Journal of Chromatography, 559 (1991) 285–294
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 472

Effect of temperature on the separation of DNA restriction fragments in capillary gel electrophoresis

ANDRAS GUTTMAN* and NELSON COOKE
Beckman Instruments, Inc., Palo Alto, CA 94304 (USA)

ABSTRACT

Two different separation modes were used in high-performance capillary gel electrophoresis to study the effect of temperature on the separation of DNA restriction fragments, isoelectrostatic (use of constant applied electric field) and isorheic (use of constant current). In both instances, the migration properties and resolution of the DNA molecules were studied as a function of column temperature between 20 and 50°C. In the isoelectrostatic separation mode, the migration time and resolution decrease as temperature increases. In the isorheic separation mode, increasing the column temperature results in a maximum migration time for all of the DNA fragments and shows a maximum resolution for the lower molecular weight fragments (<200 kilodalton).

INTRODUCTION

High-performance capillary electrophoresis (HPCE), as an instrumental approach to electrophoresis, is emerging as a powerful bioanalytical method and an effective tool for the characterization and isolation of biologically important macromolecules [1–5]. The use of narrow-bore polyacrylamide gel-filled capillaries allows extremely high efficiency separations for single- and double-stranded deoxyribonucleic acids [6–8].

Uncontrolled variations of temperature in gel electrophoresis can cause severe practical problems with respect to reproducibility, band broadening and migration properties [9–11]. For example, non-uniform column temperatures across the diameter or length of the column, caused by Joule heating, can decrease efficiency and resolution [2,12,13]. Gel-filled capillaries present an additional challenge in this regard because of the possible changes in the gel structure, such as pore size, due to high temperature [14]. In the separation of some biopolymers, such as DNA fragments, conformation changes can occur at elevated temperatures, leading to unexpected changes in migration time [15,16]. All of these problems can be avoided in HPCE by the use of column temperature control, making this technique much more efficient than conventional slab or rod gel systems. High-resolution separations can thus be achieved using constant temperature (isothermal), constant applied electric field (isoelectrostatic), constant current (isorheic), constant power (isoergic) and the use of gradients with each of those modes.

0021-9673/91/$03.50

The objective of this research was first to achieve high-resolution separations of the ϕX-174 DNA-Hae III digest restriction fragment test mixture by HPCE using gel-filled columns and then to study the effect of temperature on the electrophoretic migration properties under both constant-voltage and constant-current separation conditions.

THEORY

If a uniform electric field (E) is applied to a polyion with a net charge Q, the electrical force (F_e) is given by

$$F_e = QE \tag{1}$$

In free solution and in highly viscous media such as a gel, when a polyion is set in motion by the applied electric field, a frictional force (F_f) will act in the opposite direction:

$$F_f = f(dx/dt) \tag{2}$$

where f is the translational friction coefficient and dx and dt are the distance and time increments, respectively.

Differences in sample properties, such as shape, size or net charge, lead to differences in electrophoretic mobility and provide the basis of the electrophoretic separation. The motion of a polyion under an applied electric field can be expressed according to Newton's second law as

$$m(d^2x/dt^2) = QE - f(dx/dt) \tag{3}$$

which states that the product of the mass (m) and acceleration is equal to the difference of the electrical and frictional forces.

When the force from the applied electric field on the polyion is exactly counterbalanced by the frictional force, the particle will move with a steady-state velocity (v):

$$v = l/t_m = QE/f = \mu_e E \tag{4}$$

where l is the effective length of the capillary up to the detection point, t_m is the migration time of the solute and μ_e is the electrophoretic mobility of the polyion, which is defined as the steady-state velocity per unit field strength.

The translational friction coefficient (f) is a function of the viscosity of the surrounding media, *i.e.*, the gel, which is influenced by the temperature. For a spherical particle, when the Reynolds number is less then 0.1, the friction coefficient is given by Stokes law [17]:

$$f = 6\pi\eta r \tag{5}$$

where η is the viscosity of the surrounding medium and r is the root mean square radius of the polyion. From eqns. 4 and 5, we have

$$\mu_e = Q/f = Q/6\pi r\eta \tag{6}$$

which shows an inverse relationship between the mobility (μ_e) of the solute and the viscosity (η) of the surrounding gel–buffer medium. From eqn. 6, it is evident that the ion mobility is highly dependent on solvent viscosity, which is a function of temper-

