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# Free solution mobility of DNA molecules containing variable numbers of cationic phosphoramidate internucleoside linkages

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**Abstract**

The free solution electrophoretic mobility of an 118-base pair DNA fragment containing zero, three, six or nine cationic phosphoramidate internucleoside linkages has been measured by capillary electrophoresis. The electrophoretic mobility decreases with the increasing number of cationic phosphoramidate linkages, as expected because of the reduced negative charge on the DNA molecules. The decrease in mobility is approximately linear for DNA molecules containing three and six cationic phosphoramidate linkages, but begins to level off when nine cationic phosphoramidate linkages have been added. The mobility also varies somewhat depending on whether the modified phosphoramidate linkages are located at the 5′- or 3′-end of the DNA molecule. 

*Keywords:* Capillary electrophoresis; DNA; Phosphoramidates

## 1. Introduction

Antisense therapeutic strategies are based on the inhibition of gene expression in a sequence-specific manner using, for example, oligodeoxyribonucleotides (ODNs) which are complementary to specific mRNA sequences. The resulting DNA:RNA hybrids are substrates for ribonuclease H (RNase H), which degrades the RNA portion of the duplex and prevents translation of the targeted mRNA [1]. However, ODNs have a very short half-life in vivo, because of the rapid hydrolysis of their phosphodiester bonds by cellular endo- and exonucleases [2–5]. To increase the resistance of ODNs to cellular nucleases, and thereby increase the effectiveness of the therapeutic reagent, the phosphodiester internucleoside linkage has been modified with phosphorothioate [6], alkylphosphoramidate [1,7], cationic phosphoramidate [8] and N3′→P5′ phosphoramidate [9–11] linkages. Unfortunately, the heteroduplexes formed with ODNs that are completely modified with non-anionic internucleoside linkages are not substrates for RNase H [12–14]. ODNs containing mixed phosphoramidate and phosphodiester linkages, on the other hand, do form heteroduplexes that are

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viable substrates for RNase H [2,7,14], and so are potentially useful as therapeutic agents. DNA molecules with mixed phosphodiester and cationic phosphoramidate linkages are the subject of the present report. Such DNAs are of particular interest because the positively charged phosphoramidate linkages interact electrostatically with their target mRNAs, increasing the stability of the complexes under physiological conditions [8].

Most ODNs that have been investigated to date are relatively short. However, because of the vast excess of nontarget to target sequences in the cell, it is not clear that short ODNs will have sufficient sequence specificity to be biologically useful as antisense reagents [8]. To increase the specificity of the interaction, longer ODNs are currently being synthesized and characterized by gel electrophoresis. However, the gel electrophoretic patterns of ODNs containing cationic phosphoramidate internucleoside linkages are often difficult to interpret, because the mobility depends somewhat on whether the modified residues are located at the 3′-end of the ODN or are split between the two ends [7]. To understand whether this effect is a gel-induced anomaly, or whether the differential mobilities are due to the intrinsic electrostatic properties of the phosphoramidate derivatives, the free solution mobility of an 118-base pair (bp) DNA fragment modified by the incorporation of zero, three, six, or nine cationic phosphoramidate linkages at one or both ends has been determined by capillary electrophoresis (CE). The results indicate that the free solution mobility of DNAs containing cationic phosphoramidate linkages decreases with the increasing number of modified linkages incorporated in the fragment, and also depends on the distribution of cationic phosphoramidate linkages at the 5′- and 3′-ends of the derivatives.

## 2. Experimental

### 2.1. DNA samples

The parent DNA molecule used for these studies was a 118-bp fragment of the human 5,10-methylenetetrahydrofolate reductase (MTHFR) gene, obtained by polymerase chain reaction (PCR) amplification from isolated genomic DNA. The 118-bp fragment (called THF for brevity) contains nearly equal numbers of adenine plus thymine (A+T) and guanine plus cytosine (G+C) residues, nearly equal numbers of purine and pyrimidine residues on each strand, and a single run of four thymine residues (T tract) near the middle of the sequence. Derivatives of the 118-bp fragment containing *N*,*N*-diethylethylenediamine (DEED) linkages were synthesized by using PCR primers in the forward and/or reverse directions that contained the modified linkages. Primers containing cationic phosphoramidate linkages were synthesized using hydrogen phosphonate chemistry and purified by reversed-phase high-performance liquid chromatography (HPLC), as described previously [8]. Unincorporated primers were removed and the samples were concentrated by membrane adsorption (Qiagen). The cationic DEED–phosphoramidate linkage is shown in Fig. 1.

