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SEPARATION AND DETECTION OF DNA BY CAPILLARY ELECTROPHORESIS

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SUMMARY

The use of capillary electrophoresis for the separation and detection of nucleic acids has been investigated. Lab-model instruments have been built, using commercially available UV absorbance and fluorescence detectors which were modified for use with 50–100 μm I.D. fused-silica capillary tubing. The sensitivity of these instruments (signal-to-noise ratio = 3) was measured as 15 μg/ml for fluorescence detection of ethidium bromide-stained herring-sperm DNA and 3 μg/ml for UV absorbance detection. With the former instruments a variety of strategies has been used to attain rapid separations of bases, oligonucleotides, restriction fragments and whole phage, viral and plasmid DNAs.

INTRODUCTION

Since 1981 there has been a great increase in interest in electrophoresis in fused-silica capillaries of small inner diameter[1–4]. Capillaries of 50–100 μm I.D. have several beneficial properties, among which are rapid dissipation of Joule heat, elimination of thermal and gravitational convection, and a flat electro-endosmotic flow profile. These properties make fused-silica capillaries an ideal medium for electrophoresis in free solution[2,5]. Separations have been demonstrated for proteins, peptides, amino acids, oligonucleotides, nucleosides and several other small organic molecules[2,3,6,7]. These separations have utilized high voltages (up to 30 kV) in order to achieve rapid separation at extremely high resolution. The theoretical limit of the efficiency of capillary electrophoresis (CE) has been estimated to be over two million theoretical plates, and separations have been reported with efficiencies of nearly one million plates[8,9]. Another feature of CE is the great variety of separation strategies that may be used, including zone electrophoresis[2,9], isotachophoresis[10,11], isoelectric focusing[11,12] and micellar electrokinetic capillary chromatography (MECC)[6,7,13]. More recent work has demonstrated the feasibility of gel electrophoresis of proteins in capillaries[14].

We are interested in the separation and detection of nucleic acids by CE. Earlier work has shown that oligonucleotides and nucleic acid constituents (bases, nucleosides and nucleotides) can be efficiently separated in zone or micellar systems[6,13,15].

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However, these systems have thus far failed to separate large DNA molecules. This may be attributed primarily to the fact that nucleic acids over twenty bases in length have essentially the same charge-to-mass ratio. In this paper, we describe separations which we have achieved for nucleic acids and their constituents, ranging from nucleosides to large restriction fragments (up to 23 kb). Also detailed are the techniques we have used for the detection of nucleic acids and the properties of these various optical techniques.

EXPERIMENTAL

Materials

Nucleic acid bases and Hoechst 33258 were purchased from Sigma (St. Louis, MO, U.S.A.). Herring sperm DNA and ethidium bromide were obtained from Serva (Westbury, NY, U.S.A.). All other nucleic acids were supplied by Bethesda Research Labs (Gaithersburg, MD, U.S.A.). Acrylamide and bis-acrylamide were purchased from Bio-Rad (Richmond, CA, U.S.A.). Ammonium persulfate and N,N,N′,N′-tetramethylethylenediamine (TEMED) were supplied by International Biotechnologies (New Haven, CT, U.S.A.). All other reagents were obtained either from Fisher (Pittsburgh, PA, U.S.A.), Sigma, or Serva. Fused-silica capillary tubing was purchased from Polymicro Technologies (Phoenix, AZ, U.S.A.).

