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Separation of DNA restriction fragments by polymer-solution capillary zone electrophoresis

Influence of polymer concentration and ion-pairing reagents

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ABSTRACT

To achieve sensitive and fast separations of the DNA fragments, polymer-solution capillary zone electrophoresis (PS-CZE) is examined. A systematic study of the influence of the linear polymer concentration on the separation of restriction fragments indicates that the larger DNA fragments are resolved with greater efficiency by using a lower concentration of the polymer, while the smaller ones are better resolved with a higher polymer concentration, when added to the buffer. A specific concentration of the polymer provides a certain limit of dynamic "porosity" (sieving), which is appropriate for a given range of the DNA fragments.

Several ion-pairing reagents are examined here to improve resolutions of both small and large DNA fragments in PS-CZE. These reagents cause no interactions between the ammonium cations (reagent) and the silanol groups (capillary surface). However, they interact with the linear polymer, perhaps by hydrophobic functions, and change its physical properties, such as dynamic viscosity and sieving ability. Enhanced retention (interactions) of the DNA fragments is observed by increasing the ion-pairing contents in the polymer solution. The increased peak retention strongly suggests presence of interactions between the reagent and the DNA fragments. Different ion-pairing reagents produce different degrees of peak retentions. The interactions results from relatively *greater* hydrophobic interactions and *smaller* ionic interactions between the reagent and the polynucleotides.

The study of ethidium bromide (EdBr) additon in PS-CZE indicates that a small concentration of EdBr reduces peak widths and decreases retention of all the fragments, but has little influence on the electroosmotic flow. However, a larger EdBr concentration results in broader peak widths and causes little change in their retention. Ion-pairing and intercalating reagents interact with the DNA fragments by different mechanisms. An anion-pairing agent appears to interact predominantly by hydrophobic interactions with the purine and pyrimidine bases, while EdBr intercalates with G:C base pairs and alter the chain length.

Satisfactory separations of *both* larger and smaller fragments can be achieved by adding an appropriate ion-pairing reagent along with the linear polymer to the buffer. Critical concentrations of the linear polymer and the ion-pairing reagent must be determined in order to achieve satisfactory separations for a given range (size) of the DNA fragments.

INTRODUCTION

Non-cross-linked polymers have been used as sieving media, such as in centrifugation and electrophoresis to yield a sieving medium in capillary zone electrophoresis (CZE) [1,2]. This "polymer-solution capillary zone electrophoresis" (PS-CZE) involving the use of a free polymer in the solution has not been fully exploited for the separation of proteins and nucleic acids. The cross-linked gel matrix in classical polyacrylamide gel electrophoresis

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(PAGE) offers an anticonvective medium to prevent convection of analytes which may result from Joule heating and a sieve for size exclusion (size-dependent separation). The heat dissipation in CZE is achieved because of tiny capillary with large surface-to-volume ratio, *i.e.* Joule heat is well dissipated and natural convection is not a problem. The linear polymers offer sieving similar to that of a gel-based system. The separation of DNA fragments have recently been achieved in dilute polymer solutions with the use of a linear chain polymer, but without the aid of a cross-linked gel matrix [3–6]. Polyacrylamide [4], methylcellulose and hydroxypropylmethylcellulose [3] have been employed as sieving agents in PS-CZE for the separation of oligonucleotides and DNA fragments. The separation of nucleic acids by PS-CZE offers size-based separations, and has several advantages over capillary-gel CE (CGE). The technique is simple, fast, and offers a variety of sieving systems without requiring a change of the capillary. In order to enhance DNA separations in PS-CZE, as well as in CGE, efforts have been made to complex DNA fragments by intercalation with ethidium bromide (EdBr) [7,8]. (For full details on PS-CZE and CGE of nucleic acids, see a recent review article, ref. 9.)

The effect of linear polymer concentrations on the separation of restriction endonuclease DNA fragments is studied here. Interactions of ion-pairing reagents with DNA fragments, a linear polymer, and the capillary surface are examined. A series of ion-pairing reagents are employed for this purpose. Effects of ion-pairing reagents are compared with those of the EdBr and mechanisms of interactions of both additives are discussed here.

EXPERIMENTAL

Materials

The DNA restriction fragments mixtures, ΦX174 DNA/*Hae*III digest and pGEM-3 DNA/*Hinf*I, *Rsa*I and *Sin*I digest were obtained from Promega (Madison, WI, USA). Samples were diluted with water to 0.2 mg/ml before injection and stored at −20°C. Hydroxyethyl cellulose, tetrabutylammonium dihydrogenphosphate (TBAP) and tetrahexylammonium bromide (THAB) were from Aldrich (Milwaukee, WI, USA). Tetramethylammonium hydroxide (TMAH), triethylamine (TEA), and EdBr were from Sigma (St. Louis, MO, USA) and Fisher Scientific (Fair Lawn, NJ, USA). Buffer solutions were pre-filtered through a 0.2-μm pore filter (Gelman, Ann Arbor, MI, USA).