ature. The viscosity of the gel–buffer system decreases exponentially with temperature [18] according to

$$\eta = C_1 \cdot \exp(E_a/RT) \tag{7}$$

where C_1 is a constant, E_a is the activation energy for the viscous flow, R is the universal gas constant and T is the absolute temperature. By combining eqns. 4–7, the temperature dependence on the migration time of a polyion can be expressed as:

$$t_m = (l/EQ) \cdot \text{constant} \cdot \exp(E_a/RT) \tag{8}$$

Taking the logarithm of eqn. 8 gives a convenient linear relationship between the migration time of the solute and the reciprocal absolute temperature:

$$\ln t_m = \ln(l \cdot \text{constant}/EQ) + E_a/RT \tag{9}$$

where the first term on the right-hand side depends on the length of the capillary, the applied electric field and the charge of the solute [19]. It should be noted that the charge (Q) may be weakly dependent on temperature owing to possible conformational changes of the polyion [15]. The term E_a/RT in eqn. 9 is a function only of the temperature. As a first approximation, in the isoelectrostatic mode, only this last term is temperature dependent. In the isorheic mode when the current is kept constant, the electric field changes with temperature, and therefore both terms on the right-hand side of eqn. 9 vary with temperature.

EXPERIMENTAL

Chemicals

ϕX-174 DNA-Hae III digest (New England Biolabs, Beverly, MA, USA) was diluted with water to 50 μg/ml before injection, and was stored at −20°C when not in use. The molecular weights of the DNA fragments in this mixture range from 47 to 879 kilodalton [72–1353 base pairs (bp)]. Electrophoretic-grade acrylamide, Tris, boric acid, EDTA, ammonium peroxydisulfate and tetramethylethylenediamine (TEMED) were employed (Schwarz/Mann Biotech, Cambridge, MA, USA). All buffer and acrylamide solutions were filtered through a 0.2-μm pore size filter (Schleicher & Schüll, Keene, NH, USA) and carefully vacuum degassed.

Apparatus

The P/ACE System 2000 capillary electrophoresis apparatus (Beckman, Palo Alto, CA, USA) was used. The separations were monitored on-column at 254 nm. The temperature of the gel-filled columns was controlled to ±0.5°C by the liquid cooling system of the P/ACE instrument.

Procedures

Polymerization of the polyacrylamide was initiated by ammonium peroxydisulfate, catalyzed by TEMED and accomplished within a 0.1 mm I.D. DB-17-coated capillary (J&W, Sacramento, CA, USA) in 100 mM Tris–boric acid–2 mM EDTA (pH 8.5) (20°C) (TBE). The total length of the capillary column was 270 mm, with 200 mm to the detector. The pH of the buffer at different temperatures was determined with a Beckman ϕ-21 pH meter.

RESULTS AND DISCUSSION

Before performing these experiments, it was first important to understand the effect of temperature on the buffer pH and the resistance of the gel. The change in pH of the 100 m*M* Tris–borate–EDTA buffer employed in the range 20–50°C was determined to be only 0.4 pH unit. This did not lead to any significant difference in migration time for the large polyion molecules used in the experiments [20].

As the conductivity of the gel increases with temperature, when a constant voltage is applied to a gel-filled capillary the resistance of the column decreases as shown in Fig. 1. Therefore, in accordance with Ohm's law, the current will increase with temperature in the isoelectrostatic mode and the voltage will decrease with temperature in the isorheic mode.

Effect of temperature in the isoelectrostatic separation mode

Fig. 2 compares the separations of the ϕX-174 DNA restriction fragment mixture by capillary gel electrophoresis at different temperatures using the isoelectrostatic separation mode. It can be seen that the migration time of the DNA fragments decreases as temperature increases in accordance with the inverse relationship between the viscosity of the gel–buffer system and temperature (eqn. 9) [21,22]. Of particular interest is the relative change and reversal in peak height of the last two peaks (1078 and 1353 bp) as temperature is increased. This effect is explained by the data in Table I, where it can be seen that the column efficiency (*i.e.*, the number of theoretical plates) decreases as temperature increases. The higher molecular weight range appears to be particularly susceptible to this effect, which might be due to excessive band broadening caused by conformational changes at elevated temperature [22].