For detection either a modified Kratos Spectroflow 757 UV absorbance detector (Applied Biosystems, Foster City, CA, U.S.A.) or a modified HP1046A fluorescence detector (Hewlett-Packard, Palo Alto, CA, U.S.A.) was used. Modification of the detectors involved replacement of the flow cells with capillary holders, which immobilized the capillary in the light beams. For simplicity, these capillary holders were of similar geometry to the original flow cells. The UV detector capillary holder had a 0.50 mm × 0.15 mm slit placed adjacent to the capillary, between it and the photodiode. In the fluorescence detector, the lenses from the original flow cell were placed in the capillary holder, in order to focus the excitation and emission beams. A fused-silica capillary bridged the gap from the high-voltage electrolyte at the injection end to the ground electrolyte at the detector end. At the point where the capillary passed through the detector beam, a small segment of the polyimide coating was burned off and the capillary was cleaned with methanol, which allowed detection directly through the capillary tube. The ground electrolyte reservoir was sealed and connected to a vacuum pump (Model N79MVI, KNF Neuberger, Princeton, NJ, U.S.A.), which was used to purge, fill, and inject the capillaries, except in the polyacrylamide gel experiments, where injection was accomplished by electrophoresis. Power supplies (±30 kV) were obtained from either Hipotronics (Brewster, NY, U.S.A.) or Gamma High-Voltage Research (Mt. Vernon, NY, U.S.A.).

Methods

New capillaries were cleaned by sequentially rinsing for 5–10 min, first with water, then with 0.1 *M* sodium hydroxide, again with water, and finally with the buffer to be used for the subsequent separation. This procedure was also used to recondition capillaries after every 2–4 runs. For gel electrophoresis, tubes were treated with 0.4% (v/v) 3-methacryloxypropyltrimethoxysilane in water, adjusted to pH 3.5 with acetic acid[16].

Polyacrylamide gels were prepared by a standard method[17]. Monomer mix was degassed under vacuum in an ultrasonic bath. Gel mixture was drawn into the capillary under vacuum immediately after the addition of TEMED and ammonium persulfate.

For cetyltrimethylammonium bromide (CTAB)-MECC and gel electrophoresis applications the high voltage (injection end of capillary) was at a negative potential. All other applications utilised high voltage at positive potentials.

Commonly used buffers were TPE (80 m*M* Tris-phosphate, 2 m*M* EDTA, pH 8.0), and a 1:10 dilution of TBE (9 m*M* Tris, 9 m*M* boric acid, 0.2 m*M* EDTA, pH 8.0). All solutions were made from water which had been highly purified by ultrafiltration (Barnstead NANOpure II System, Sybron Corp., Newton, MA, U.S.A.).

UV absorbance detection was carried out at 260 nm. Fluorescence detection was performed at excitation and emission wavelengths of 250 nm and 585 nm, respectively, except as noted.

RESULTS AND DISCUSSION

In general, CE separations have been characterized by high speed and high resolution. Another potential benefit of CE is the ability to obtain real-time quantitative analysis. In order to test this ability for nucleic acid analyses, calibration curves were plotted for the absorbance and fluorescence detectors. Serial dilutions of herring-sperm DNA in TPE were drawn by vacuum through capillaries inserted into the detectors. The absorbance detector displays a linear response over three orders of magnitude, up to 2.0 mg DNA/ml (data not shown). Its sensitivity limit is 3 μg/ml (measured at 3:1 signal-to-noise ratio in a 50-μm I.D. capillary). Since the detection volume of this detector is around 1 nl (based on 0.5 mm slit length and 50 μm I.D.), this limit would correspond to detection of peaks containing as little as 3 pg of DNA. However, since this measurement was made in a static system, it must be considered the idealized limit for this detector. During actual separations, higher noise levels were observed, and this resulted in a somewhat higher detection limit, which was dependent on the particular buffer system being used. This additional noise may be due to electrical impulses transmitted to the detector when the high voltage is applied, or to effects of the buffers. It should be noted also that some buffers may attenuate the response, in particular those buffers which contain additives that significantly alter the refractive index of the aqueous solution or chaotropic agents which affect the structure of nucleic acid, such as urea.