Capillary zone electrophoresis system

The CE equipment, designed in this laboratory [10], consisted of the following four basic components: (a) A polyimide-coated fused-silica capillary tubing [80 cm × 75 μm I.D. × 280 μm O.D., 30 (or 50) cm effective length]; anodic reservoir electrically grounded. (b) A specifically designed on-line Z-shaped flow cell with a light path of 3 mm (LC Packing, San Francisco, CA, USA). (c) A variable-wavelength UV monitor (Spectroflow 757; Kratos Analytical Instruments, Ramsey, NJ, USA), which was modified to accommodate the above mentioned flow cell. (d) A high-voltage power supply (0 to 30 kV) with features of programmable, reversible-polarity output (Bertan, Hicksville, NY, USA). The direction of the solution flow during the CE was changed by simply changing the electrode polarities. The electric current was monitored by a digital multimeter (John Fluke, Everett, WA, USA). An elution pump (Waters, Milford, MA, USA) was used for washing the column and initiating the sample injection. The signals from the detector were fed to a strip-chart recorder for instant monitoring of the results. In addition, data were acquired with an IBM-compatible personal computer by using an analog-to-digital interface, and retrieved with the help of Nelson Analytical (Cupertino, CA, USA) software. Sample was injected from the anode side and the detector installed on the cathode side. The operation temperature was controlled at 20 ± 0.5°C.

Diverse methods

The electroosmotic flow measurement was carried out according to the method of Lukacs and Jorgenson [11]; phenol was used as a neutral marker. The injected volume was established by introducing a standard solution of adenosine and determining its concentration from the peak area as a function of time. The specific viscosity of the solutions containing polymer of different concentrations was measured by using an Ostwald

viscometer in relation to pure water at 20 ± 0.5°C [5,12].

Equipment operation

The capillary column was first washed by pumping a buffer through it and allowing the pressure to reach a constant value. The liquid flow was then disconnected and the capillary dipped into the sample contained in a miniature conical polyethylene tube. The positive liquid flow current generated by the pump and the gravitational force were enough to allow introduction of the sample into the fine capillary column. The sample flow was allowed for a specific time (between 5 and 20 s). This method of sample application was found to be very reproducible. For example, a 20-s injection introduced 7.9 nl of the sample into the capillary column. This value having a standard deviation of 0.05, was based on six different injections.

RESULTS

The effect of a linear polymer concentration on the separation of DNA fragments

The separation of DNA fragments derived from ΦX174 digested with restriction endonuclease *Hae*III is shown in Fig. 1. Panels a, b and c show these separations while using an increasing amount of hydroxyethylcellulose (HEC). The results indicate that larger DNA fragments [1353 base pairs (bp) to 872 bp, peaks 1 to 3] are resolved with greater efficiency by employing a lower HEC concentration (0.25%), while the smaller ones (234 bp to 72 bp, peaks 7 to 11) are resolved with significantly enhanced resolutions by adding a higher polymer concentration to the buffer (0.50% to 0.75% HEC). The polymer provides a certain range of dynamic "porosity" (sieving effect) for the DNA fragments. Though larger fragments fail to resolve with a higher concentration of the polymer, the smaller ones undergo fractionation. Similarly, larger fragments tend to fractionate with a lower polymer concentration, but the smaller ones fail to resolve.

The effect of ion-pairing reagents in CZE

The effect of ion-pairing reagents on electroosmotic flow, and also on the emergence of unresolved DNA fragments from the capillary, is shown in Table I. None of the four ion-pairing reagents alters the electroosmotic flow in CZE,