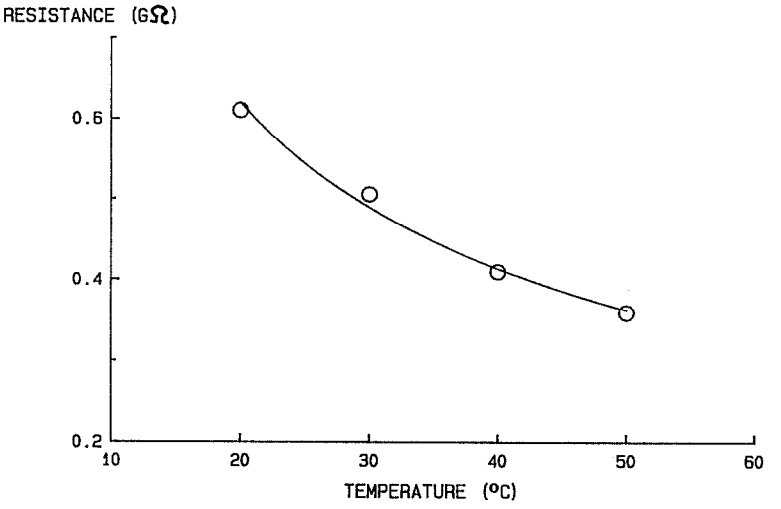

Fig. 1. Relationship between the temperature and the resistance of the gel-filled capillary column under a constant applied electric field. Conditions: isoelectrostatic mode, 400 V/cm; polyacrylamide gel column, effective length 20 cm, total length 27 cm; buffer, 0.1 *M* Tris–boric acid–2 m*M* EDTA (pH 8.5).

