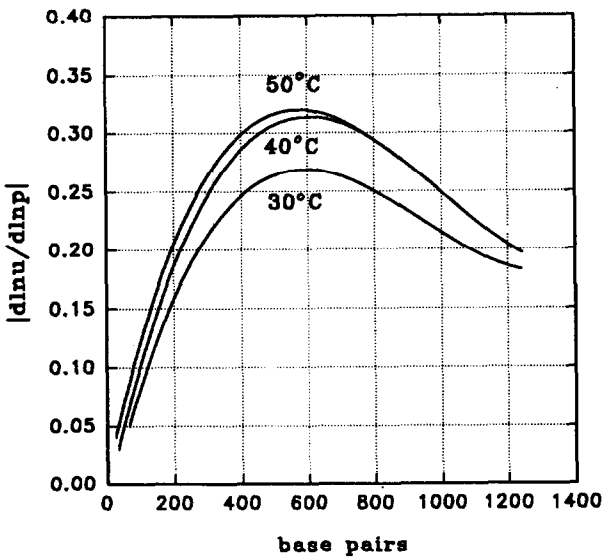

Fig. 4. Dependence of the selectivity on the number of base pairs of restriction fragments (pBR-322 DNA Msp I digest) and a 123 bp ladder of DNA fragments. Conditions: temperature, 30, 40 and 50°C; $E = 4.9$ kV/m; 1% SeaPrep agarose. The plots are polynomial fits of the selectivities calculated from the raw experimental data (eqn. 5).

Msp I digest) and 123 bp ladder of double-stranded DNA at an electric field strength of 4.9 kV/m in 1% SeaPrep agarose.

The increase in selectivity for molecular sizes up to about 400 bp is in accordance with the sieving model (eqn. 10). The existence of maxima at 600 bp indicates the change in the character of molecular migration through the agarose network. Under the optimum selectivity conditions, molecules in the size range *ca.* 400–800 bp are separated. This regime of migration is called entropically regulated transport [28,29]. The transport of molecules larger than 800 bp starts to be driven by another mechanism. It is probably reptation where the selectivity is independent of the molecular size according to eqn. 11. However, there are no other data for the selectivity of the separation of longer molecules to prove the reptation character of migration.

The selectivity of the separation of about 600 bp molecules is the optimum for the whole range of temperatures. This can be interpreted as the absence of a temperature effect on the shape of a polymer coil.