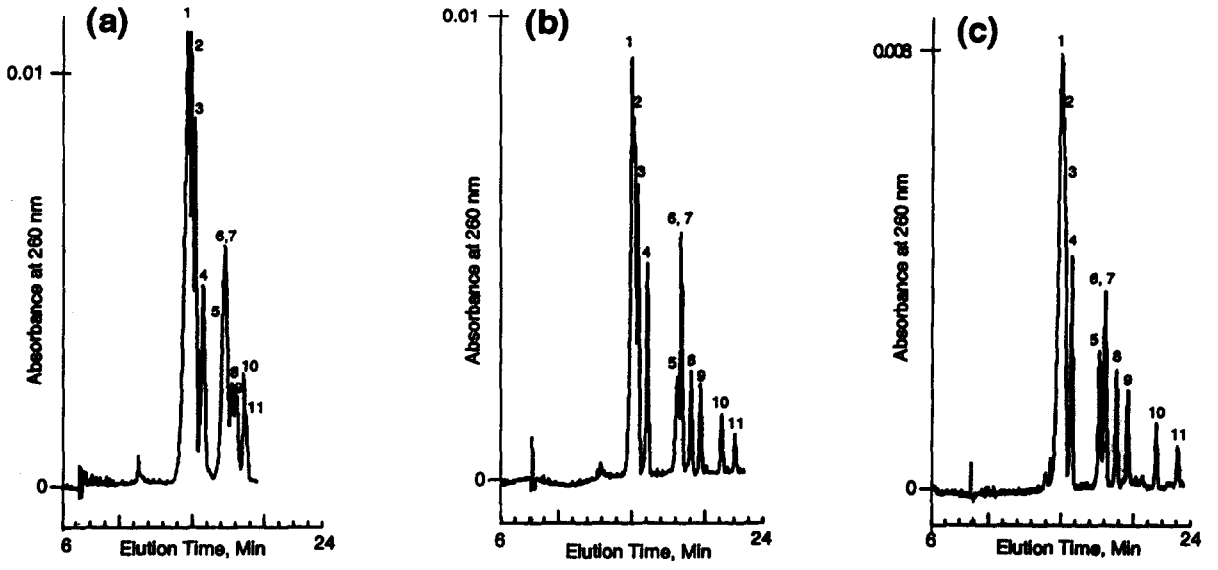


Fig. 1. The separation of ΦX174 DNA/*Hae*III fragments with different concentrations (w/v) of hydroxyethylcellulose (HEC): (a) 0.25%, (b) 0.5% and (c) 0.75%. Buffer, in addition to HEC, contained 50 m*M* Tris–borate, 2 m*M* EDTA and 7 *M* urea, pH 8.85. The column was an untreated capillary, 80 cm × 75 μm I.D. (effective length, 50 cm); applied voltage, 250 V/cm; detection at 260 nm, 20°C. Approximately 1.6 ng of the sample was injected. The peak numbers correspond to various DNA fragments of the listed base pairs (peak No.): 1353 (1), 1078 (2), 872 (3), 603 (4), 310 (5), 281, 271 (6,7), 234 (8), 194 (9), 118 (10) and 72(11).

TABLE I

EFFECT OF ION-PAIRING REAGENTS IN CZE

Ion-pairing reagent[a]	Electroosmotic flow[b] (±0.05)	Retention time (min)[c] (±0.1)
None[a]	4.3	20.4
Triethylamine, 5 m*M*	4.2	23.8
Tetramethylammonium hydroxide, 5 m*M*	4.3	23.2
Tetrabutylammonium dihydrogenphosphate, 5 m*M*	4.3	26.4
Tetrahexylammonium bromide, 5 m*M*	4.3	21.4

[a] Buffer contained 50 m*M* Tris–borate, 2 m*M* EDTA and 7 *M* urea, pH 8.85.
[b] Electroosmotic flow is expressed in $cm^2\ V^{-1}\ s^{-1} \times 10^{-4}$.
[c] Emergence of unresolved pGEM DNA fragments.

suggesting that no interaction occurs between the ammonium cations (weak acid) and silanol groups (weak base). The bulky alkyl chains can prevent this interaction with the capillary surface.

Polymer addition progressively decreases the electroosmotic flow, but dramatically increases the solution viscosity. For example, three HEC concentrations (0.25, 0.50 and 0.75%) produced electroosmotic flow values of 4.13, 3.89 and $3.71 \cdot 10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$, respectively; and relative viscosity of 1.66, 3.82 and 7.26, respectively. The loss in electroosmotic flow is apparently caused by an increase in the viscosity, thus lowering the "bulk movement" of the solution in CZE.

Different reagents retain the DNA fragments for different times in CZE, although the ion-pairing reagents have no resolving power (all fragments emerge unresolved). In each case, the fragments are retained for a longer period of time than the buffer itself. The increased reten-

TABLE II

EFFECT OF ION-PAIRING REAGENTS ON ELECTROOSMOTIC FLOW IN PS-CZE

Ion-pairing reagent[a]	Concentration (m*M*)	Electroosmotic flow[b]
None[a]	0	3.9
Triethylamine	3	3.6
Triethylamine	5	3.6
Triethylamine	7	3.6
Tetramethylammonium hydroxide	1	3.8
Tetramethylammonium hydroxide	3	3.6
Tetramethylammonium hydroxide	5	3.6
Tetrabutylammonium dihydrogenphosphate	1	3.8
Tetrabutylammonium dihydrogenphosphate	3	3.6
Tetrabutylammonium dihydrogenphosphate	5	3.4
Tetrabutylammonium dihydrogenphosphate	7	3.2
Tetrahexylammonium bromide	1	3.7
Tetrahexylammonium bromide	3	3.5
Tetrahexylammonium bromide	5	3.5

[a] Buffer contained 50 m*M* Tris–borate, 2 m*M* EDTA, 7 *M* urea and 0.5% hydroxyethylcellulose, pH 8.85.
[b] Electroosmotic flow is expressed in $cm^2\ V^{-1}\ s^{-1} \times 10^{-4}$.

tion strongly suggests interactions between the reagent and the DNA fragments (for nature of this interaction, see Discussion).

The effect of ion-pairing reagents in PS-CZE

The ion-pairing reagents exhibit no influence on the electroosmotic flow as observed in Table I. However, some reduction in the electroosmotic flow is observed when the reagent is added with a linear polymer (0.5% HEC) to the buffer. This loss in electroosmotic flow (2 to 22%), as shown in Table II, is directly related to the amount of the ion-pairing reagent present in the polymer solution.