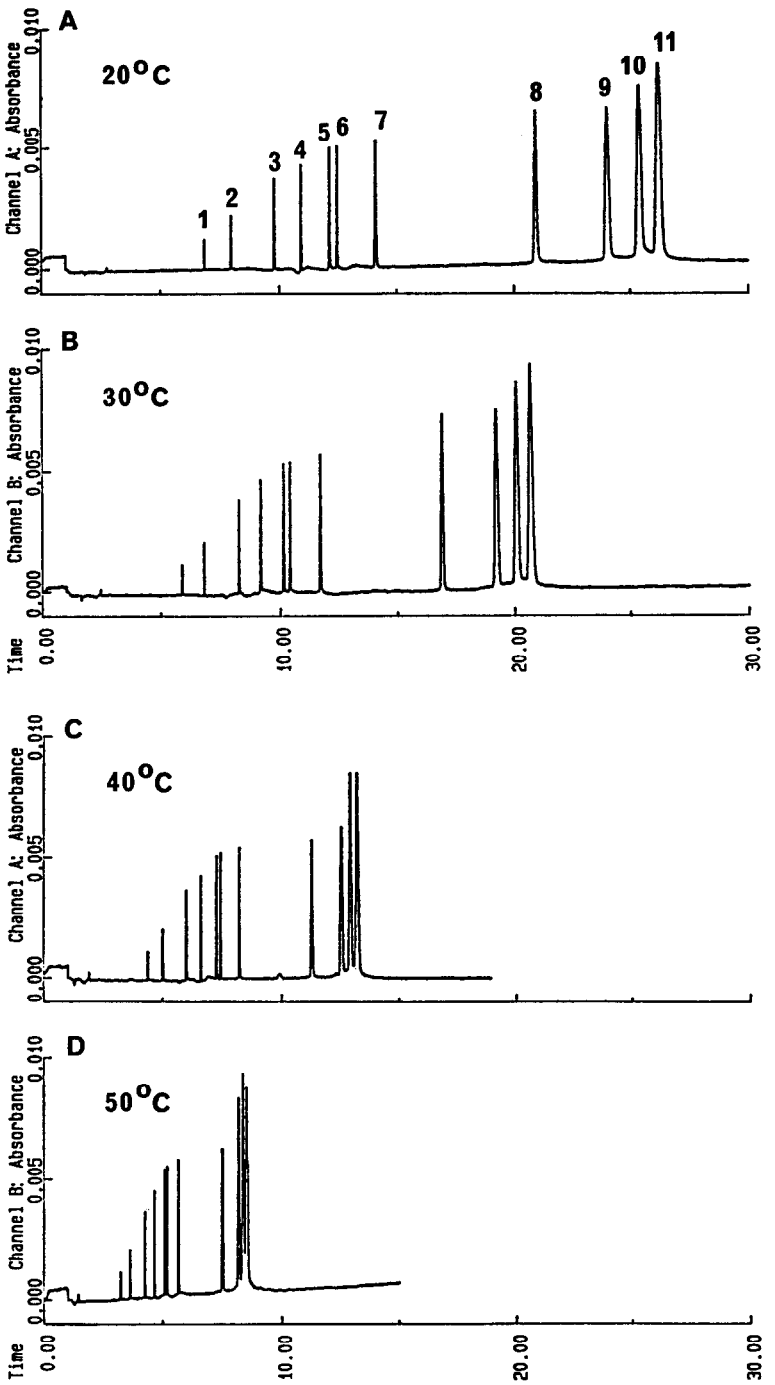

Fig. 2. Separation of the ϕX-174 DNA restriction fragment mixture by capillary gel electrophoresis at different temperatures in the isoelectrostatic separation mode: (A) 20; (B) 30; (C) 40; (D) 50°C. Peaks: 1 = 72; 2 = 118; 3 = 194; 4 = 234; 5 = 271; 6 = 281; 7 = 310; 8 = 603; 9 = 872; 10 = 1078; 11 = 1353 base pairs. Conditions as in Fig. 1. Injection, 2 s, 45 mW. Sample, 50 μg/ml of ϕX-174 DNA-Hae III digest.

TABLE I

PEAK-HEIGHT RATIOS (NORMALIZED TO PEAK AREA) AND THEORETICAL PLATE NUMBERS OF THE 1078 AND 1353 BASE PAIR FRAGMENTS OF ϕX-174 DNA-Hae III DIGEST USING THE ISOELECTROSTATIC AND ISORHEIC MODES

Conditions as in Figs. 2 and 4.

Temperature (°C)	Normalized peak-height ratio (1353/1078 bp)		Theoretical plates × 10^3 (1353 bp)	
	Constant voltage	Constant current	Constant voltage	Constant current
20	1.07	1.12	75	61
30	1.03	0.92	68	39
40	1.00	0.79	60	25
50	0.95	0.72	48	18

The logarithm of migration time for different DNA fragments is plotted in Fig. 3 as a function of reciprocal temperature and shows a linear relationship. These data agree well with eqn. 9, where the first term on the right-hand side is assumed to be constant and therefore the term E_a/RT describes the migration properties of the DNA.

Effect of temperature in the isorheic separation mode

According to Ohm's law, the voltage is directly proportional to the resistance when the current is kept constant. As the resistance is a function of temperature (Fig. 1), the apparent electric field will be determined to a large extent by the temperature of the column. Further, as the viscosity of the gel–buffer system decreases with tem-

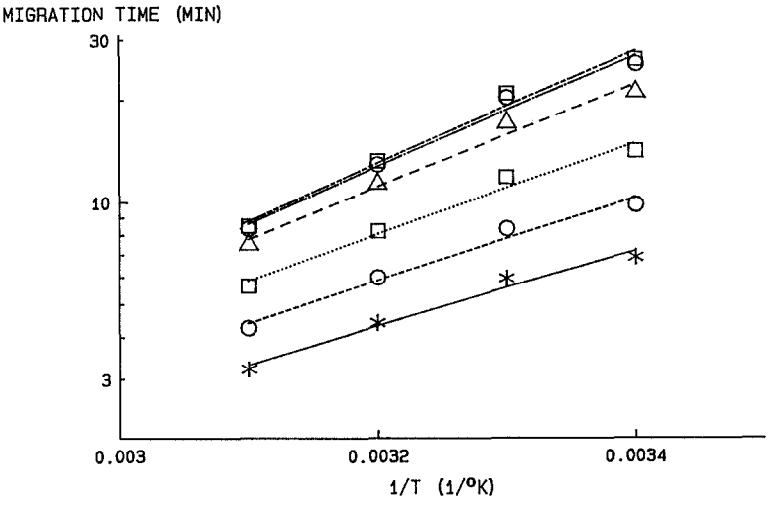

Fig. 3. Semi-logarithmic plot of the electrophoretic migration times of DNA restriction fragments as a function of the separation temperature in the isoelectrostatic mode. Conditions as in Fig. 2. Lines correspond to different sized DNA fragments: from top to bottom, 1353, 1078, 603, 310, 194 and 72 base pairs.