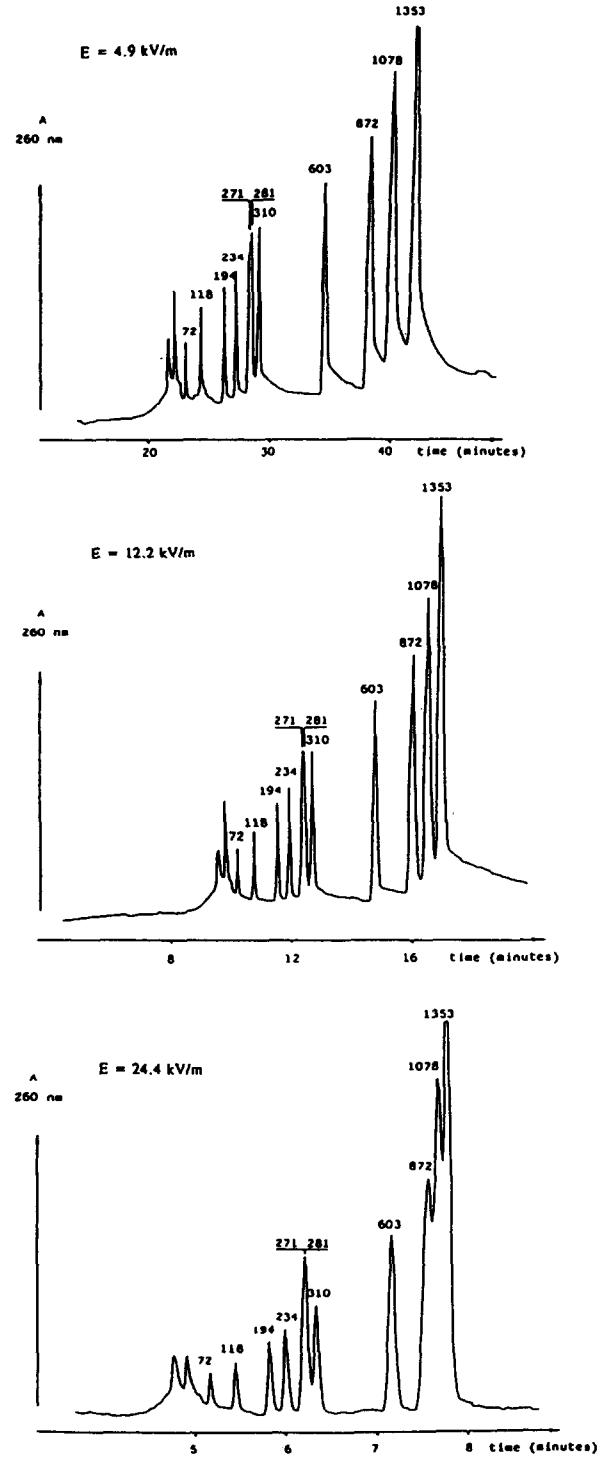

Fig. 5. Three electropherograms of restriction fragments (ϕX-174 DNA Hae III digest) at three voltages, 2 kV (E = 4.9 kV/m), 5 kV (E = 12.2 kV/m) and 10 kV (E = 24.4 kV/m), at 50°C in 1% SeaPrep.

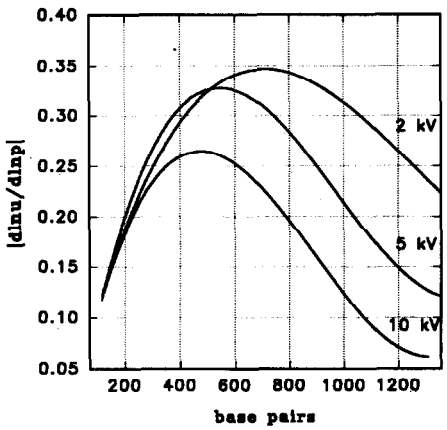

Fig. 6. Dependence of the selectivity on the number of base pairs of restriction fragments (ϕX-174 DNA Hae III digest) under the same conditions as in Fig. 5. The data for the plots are treated as in Fig. 4.

The effect of the voltage on the migration of DNA molecules can be elucidated from Figs. 5 and 6. Fig. 5 depicts clearly the negative effect of the electric field strength. There are three separations of restriction fragments (ϕX-174 DNA Hae III digest) at electric field strengths 4.9, 12.2 and 24.4 kV/m at 50°C in 1% SeaPrep agarose. It can be concluded that the analysis could be accelerated by higher voltage but at the expense of resolution.

The dependences of the selectivity on the number of base pairs for three values of the electric field strength, 4.9, 12.2 and 24.4 kV/m, are shown in Fig. 6. The three curves were calculated using least-squares three-order polynomial fits of the selectivity to the field strength. The selectivity (eqn. 5) was calculated from the raw experimental data of the separations shown in Fig. 5.

The decrease in the selectivity with applied voltage is followed by a shift of the maxima of the curves to lower numbers of base pairs. This phenomenon can be explained by the biased reptation model [30–32]. The random coil conformation of DNA molecules is stretched by the electric field. The coil becomes more elongated as the strength of the electric field increases and, in the limiting case, the coil becomes a rod [33–36]. Under such a condition the molecules of different lengths migrate at the same velocity and the selectivity of the system approaches zero. The shift of the maxima is an indication that the elongated coils behave as coils of longer molecules.

The effect of temperature on the selectivity of the separation of DNA fragments of 123 bp ladder in 1% AcrylAide and 1% SeaPrep agaroses is shown in Fig. 7. The data were treated in the same way as in Figs. 4 and 6. For the sake of clarity, only the fragments at which the selectivity is increasing with size (*cf.*, Fig. 4) are included here.