Eight different THF derivatives were studied, with the designations and arrangement of phosphoramidate linkages shown in Table 1. Because DNA polymerase probably does not read through cationic phosphoramidate linkages, the modified THF derivatives are assumed to be double-stranded up to the position of the modified linkages, which are believed to exist as single-stranded dangling ends. The number of double-stranded base pairs in each modified THF derivative is indicated in Table 1, along with the size and arrangement of the cationic dangling ends. The THF derivatives were characterized by electrophoresis in 3.0% agarose gels (Gibco BRL Ultrapure Agarose), cast and run in 40 m*M* Tris–acetate–EDTA (TAE) buffer, pH 8.0 [15]. A photograph of a typical gel is shown in Fig. 2.

Plasmid pUC19 (2686 bp) was used as a mobility

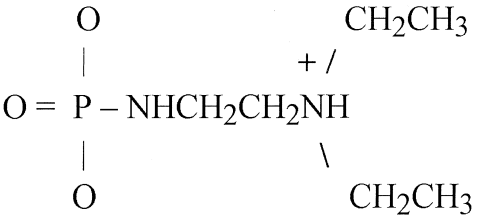

Fig. 1. Schematic diagram of the *N*,*N*-diethylethylenediamine (DEED)–phosphoramidate linkage.

Table 1
The THF derivatives studied with designation and arrangement of phosphoramidate linkages

| DNA sample | No. bp[a] | Modification pattern[b] | Terminal sequences[c] |
|---|---|---|---|
| THF1 | 118 | 0, 0 | TTGAGGCTGACC/ /ATGCACCGACAT<br>AACTCCGACTGG/ /TACGTGGCTGTA |
| THF2 | 115 | 0, 3 | TTGAGGCTGACC/ /ATGCACCGA<br>AACTCCGACTGG/ /TACGTGGCT+G+T+A |
| THF3 | 112 | 0, 6 | TTGAGGCTGACC/ /ATGCAC<br>AACTCCGACTGG/ /TACGTG+G+C+T+G+T+A |
| THF4 | 115 | 3, 0 | T+T+G+AGGCTGACC/ /ATGCACCGACAT<br>TCCGACTGG/ /TACGTGGCTGTA |
| THF5 | 112 | 3, 3 | T+T+G+AGGCTGACC/ /ATGCACCGA<br>TCCGACTGG/ /TACGTGGCT+G+T+A |
| THF6 | 109 | 3, 6 | T+T+G+AGGCTGACC/ /ATGCAC<br>TCCGACTGG/ /TACGTG+G+C+T+G+T+A |
| THF7 | 112 | 6, 0 | T+T+G+A+G+G+CTGACC/ /ATGCACCGACAT<br>GACTGG/ /TACGTGGCTGTA |
| THF8 | 109 | 6, 3 | T+T+G+A+G+G+CTGACC/ /ATGCACCGA<br>GACTGG/ /TACGTGGCT+G+T+A |

[a] Number of base pairs linked by phosphodiester bonds between residues.
[b] Number of cationic phosphoramidate linkages at the 5′- and 3′-ends, respectively.
[c] (+) Represents a cationic phosphoramidate linkage between two adjacent nucleotide residues.

marker. Linearized pUC19 was prepared by digestion with the restriction enzyme *Aat*II according to the manufacturer's instructions. After digestion, the DNA was ethanol precipitated, dissolved in T0.1E buffer (10 m*M* Tris–HCl, pH 8.1, plus 0.1 m*M* EDTA), and stored at −20°C. The linearized product

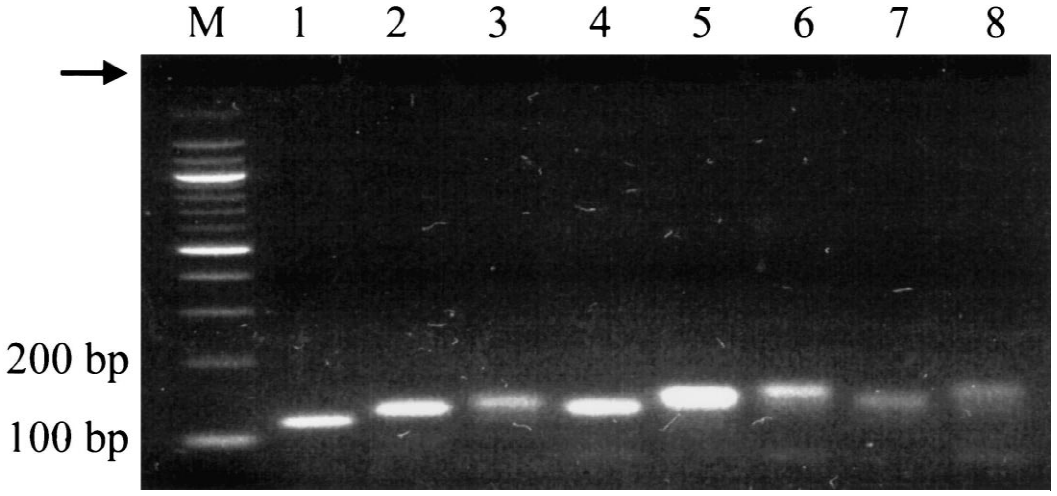


Fig. 2. Electrophoresis of samples THF-1 to THF-8 (lanes 1 to 8, respectively) in 3.0% agarose gels cast and run in TAE buffer. The position of the wells is indicated by the arrow on the left; the sizes of two bands in the marker lane, M, are indicated.

was characterized by electrophoresis in 1.0% agarose gels cast and run in TAE buffer, pH 8.0.