Fig. 1 shows the response of the fluorescence detector to DNA in varying concentrations of ethidium bromide (EB) and also in 20 μg/ml Hoechst 33258 (H33258). The latter is a nuclear stain which is seldom used in electrophoresis, since its emission is at a wavelength which is not as easily seen by the naked eye as that of EB. For EB staining, the greatest sensitivity is obtained at low concentrations of the dye. However, with increasing concentrations of DNA, the dye solution becomes saturated with DNA, and linearity is lost. Thus, for higher DNA concentrations, higher concentrations of EB are required in order to obtain a linear response. This is contrary to the results for gel staining in which a linear response is suggested for low EB concentration over a wide range of DNA concentrations[18]. This may be attributed to the large volume excess of EB solution used in these procedures. In contrast to

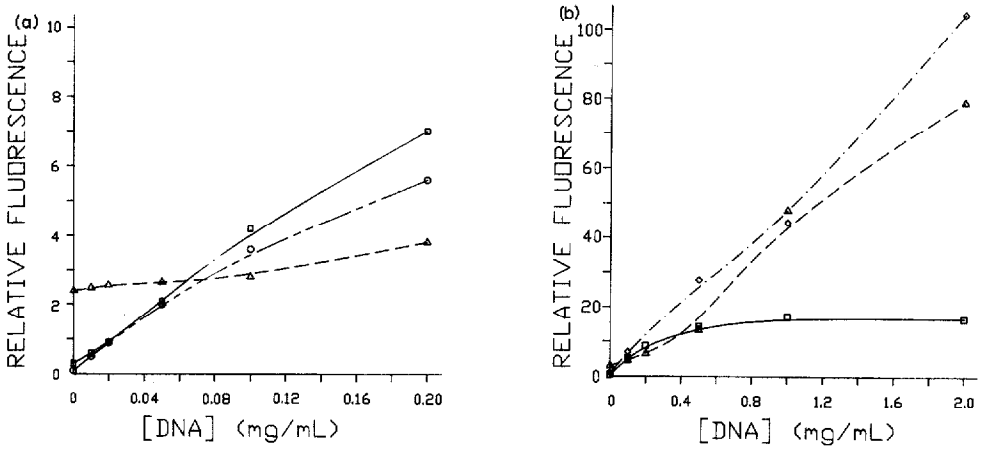

Fig. 1. Response of modified HP1046A fluorescence detector to DNA for various dye concentrations. Serial dilutions of herring-sperm DNA in TPE, with dye, were drawn through the detector under vacuum in a 75-μm-I.D. capillary. (a) Low concentration range. (b) High concentration range. Fluorescence measurements for H33258 stained samples at excitation and emission wavelengths of 232 and 458, respectively. (○) EB 10 μg/ml; (□) EB 20 μg/ml; (△) EB 200 μg/ml; (◇) H33258 20 μg/ml.

EB, staining with H33258 apparently shows a wide linear range, and thus merits further investigation. Sensitivity levels for fluorescence were measured as 15 μg/ml DNA for 10 μg/ml EB or 30 μg/ml DNA in 20 μg/ml H33258. For comparison, sensitivity was also measured for fluorescein as $7 \cdot 10^{-7}$ *M* (260 ng/ml), using broad-band UV excitation and monitoring emission at 515 nm.

These results, in combination with other data we have obtained, suggest that greater sensitivity and linearity of response is obtained by using absorbance detection, whereas fluorescence detection of stained DNA has the benefits of reduced background noise and elimination of certain artifacts (such as peaks caused by protein contaminants). The lower sensitivity of absorbance detection is surprising, considering that fluorescence detection of EB-stained gels is the most popular detection method for DNA gel electrophoresis. However, the popularity of this technique is principally due to its simplicity compared to densitometric analysis. Furthermore, the high absorptivity of nucleic acid polymers is well documented, and is the basis for the most common technique for accurate quantification of dilute DNA samples of manageable volume[18]. On the other hand, the high sensitivity limit obtained for EB staining may be caused by inadequacies in the optics of this design. Future detectors should include tighter focusing of the excitation beam into the detection volumes as well as elimination of scattered light which may contribute to background. It will be interesting to see if this sensitivity level will be significantly improved in upgraded detectors.