The effect of ion-pairing reagents on the retention of DNA fragments in PS-CZE

The separation of eleven DNA fragments, derived from ΦX174/*Hae*III, are examined in the presence of four different ion-pairing reagents, and with several concentrations of each reagent. The results, shown in Fig. 2, confirm enhanced retention (interactions) of DNA fragments with increasing amounts of the ion-pairing reagent in the polymer solution. Different ion-pairing reagents produce different degrees of retention enhancement. Once a critical concentration of the reagent is achieved (such as 5 m*M* TBAP), no significant enhancement in their resolutions can be attained by further addition of the reagent (Fig. 2a).

TEA, in excess of 1 m*M* concentration, fails to show any improvement in resolution (Fig. 2b). Though the addition of TMAH and THAB enhances resolution, TBAP provides the most dramatic improvement in resolutions for these DNA fragments (compare Fig. 2c and d with a).

The effect of intercalation of G:C base pairs with EdBr on electroosmotic flow and retention of DNA fragments in PS-CZE

The results in Table III indicate that a small concentration of EdBr reduces peak widths and decreases retention of all the fragments, but has little influence on the electroosmotic flow. However, a larger EdBr concentration, such as 0.15 m*M*, results in broader peak widths and causes little change in their retention. With EdBr as an additive, the larger DNA fragments fail to resolve and all the fragments emerge earlier than in the absence of EdBr. Though the separation of the large DNA fragments is not favorably influenced, the fragments of 271 bp and 281 bp are partially resolved in the presence of >60 μM EdBr. In general, small concentrations of EdBr tend to make the peaks sharper, but a larger concentration produces broader peaks. The ion-pairing reagent, such as TBAP, alone appears to resolve both larger and smaller DNA fragments and produces moderate peak widths, but retains the peak longer. A combination of EdBr and TBAP slightly decreases the electroosmotic flow, broadens all the peaks, and prolongs peak emergence from the column.

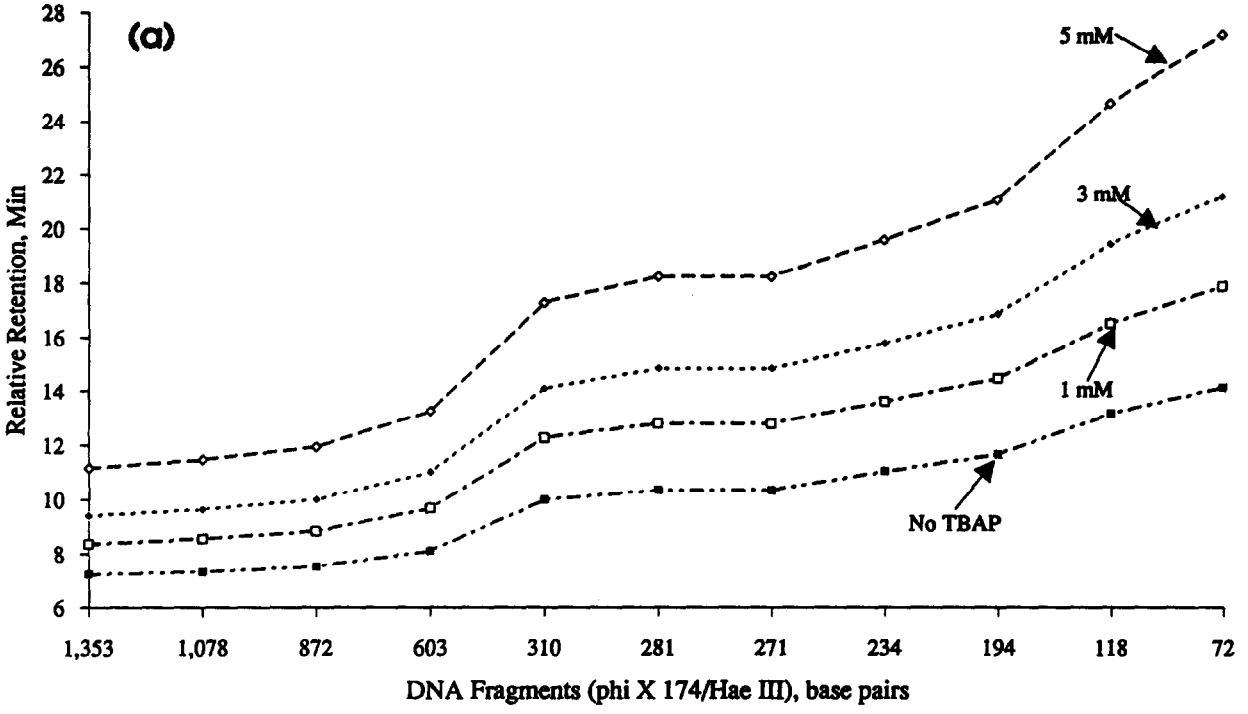
Examples of the separation of DNA fragments with a combination of an ion-pairing agent and a linear polymer by PS-CZE

Results in Fig. 3 indicate improvement in the resolution of the DNA fragments with the addition of an ion-pairing reagent, such as TBAP. Satisfactory separations of *both* larger and smaller DNA fragments are observed with the addition of an appropriate ion-pairing reagent along with the linear polymer to the buffer. A lower concentration of the reagent (3 m*M* TBAP) can be employed if the capillary length is increased (Fig. 3a). Conversely, the use of a shorter capillary (30 cm) requires a higher concentration of the reagent (Fig. 3b).