The character of the presented curves is determined by the structural changes of the agarose and by conformational changes in the polynucleotide molecule. When working with SeaPrep at 20°C (the gelling point is 17°C), an increase in

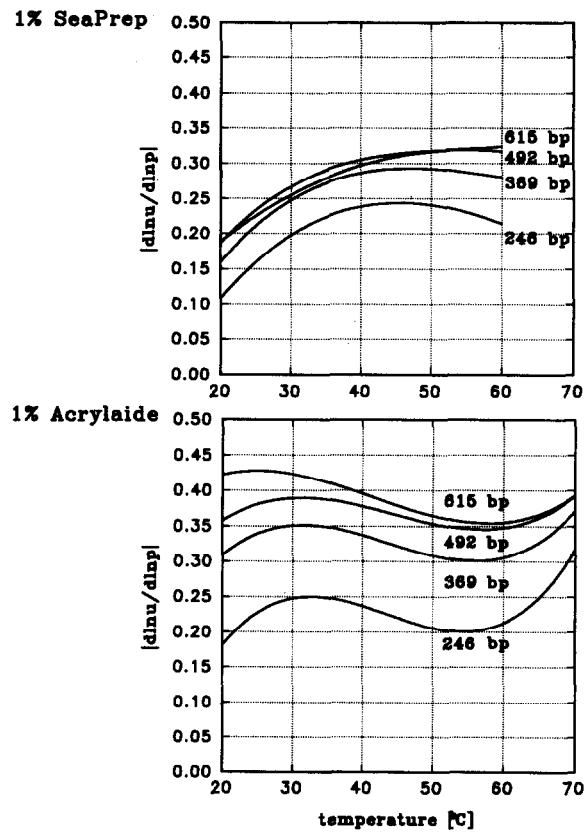

Fig. 7. Comparison of the selectivity dependence on temperature for 1% SeaPrep and 1% AcrylAide agaroses at $E = 4.9$ kV/m. The number of base pairs of selected fragments of 123 bp ladder is indicated on each curve. The data for the plots are treated as in Fig. 4.

the viscosity and therefore the beginning of gelation was obvious. Nevertheless, the selectivity is the lowest at this temperature. In contrast, there is no significant decrease in the selectivity of SeaPrep at temperatures above 50°C, which is its nominal melting point. The AcrylAide agarose exhibits a higher and more varied selectivity versus temperature than that of the SeaPrep. The only available data about AcrylAide agarose is the gelling temperature, given as less than 0°C. With the help of this information, it is possible to explain the different appearances of the two dependences as a shift of the structural transitions of AcrylAide toward the lower temperatures. Thus the graphs belonging to the SeaPrep agarose in the temperature range 20–60°C can be compared with the graphs for AcrylAide shifted downwards by 20°C.