A variety of methods has been utilised for the separation of nucleic acids and their constituents, the strategy used being primarily dependent upon the size range being analysed. Fig. 2 shows a MECC separation of nucleic acid bases in a sodium dodecyl sulphate (SDS)-containing buffer. The mechanism of this separation method is well understood, and has been documented elsewhere[6,19,20]. Briefly, the separation

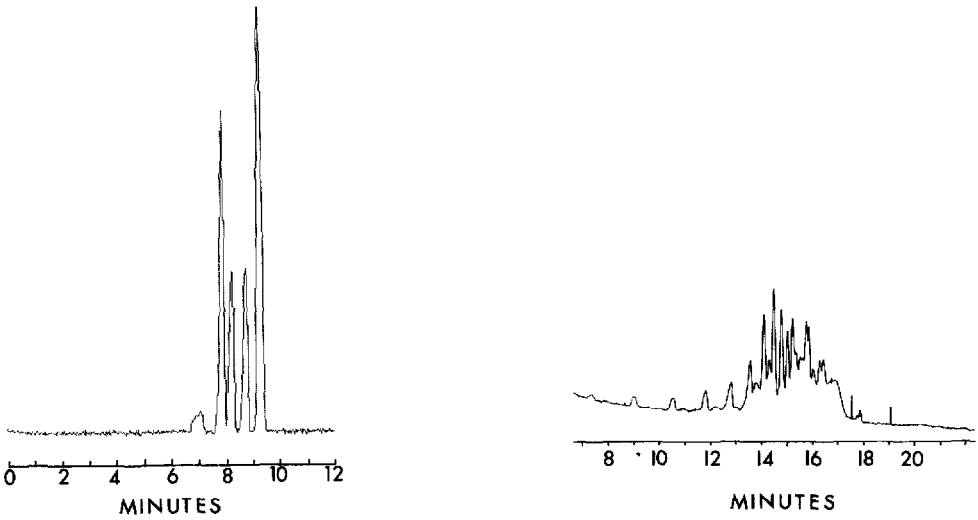

Fig. 2. MECC of nucleic acid bases. Peaks are from left to right: uracil, cytosine, thymine and adenine. Approximately 5 ng each was injected in 5 nl. Electrophoresis at 24 kV, 57 μA, in 25 m*M* $Na_2B_4O_7$–50 m*M* NaH_2PO_4–0.1 *M* SDS (pH 7.0), in a 50 cm × 50 μm I.D. capillary. Detection by UV absorbance.

Fig. 3. Zone electrophoresis of BRL oligo dT ladder. Approximately 12 ng was injected in 50 nl. Electrophoresis at 16 kV, 36 μA, in 50 m*M* phosphate buffer, in a 50 cm × 50 μm I.D. capillary. Detection by UV absorbance.

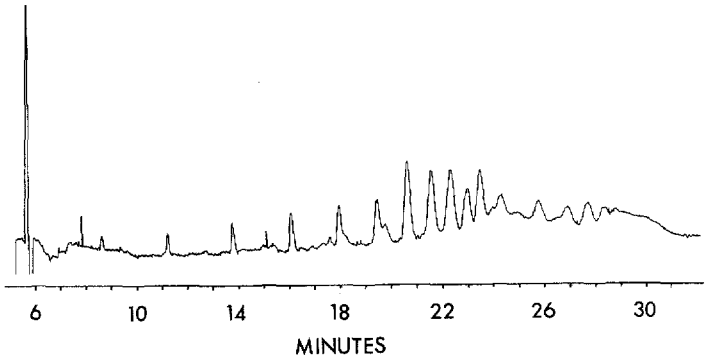

Fig. 4. Same as Fig. 3, except that buffer included 5% ethylene glycol, and electrophoresis conditions were 17 kV and 50 μA.

is based on the ability of the bases to partition into a SDS micelle. Since bases are uncharged at neutral pH, and SDS micelles are negatively charged, bases which partition more efficiently into the micelles (*e.g.*, purines) will be retarded, due to the electrophoretic motion of the micelles against the electroosmotic flow. In addition to bases, MECC has been shown to efficiently separate nucleosides and nucleotide phosphates[6,13], and oligonucleotides[6], as well as basic, acidic and (net) uncharged peptides[21].