Another example of the separation of DNA fragments (pGEM-3 digested with restriction endonucleases *Hind*I, *Rsa*I and *Sin*I) by PS-CZE involving a combination of an ion-pairing agent and a linear polymer is shown in Fig. 4. The addition of the ion-pairing reagent, such as TBAP, improves resolution of *both* larger and smaller DNA fragments, ranging from 36 to 2645 bp.

Comparison of separations achieved by two different detection methods

A flow cell of Z-type (light path, 3 mm) used in this study, offers significant detection sensitivity by UV absorption, for example the detection of 8 ng (0.8 fmol or 71 n*M*) DNA fragments. However, the fragments differing by a small chain length (such as 179 bp from 222 bp fragments) could not be resolved while employing a


(a)
5 mM
3 mM
1 mM
No TBAP
Relative Retention, Min
28
26
24
22
20
18
16
14
12
10
8
6
1,353
1,078
872
603
310
281
271
234
194
118
72
DNA Fragments (phi X 174/Hae III), base pairs

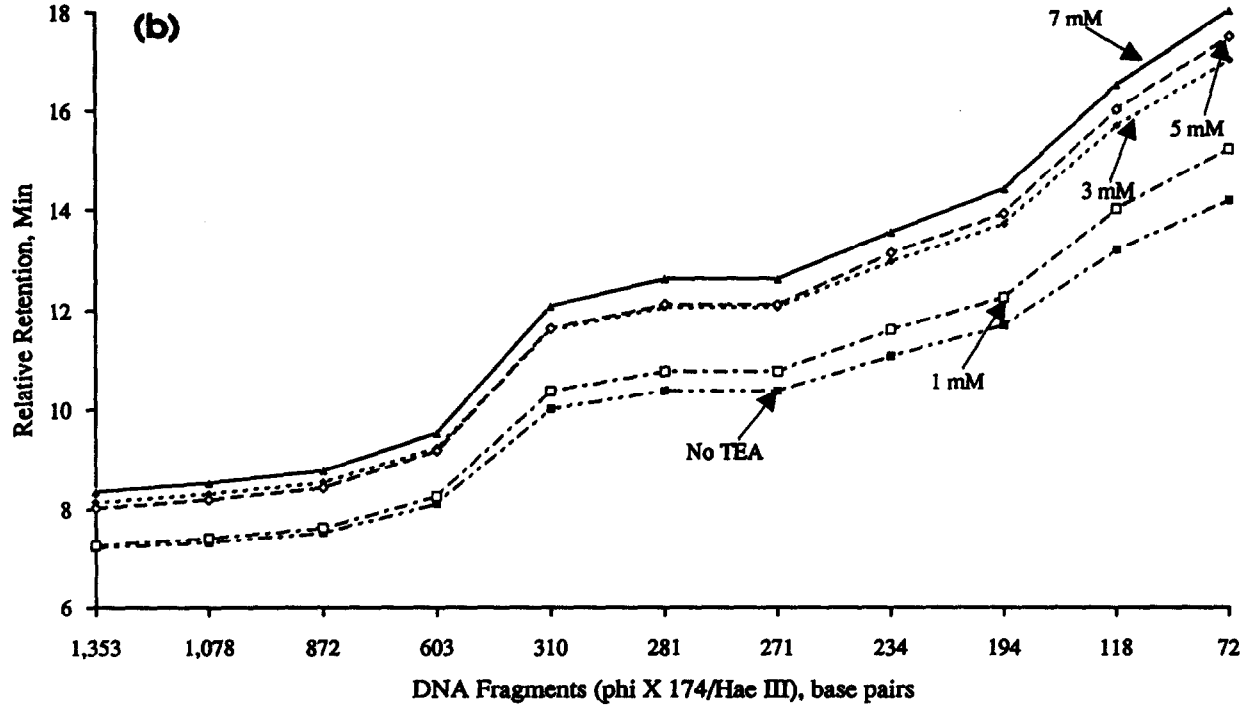
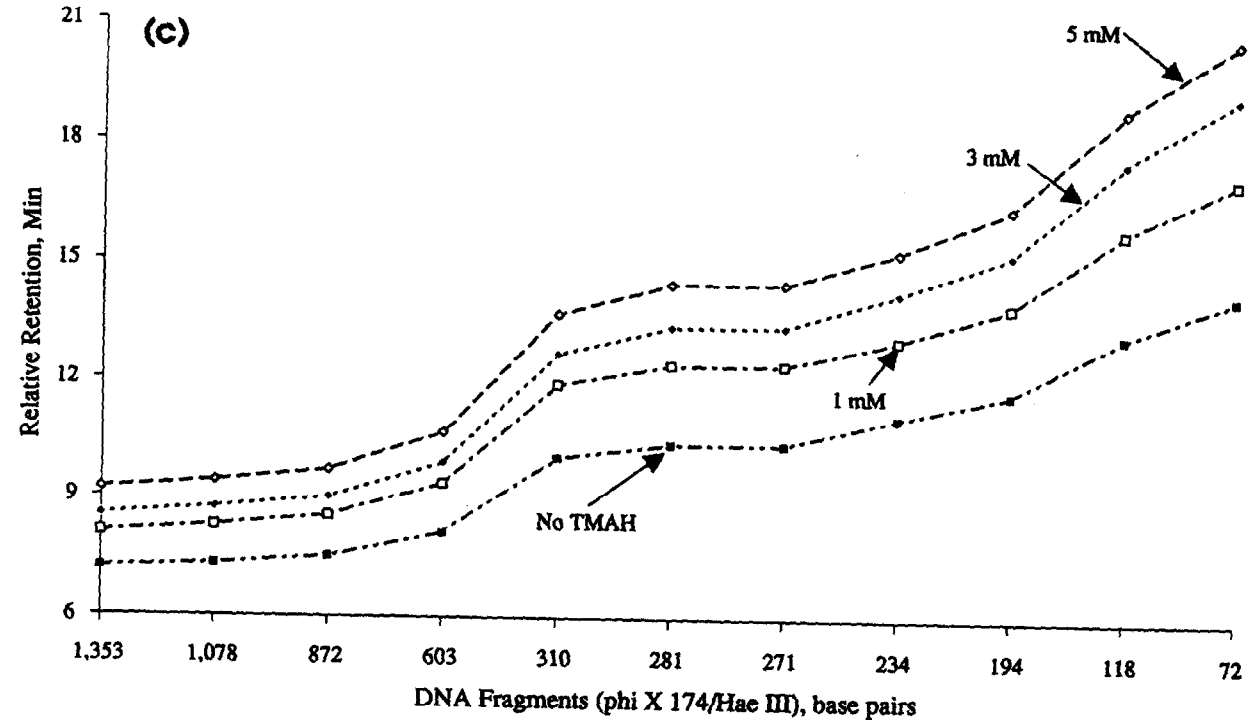