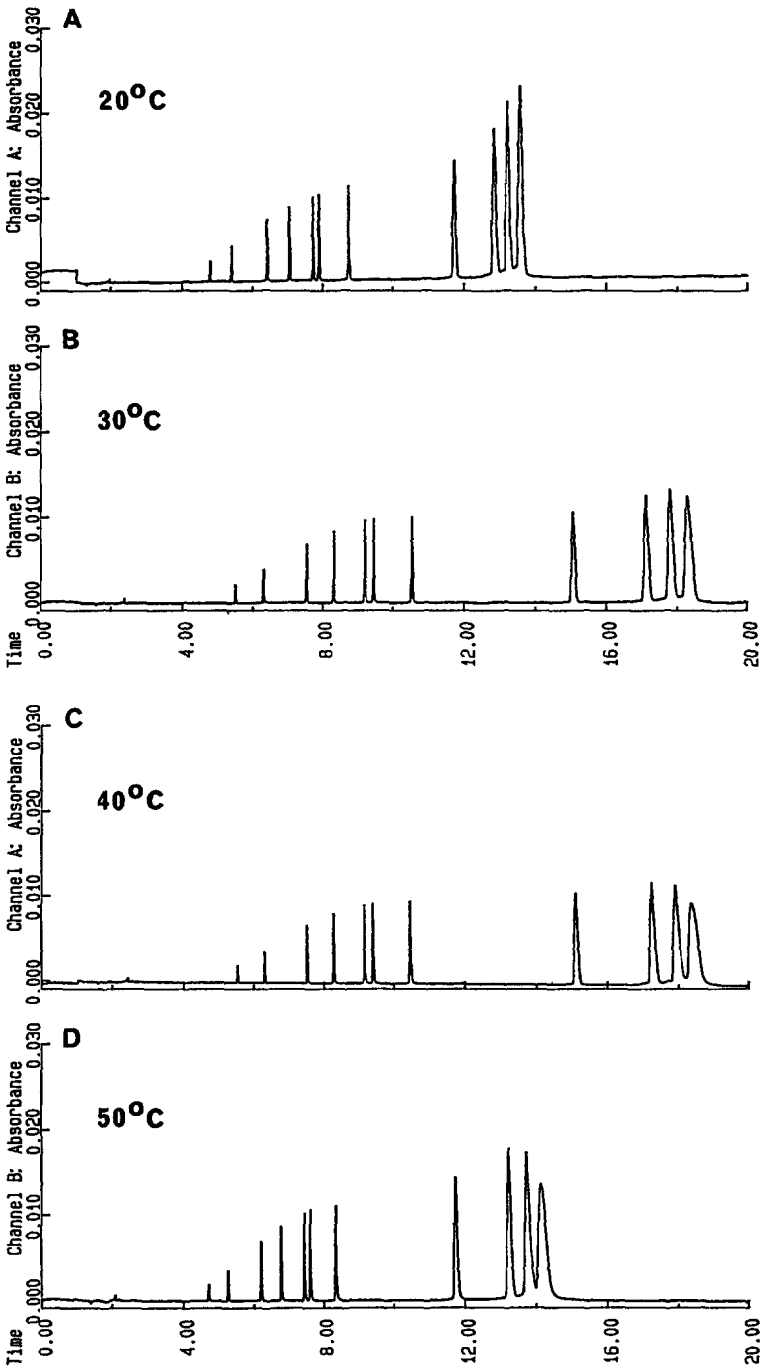

Fig. 4. Separation of the ϕX-174 DNA restriction fragment mixture by capillary gel electrophoresis at different temperatures in the isorheic separation mode: (A) 20; (B) 30; (C) 40; (D) 50°C. Conditions: isorheic mode, 20 μA; otherwise as in Fig. 2.

perature (eqn. 7), it also has an effect on the mobility of the sample components.

Fig. 4 compares the separations of the restriction fragment test mixture at different temperatures in the isorheic separation mode. In comparing these separations with those in Fig. 2, a different migration behavior is observed as the temperature is increased. In this instance, the migration time first increases with temperature, and then decreases above 40°C. This behavior can be understood by plotting the logarithm of migration time for different DNA fragments as a function of temperature, as shown in Fig. 5, and by referring back to eqn. 9. In this instance the two terms on the right-hand side of eqn. 9 combined describe the DNA migration properties. At low temperature (20°C), both the voltage and viscosity are high. The effect of the higher viscosity (eqn. 9, E_a/RT term) on migration will dominate over the voltage effect (eqn. 9, first term on the right-hand side) and lead to slower migration. On increasing the temperature of the gel-filled capillary column (30–40°C), the effect of voltage and viscosity on migration will be partially balanced, leading to a maximum migration time. At high temperature (50°C), the effect of the lower voltage on migration will prevail over the lower viscosity, leading to slower migration.