Separation of large DNAs (*i.e.*, plasmids, restriction fragments and larger molecules) in capillaries has proven to be a formidable problem. Early conjecture was

that separations of nucleic acids over eight bases long would not be possible without the "sieving effect of a high density gel"[15]. (Unlike size-exclusion chromatography, gel electrophoresis apparently utilizes a sieving mechanism, whereby smaller polymers move more quickly and larger ones are more highly retained.) However, the separation of oligonucleotides by CE with continuous, non-partitioning buffers, as well as more complex systems, has been successfully accomplished. Fig. 3 shows a separation of oligodeoxythymidines ranging in size from 4 to 22 bases. In Fig. 4, the same electropherogram is shown with the addition of 5% ethylene glycol, which increases the viscosity and thus reduces the electroosmotic flow. It can be seen that, while the total elution time is increased, the later-eluted peaks are significantly better resolved. The mechanism of these separations is not yet fully understood, but is likely to involve a combination of mobility and viscous drag. Nevertheless, separations of larger DNAs have been difficult and elusive.

Early attempts in this laboratory have involved the use of simple buffers, such as 10 m*M* Tris-borate. It was felt that the differences in viscous drag could cause sufficient variations in migration velocities of fragments of differing sizes. Fig. 5 shows a separation of SV40 DNA in a 1:10 dilution of TBE. In this figure, two major peaks can clearly be distinguished, as well as a smaller, early-eluted peak and a range of peaks of intermediate migration rate. Although these fractions have not yet been isolated and analysed, it may be conjectured that the three principal peaks correspond to linear, open-circular, and supercoiled forms. It is well known that the tertiary structure of these different forms of DNA causes large differences in the Stokes radius of these molecules, and this results in increasing viscous drag as follows:

$$\text{linear} \geqslant \text{open-circular} > \text{supercoiled}.$$

Since all of these DNAs have the same mass and essentially the same charge, it seems likely that the resultant differences in frictional forces are the cause of the observed separation. (These tertiary structure differences are known to affect migration rates

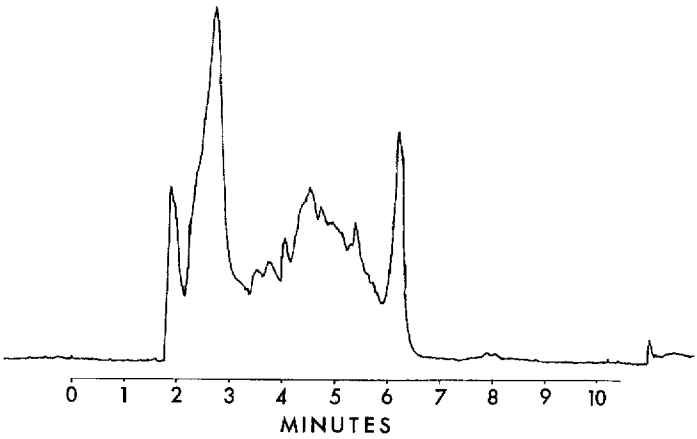

Fig. 5. SV40 RF DNA electrophoresed in a 50 cm × 75 μm I.D. capillary, containing a 1:10 dilution of TBE and 100 μg/ml EB. Electrophoresis at 30 kV and 10 μA. Sample, *ca.* 40 ng of DNA. Detection by fluorescence.

for agarose gel electrophoresis, as well as sedimentation coefficients for ultracentrifugation[22].) The source of the range of intermediate peaks is not known. Attempts at performing the same separation with viscous buffers, such as 5% ethylene glycol or 5% glycerol, or buffers containing high-molecular-weight reagents, such as Ficoll or polyvinylpyrrolidone, have resulted in reduced resolution, and also, in certain instances, the appearance of artifacts, or precipitation, leading to loss of current.