(b)
7 mM
5 mM
3 mM
1 mM
No TEA
Relative Retention, Min
18
16
14
12
10
8
6
1,353
1,078
872
603
310
281
271
234
194
118
72
DNA Fragments (phi X 174/Hae III), base pairs

Fig. 2.



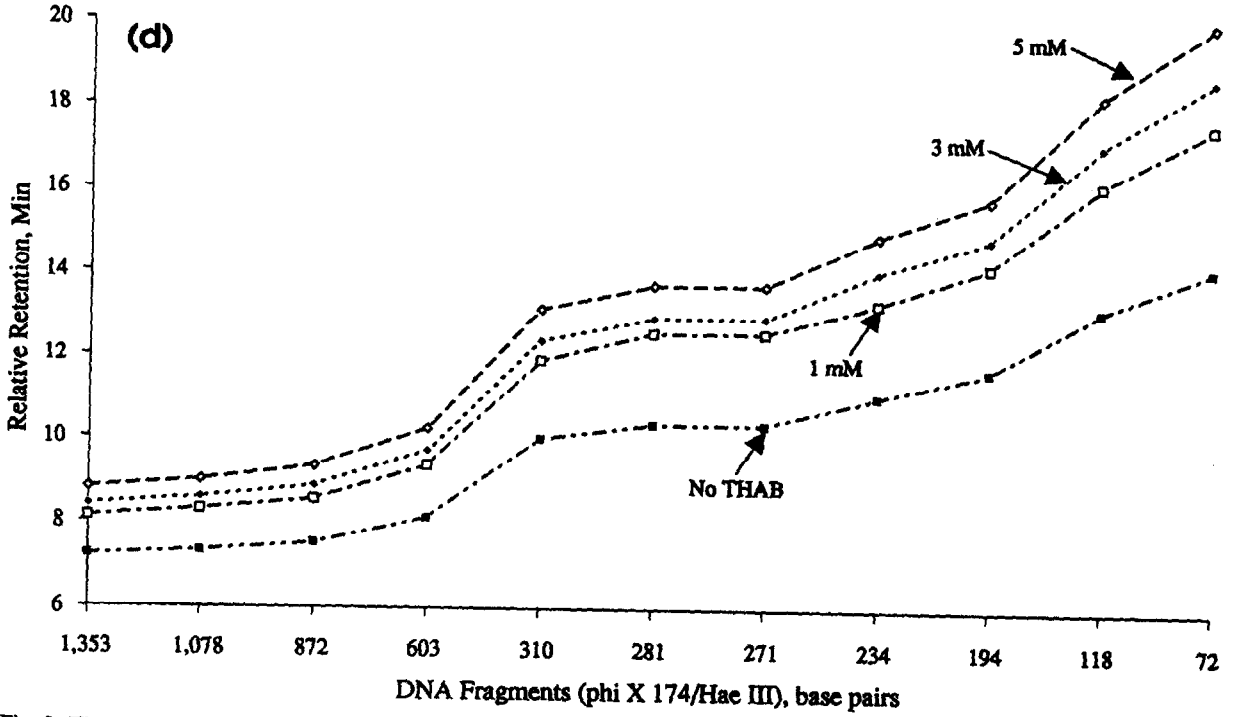


Fig. 2. The emergence of ΦX174 DNA/*Hae*III fragments in the presence of different ion-pairing reagents: (a) TBAP, (b) TEA, (c) TMAH and (d) THAB. The buffer contained 0.5% HEC; for other experimental details, see caption to Fig. 1. The relative retention time is expressed as $t_R - t_0$, where t_0 is the front peak of benzene and t_R is the retention of the DNA fragment.

TABLE III

EFFECT OF ETHIDIUM BROMIDE ADDITION ON THE SEPARATION OF DNA FRAGMENTS

Additive[a]	Concentration	Electroosmotic flow[b]	Peak width (min)[c], DNA fragments			Relative retention time (min)[d], DNA fragments		
			603 bp	194 bp	72 bp	603 bp	194 bp	72 bp
None	0	3.5	0.36	0.32	0.36	10.2	17.0	21.6
Ethidium bromide	1 μM	3.4	0.28	0.30	0.26	10.5	16.9	22.6
Ethidium bromide	60 μM	3.4	0.26	0.17	0.22	8.2	13.4	19.2
Ethidium bromide	150 μM	3.3	0.37	0.42	0.23	7.9	13.0	19.2
Ethidium bromide and TBAP	60 μM 5 mM	3.1	0.38	0.33	0.37	10.7	18.2	27.5
TBAP	5 mM	3.4	0.31	0.28	0.31	13.3	21.1	27.2

[a] Buffer contained 50 mM Tris–borate buffer, 2 mM EDTA, 7 M urea, 0.5% hydroxyethyl cellulose, and any additive, pH 8.85.
[b] Electroosmotic flow is expressed in $cm^2\ V^{-1}\ s^{-1} \times 10^{-4}$.
[c] Peak widths at half-maximum height of the fragments derived from ΦX174 DNA.
[d] Relative retention time is expressed as $t_R - t_0$, where t_0 is the front peak of benzene and t_R is the retention of the DNA fragment.

flow cell of such a large volume (13.3 nl). A commercially available detector (Isco Model CV[4]) having a light path of the capillary diameter (75 μm) also produced identical separations, but exhibited approximately five times less sensitivity. However, the plate numbers were substantially increased (such as, from 33 000 to 70 000) with the use of the narrow-bore light path detector.

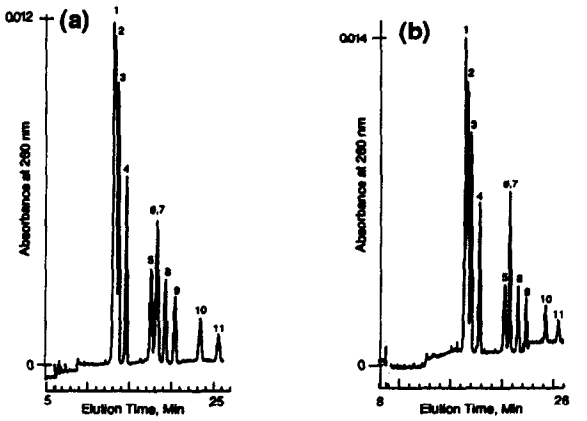


Fig. 3. The separation of ΦX174 DNA/*Hae*III fragments with different TBAP concentrations and column lengths: (a) 3 mM TBAP, 50 cm effective column length and (b) 5 mM TBAP, 30 cm effective column length. The buffer contained 0.5% HEC; for other experimental details and peak identities, see caption to Fig. 1.