It can also be seen in Table I that the peak-height ratios for the higher molecular weight fragments in the isorheic mode decrease more (0.4 unit) than in the isoelectrostatic mode (0.12 unit). This is probably due to a greater decrease in efficiency, which can be explained by the combined effects of the decreasing apparent electric field [2] and possible conformational changes [22] with elevated temperature.

Effect of temperature on resolution

Referring back to Fig. 2, it is worth noting that good resolution is obtained between the 271 bp (peak 5) and 281 bp (peak 6) fragments. In other capillary electrophoretic systems where viscous hydrophilic polymers were used as sieving agents,

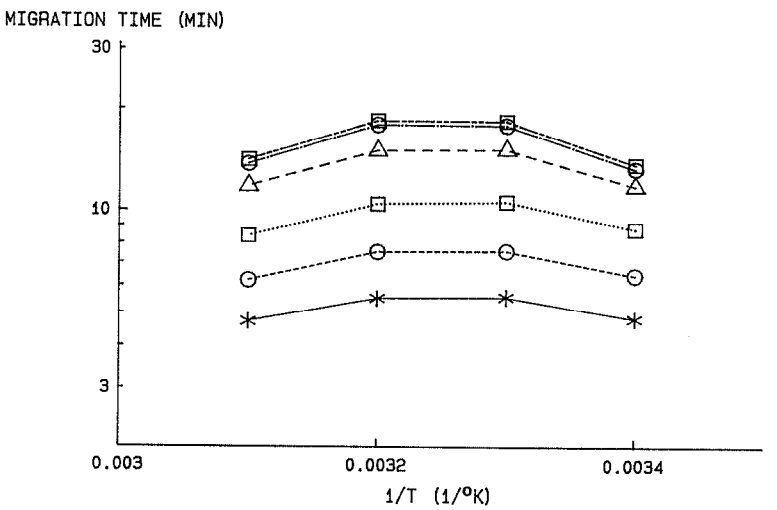

Fig. 5. Semi-logarithmic plot of the electrophoretic migration time of DNA restriction fragments as a function of the separation temperature in the isorheic separation mode. Conditions as in Fig. 4. Lines correspond to different sized DNA fragments: same order as in Fig. 3.

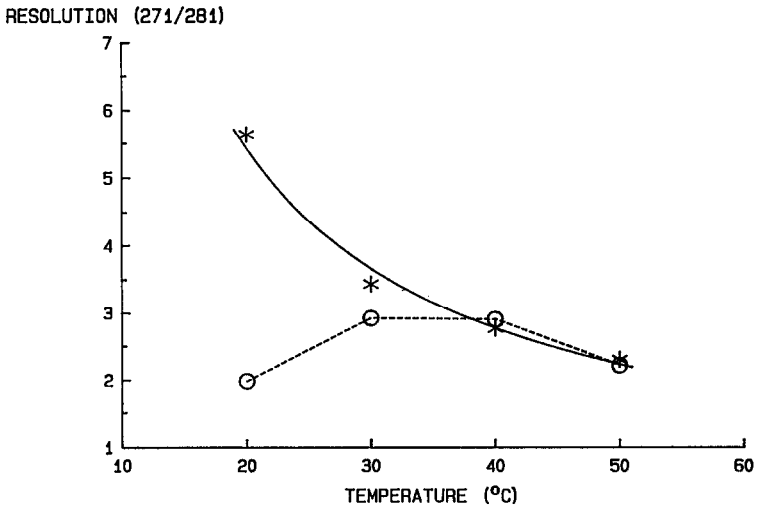

Fig. 6. Resolution between the 271 and 281 base pair DNA fragments as a function of the separation temperature using the (*) isoelectrostatic and (○) isorheic separation modes. Conditions as in Figs. 2 and 4.

these fragments could only be separated with the addition of special additives, such as intercalating agents, to the buffer [23]. Within the temperature range used in this study, a good separation of these two fragments was achieved in both the isoelectrostatic and isorheic separation modes.