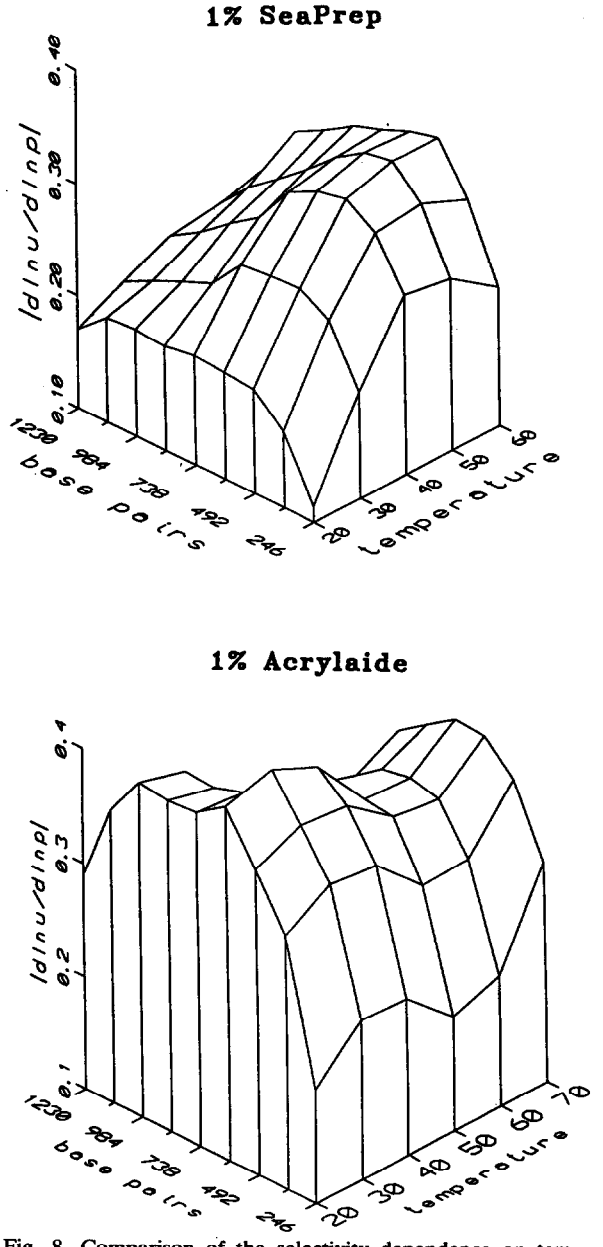

Fig. 8. Comparison of the selectivity dependence on temperature (°C) and number of base pairs for 1% SeaPrep and 1% AcrylAide at $E = 4.9$ kV/m. The selectivities are calculated from the raw experimental data (eqn. 5).

Fig. 8 presents views of the raw experimental data in a three-dimensional plot of the dependence of the selectivity on the molecule size and temperature. These data are the same as those partially used in Fig. 7.

The only explanation for the high selectivity of both agaroses at a temperature near the melting point is the existence of the spinodally decomposed sol and the early structures of the gel. On the other hand, the surprisingly high selectivity even at 60 and 70°C could be due to a closer mutual contact of the polynucleotide and agarose molecules. This is caused either by a large thermal movement or by the beginning of the double helix→single helix transition of the DNA molecule. In this instance even the completely unstructured agarose sol still retains good separation properties. To prove these hypotheses is the object of further study.

CONCLUSIONS

Giddings *et al.*'s definition of the selectivity (eqn. 5) proved to be a universal and very useful criterion of the quality of the separation of polynucleotides by electrophoresis. The selectivity reflects sensitively the changes in the character of the transport of a polyelectrolyte through a separation medium.

With the help of the selectivity definition (eqn. 5), the proportional relationships for the selectivity of the sieving and reptation models were derived. While the selectivity of the sieving model is nearly linearly proportional to the

number of base pairs (eqn. 10), the selectivity of reptation is independent of the size of a molecule (eqn. 11). Hence, from the point of view of selectivity, the sieving mechanism is similar to partition chromatography and reptation is similar to gel permeation chromatography or field-flow fractionation.

The experimental results show (Figs. 7 and 8) that it is possible to work with low-melting-point agaroses (SeaPrep, AcrylAide) even at temperatures above the melting point without any decrease in selectivity. The speed of an analysis of DNA fragments in a polymer separation medium can be increased at higher temperatures without any significant decrease in resolution, while the application of a higher voltage leads to decreased resolution (Figs. 4, 5 and 8).

The dependence of the selectivity on the molecular size (Fig. 4) indicates that molecules of different sizes migrate through the 1% agarose network under three different regimes. The molecules in the size range up to *ca.* 400 bp migrate in the sieving model. Under the optimum selectivity conditions molecules in the size range ca. 400–800 bp are separated in the regime of entropically regulated transport. The transport of molecules larger than 800 bp starts to be driven by a reptation mechanism.

It seems that the best conditions for the separation of polyelectrolytes are in a medium where the regions of close mutual contact exist together with regions where a molecule is allowed to relax freely. The results show that the spinodally decomposed sol, at a temperature close to the melting point, might be a medium with this feature.

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