DISCUSSION

DNA fragments in PS-CZE (uncoated capillaries) migrate upstream (towards the injector),

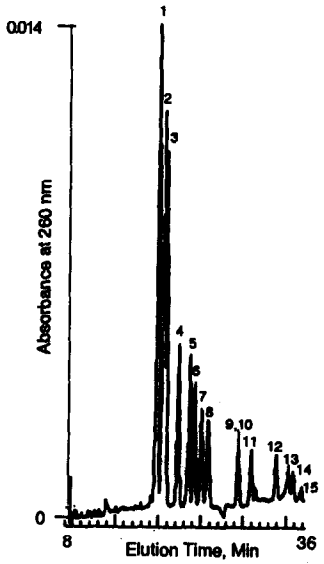


Fig. 4. The separation of pGEM-3 DNA (digested with restriction endonucleases *Hind*I, *Rsa*I and *Sin*I) fragments. The buffer contained 0.5% HEC and 5 mM TBAP; for other experimental details, see caption to Fig. 1. Approximately 8 ng of the sample were injected. The peak numbers correspond to various DNA fragments of the listed base pairs (peak No.): 2645 (1), 1605 (2), 1198 (3), 676 (4), 517 (5), 460 (6), 396 (7), 350 (8), 222, 179 (9,10), 126 (11), 75 (12), 65 (13), 51 (14), 36 (15).

against electroosmotic flow due to the anionic charges of the phosphate groups which are present in the DNA molecule. The electrophoretic mobility and electroosmotic flow are of unequal strength; the electroosmotic flow exerts a greater velocity and tends to *sweep* or *comb* the molecules towards the detector (cathode). Consequently, the larger fragments exit prior to the smaller ones even though the former fragments exhibit a greater degree of negative (phosphate) charge than the smaller fragments.

The interaction between the ion-pairing reagent and the DNA fragments can occur in two ways. One involving an ionic interaction between the phosphate anions (polynucleotides) and the ammonium cations of the reagent. The other attraction can involve hydrophobic interactions between the purine and pyrimidine bases and the alkyl chains of the reagent. These interactions, singly or in combination, produce a DNA–reagent complex, having a greater molecular mass and diminished anionic character, *i.e.* electrophoretic migration. Though a loss in the electrophoretic migration should decrease the peak retention, the electroosmotic flow remains unaltered. Therefore, the enhanced retention is the result of relatively *greater* hydrophobic interactions and *smaller* ionic interactions. For example, TEA and TMAH influence DNA separations similarly; both reagents have similar alkyl chains (hydrophobicity), while they exhibit significantly different cationic charges at pH 8.8.

The ion-pairing reagents apparently interact with the linear polymers, perhaps by hydrophobic interactions, and change their physical properties, such as dynamic viscosity and dynamic "porosity". Besides the concentration effect, different ion-pairing reagents appear to influence the electroosmotic flow differently; for example, compare the effect of TEA with that of TBAP in Table II.

A critical concentration of the linear polymer must be determined for satisfactory separation of a given range (size) of the DNA fragments. Though an increase in the polymer concentration enhances resolution of the smaller fragments, increased viscosity can make the operation impractical. A moderate concentration of 0.5% HEC (relative viscosity of 3.82) appears to be appropriate for the separation of both ranges of the DNA fragments from the two organisms used in this study. Nevertheless, no polymer concentration can resolve DNA fragments exhibiting only very small mass differences (such as 281 bp and 271 bp, peaks 6 and 7), at least under our detection system (3-mm flow cell, 13-nl volume).

Schwartz *et al.* [13] have studied the separation of DNA fragments with a polysiloxane-coated capillary and polymeric buffer additives. An addition of EdBr to the buffer solution results in longer migration times of the DNA fragments and better peak resolutions. The authors propose that in addition to the sieving effect, the linear polymer may cause an additional dynamic coating on the capillary surface, and consequently can suppress the electroosmotic flow. The results in Figs. 1 and 2 indeed confirm a loss in the electroosmotic flow when a linear polymer is added to the buffer.

An enhancement in the separation of the pBR322 plasmid fragments was observed with the addition of EdBr to a separation medium containing 0.5% HEC. DNA fragments differing only by a few base pairs can be resolved by the inclusion of EdBr [8]. Guttman and Cooke [7] have also observed enhancement in the separation of DNA fragments, ranging from 20 to 2000 bp by CGE with the addition of EdBr.

Our results in Table III confirm these observations in quantitative terms. The consequences of the two additives, an ion-pairing agent and an intercalating agent are also compared in Table III. These two reagents interact with the DNA fragments by different mechanisms. An anion-pairing agent appears to interact predominantly by hydrophobic interactions with the purine and pyrimidine bases, while EdBr intercalates with G:C base pairs and alters the chain length. EdBr can fill one site for every other base pair, thus increasing the DNA length (fully saturated DNA chain is increased by 50%) [14,15]. This increase in the chain length caused by EdBr appears to be the reason of early emergence of DNA fragments from the capillary column.

Application of different types of detection methods, *i.e.* light paths of 3 mm or 75 μm, surprisingly produced almost identical resolutions of the DNA fragments (Fig. 3b and Fig. 4), but the narrow-bore detector produced higher

efficiency (small peak widths). These results are thus repeatable irrespective of the instrument, origin of capillaries or type of detector.

In conclusion, a critical concentration of the linear polymer must be determined appropriate for the given range of DNA fragments. An ion-pairing reagent improves the resolution of the DNA fragments, but different reagents yield different degrees of enhancement. EdBr as an additive in PS-CZE, also improves resolution of the fragments, but by a different mechanism.

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