Fig. 6 shows that the resolution between these two fragments is also a function of temperature. In the isoelectrostatic separation mode, the resolution in the lower molecular weight range decreases with temperature similarly to that observed with the higher molecular weight fragments (Table I). However, in the isorheic separation mode the resolution values of these low-molecular-weight fragments go through a maximum, in contrast to what was observed for the high-molecular-weight fragments.

CONCLUSIONS

According to theory, the migration of polyions in electrophoresis is driven by the potential difference between the two electrodes. In the isoelectrostatic and isorheic separation modes, the current and voltage are determined by the resistance of the capillary gel column at a given temperature, respectively.

Migration times decrease with increasing temperature in the isoelectrostatic mode and show a maximum in the isorheic mode. The resolution between the lower molecular weight DNA fragments (<300 bp) decreases in the isoelectrostatic separation mode and shows maxima in the isorheic mode at elevated temperature. However, the efficiency in the higher molecular weight range (>1000 bp) decreases in both modes with increasing temperature.

We are continuing these studies to determine the effect of other variables such

as column length, applied electric field, molecular weight of the polyion and the constant power separation mode (isoergic) in capillary gel electrophoresis.

ACKNOWLEDGEMENTS

The authors thank Professor Barry L. Karger and Dr. Herb Schwartz for stimulating discussions and for reviewing the manuscript. The help of Phyllis Browning in the preparation of the manuscript is also highly appreciated.

REFERENCES

1 J. W. Jorgenson and K. D. Lukacs, *Science (Washington, D.C.)*, 222 (1983) 266.
2 B. L. Karger, A. S. Cohen and A. Guttman, *J. Chromatogr.*, 492 (1989) 585.
3 S. Hjerten, *J. Chromatogr.*, 270 (1983) 6.
4 R. A. Wallingford, and A. G. Eving, *Anal. Chem.*, 58 (1988) 258.
5 M. J. Gordon, X. Huang, S. L. Pentoney, Jr., and R. N. Zare, *Science (Washington, D.C.)*, 242 (1988) 224.
6 J. A. Lux, H. F. Yin and G. Shomburg, *J. High. Resolut. Chromatogr.*, 13 (1990) 436.
7 A. Guttman, A. S. Cohen, D. N. Heiger and B. L. Karger, *Anal. Chem.*, 62 (1990) 137.
8 D. N. Heiger, A. S. Cohen and B. L. Karger, *J. Chromatogr.*, 516 (1990) 33.
9 R. J. Nelson, A. Paulus, A. S. Cohen, A. Guttman and B. L. Karger, *J. Chromatogr.*, 480 (1989) 111.
10 R. West, *Biopolymers*, 26 (1987) 607.
11 G. W. Slater and J. Noolandi, *Electrophoresis*, 9 (1988) 643.
12 S. Terabe, K. Otsuka and T. Ando, *Anal. Chem.*, 57 (1985) 834.
13 E. Grushka, R. M. McCormick and J. J. Kirkland, *Anal. Chem.*, 61 (1989) 241.
14 T. Tanaka, *Sci. Am.*, 244 (1981) 124.
15 C. R. Cantor, C. L. Smith and M. K. Mathew, *Annu. Rev. Biophys. Chem.*, 17 (1988) 287.
16 G. W. Slater and J. Noolandi, *Biopolymers*, 25 (1986) 431.
17 P. Grossmann and D. S. Soane, *Anal. Chem.*, 62 (1990) 1592.
18 F. W. Billmeyer, Jr., *Textbook of Polymer Science*, Wiley, New York, 3rd ed., 1984.
19 A. Guttman and N. Cooke, *Appl. Theor. Electrophor.*, in press.
20 A. Guttman, A. Arai, A. S. Cohen and B. L. Karger, presented at the *14th International Symposium on Column Liquid Chromatography, Boston, May 20–25, 1990*, paper P414.
21 B. L. Karger, A. S. Cohen, D. N. Heiger and K. Ganzler, presented at the *3rd International Symposium on High Performance Capillary Electrophoresis, San Diego, CA, February 3–6, 1991*, paper LM-4.
22 N. C. Stellwagen, *Biochemistry*, 22 (1983) 6186.
23 H. E. Schwartz, K. Ulfelder, F. J. Sunzeri, M. P. Busch and R. G. Brownlee, *J. Chromatogr.*, 559 (1991) 267.