Attempts to separate large linear DNAs by this mechanism have thus far failed, presumably due to the direct proportionality between charge, drag, and mass. In one series of experiments, in a capillary treated with 3-methacryloxypropyltrimethoxysilane, Hind III fragments of λ-DNA were electrophoresed for varying amounts of time, ranging from 1 to 40 min. In this tube, the electroosmotic flow was only slightly greater than the electrophoretic velocity of the DNA, and this resulted in an extremely slow net migration of the sample. After electrophoresis, the sample was drawn past the detector under vacuum. Even after 40 min of electrophoresis, only a single peak was observed (data not shown).

Since these mechanisms had failed to resolve linear DNAs, it was our opinion that the remaining options for these separations were either gel electrophoresis or chromatography based on specific complexation. Presently, gel electrophoresis is the method of choice for separating linear DNAs. Furthermore, gel electrophoresis in capillaries has been demonstrated to be an effective way of obtaining rapid size separations of proteins[14] and oligonucleotides[23]. Fig. 6 shows a separation of the Hae III restriction fragments of φ-X DNA in a polyacrylamide gel. In this electrophe-

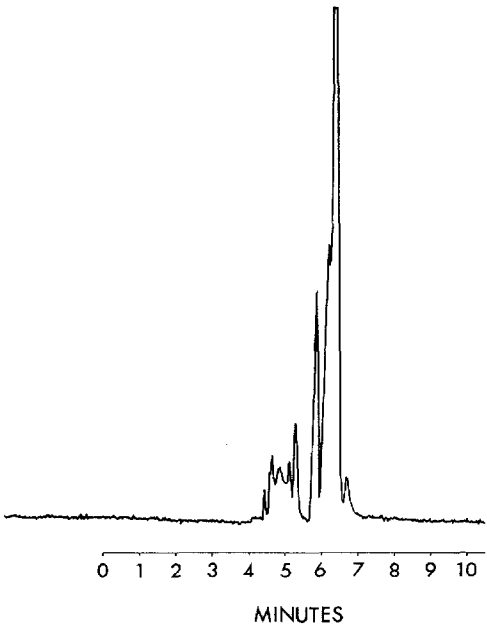

Fig. 6. Hae III fragments of φ-X DNA, electrophoresed in a 3%T, 5%C polyacrylamide gel-filled 20 cm × 100 μm I.D. capillary. Electrophoresis at 10 kV and 10 μA in 0.1 X TBE, containing 10 μg/ml EB. Detection by fluorescence.

rogram, nine different fractions can clearly be distinguished. Elution time is quite rapid as compared to conventional polyacrylamide gel electrophoresis. However, compared to the latter technique, a significant compression of DNA bands is observed. This is probably due to the high voltage applied, and may be a phenomenon similar to the compression of high-molecular-weight DNAs in agarose gels. A possible solution of this problem, which has not yet been investigated, is the use of pulsed field inversion.

Additional problems have also been encountered in these experiments. First, the effective separation range of polyacrylamide gels is around 2000 base pairs, whereas larger molecules require a larger pore matrix, such as agarose, for efficient separation. However, agarose gels have proven to be a poor medium for CE, since they are relatively unstable at high temperature and field strength. Thus, separations in agarose gels have been extremely difficult to reproduce. Additionally, the investigation of separations in acrylamide gels has been hindered by the difficulty of preparing gels of adequate quality, and associated problems with reproducibility from gel to gel. Further experiments in this direction will most likely focus on alternative gel matrices as media for capillary gel electrophoresis.

The final apparent candidate for the separation of linear DNAs is specific complexation. Many cations, including magnesium and tetra-alkylammonium ions have displayed strong interactions with DNA and nucleic acid constituents. Most intriguing has been the success of reversed-phase ion-pair high-performance liquid chromatography (HPLC) in the separation of DNA restriction fragments[24]. In light of the analogies between MECC and reversed-phase (RP)-HPLC, it seems likely that a mechanism similar to ion-pairing chromatography may be utilised to enable the separation of DNA restriction fragments in micellar buffer systems. There are two prime choices for such buffer systems: either a SDS-micelle buffer, containing tetraalkylammonium ions, or a CTAB-micelle buffer, in which CTAB would serve both as micelle and as ion-pairing reagent. Previous work has demonstrated that CTAB at concentrations above $3.5 \cdot 10^{-4}$ *M* effectively reverses the electroosmotic flow[25]. Thus, using a negative potential at the injection end of the capillary, a CTAB-micelle system will behave like a SDS system. We have chosen the CTAB system because of its inherent simplicity compared to a multi-component system. Fig. 7 shows a separation of φ-X DNA Hae III fragments in a phosphate–borate buffer containing 20 m*M* CTAB and 4 *M* urea. Our experience has shown that inclusion of urea enhances the resolution of this separation. This was an expected result, since partial unzipping of the DNA helix will allow hydrophobic interaction between the interior of the micelles and the bases as well as ion pairing between the surface of the micelles and the phosphate backbone.

Several features of the electropherogram in Fig. 7 are notable. First, the separation is very rapid, and is completed in less than 8 min. Second, it is extremely efficient. The number of theoretical plates for this separation was estimated as 1 000 000 plates, on the basis of the formula

$$N = 5.54\,(t/w)^2$$

where t is the elution time and w is the full peak-width measured at half-maximum[1]. Finally, it should be noted that all fractions are eluted in a total elapsed time of less than 1 min. This is a reflection of the minuteness of the differences which are the basis

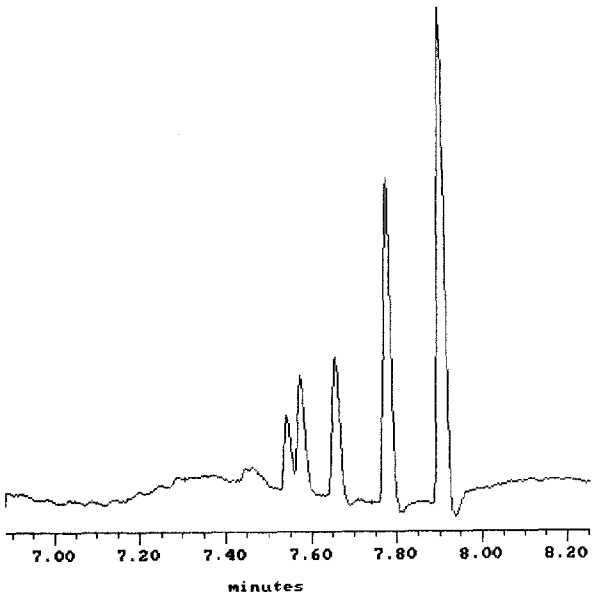

Fig. 7. Hae III fragments of φ-X DNA, electrophoresed in 20 m*M* CTAB–10 m*M* NaH_2PO_4–6 m*M* $Na_2B_4O_7$–4 *M* urea (pH 7.0). Previous to injection, DNA was heated to 95°C for 2 min. Electrophoresis at 15 kV and 12 μA in a 40 cm × 50 μm I.D. capillary. Sample, *ca.* 20 ng in 25 nl. Detection by UV absorbance.

for this separation. Similarly, in ion-pair RP-HPLC of restriction fragments, all fractions are generally eluted within a tight range of acetonitrile percentage[24]. It is interesting to note that in recent experiments, Cohen and Karger have obtained similar high-efficiency separations of Hind III fragments of λ-DNA in a SDS–urea system containing Tris–borate[26]. These separations are especially intriguing and are currently under intensive investigation.

CONCLUSIONS

CE can potentially be used to separate nucleic acids, ranging from small oligonucleotides to linear and circular DNAs of greater than 2000 base pair length. Separations have been demonstrated for these molecules, although in most cases the separation mechanisms are thus far poorly understood. A better understanding of these mechanisms will come with continued investigation in this and other labs. Linearity of absorption and fluorescence detection has been demonstrated for static systems. However, this capability has yet to be shown to yield quantitative results under actual operating conditions. This topic is currently under investigation. Although no work has been done on separations of RNAs in this lab, due to the difficulties involved with the handling of RNA samples, it is assumed that the same techniques should also result in successful separations of oligoribonucleotides and RNA molecules.

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