### 2.2. Capillary electrophoresis

Capillary zone electrophoresis was carried out with a Beckman P/ACE System 2100 apparatus (Fullerton, CA, USA), operated in the reverse polarity mode with UV detection at 254 nm. Elution times and peak profiles were analyzed with the Gold software program. The capillary, which had a length of 36.65 cm (29.85 cm to the detector) and an internal diameter of 75 μm, was coated with poly(*N*-acryloylaminopropanol) [16,17] to minimize the electroendoosmotic flow (EOF) of the solvent. The EOF, which was determined by direct measurement with 8 m*M* acrylamide, was found to be $5.8 \cdot 10^{-6}$ $cm^2$ $V^{-1}$ $s^{-1}$ (elution time=7.2 h at $E=200$ V/cm). Hence, the EOF could be neglected when calculating DNA mobilities.

The running buffer used for all experiments was 40 m*M* TAE buffer, pH 8.0 [15]. No sieving liquid polymers were used; the capillary was filled with buffer alone. The DNA samples were injected into the capillary by a 1 s injection at low pressure (0.5 p.s.i., 0.007 MPa), using methods described previously [18]. Between runs, the capillary was rinsed with running buffer for 3 min at high pressure (0.28 MPa, >120 column volumes). The capillaries were flushed with distilled deionized water for 30 min at the end of each day and filled with deionized water overnight.

DNA electrophoretic mobilities, $\mu$, were calculated from Eq. (1):

$$\mu = d/Et \tag{1}$$

where $d$ is the distance to the detector (in cm), $E$ is the electric field strength (in V/cm) and $t$ is the time required for the sample to migrate to the detector (in s). All measurements were carried out at 25.0±0.1°C, using $E=100$ or 150 V/cm. The observed mobilities were independent of electric field strength within this range. The mobilities of the various THF derivatives were measured with and without linear pUC19 DNA in the solution as a mobility marker. Mobility differences, $\Delta\mu$, between the linear pUC19 and the THF derivatives were calculated from Eq. (2):

$$\Delta\mu = \mu_{\mathrm{pUC}} - \mu_{\mathrm{thf}} \tag{2}$$

where $\mu_{\mathrm{thf}}$ is the mobility of the THF derivative and $\mu_{\mathrm{pUC}}$ is the mobility of linear pUC19. The mobility differences observed for the various THF derivatives could usually be determined within ±0.5%. The mobilities of the THF derivatives were then normalized for small day-to-day variations in the condition of the capillary by calculating the average value of the mobility of linear pUC19 observed in different runs on different days, $\langle\mu_{\mathrm{pUC}}\rangle$, and subtracting the measured values of $\Delta\mu$ according to Eq. (3):

$$\mu_{\mathrm{THF}} = \langle\mu_{\mathrm{pUC}}\rangle - \Delta\mu \tag{3}$$

where $\mu_{\mathrm{THF}}$ is the normalized mobility of the particular THF derivative. The average mobility of pUC19 was found to be $(3.769\pm0.030)\cdot10^{-4}$ $cm^2$ $V^{-1}$ $s^{-1}$, constant within ±1% during the entire course of the measurements.

The number of theoretical plates in the separation was estimated from the length of the capillary to the detector, $L$, and the total variance of the zone, $\sigma_{\mathrm{T}}^2$, according to Eq. (4):

$$N = L^2/\sigma_{\mathrm{T}}^2 \tag{4}$$

where:

$$\sigma_{\mathrm{T}}^2 = \frac{1}{8\ln 2}\cdot\left(\frac{w_{\mathrm{h}}L}{t}\right)^2 \tag{5}$$

and $w_{\mathrm{h}}$ is the full peak width at half height and $t$ is the elution time [19].

## 3. Results

The eight THF derivatives studied here were characterized by agarose gel electrophoresis, as shown in Fig. 2. The derivatives were reasonably monodisperse, since a single major peak was observed for each sample. However, faint bands corresponding to low-molecular-mass impurities, possibly primer dimers, can be observed for fragments THF-6 and THF-8 (lanes 6 and 8). The mobilities of the various THF derivatives decreased with the increasing number of cationic phosphoramidate residues, as expected from the decrease in net negative charge of the DNA molecules.

Typical CE electropherograms observed for the

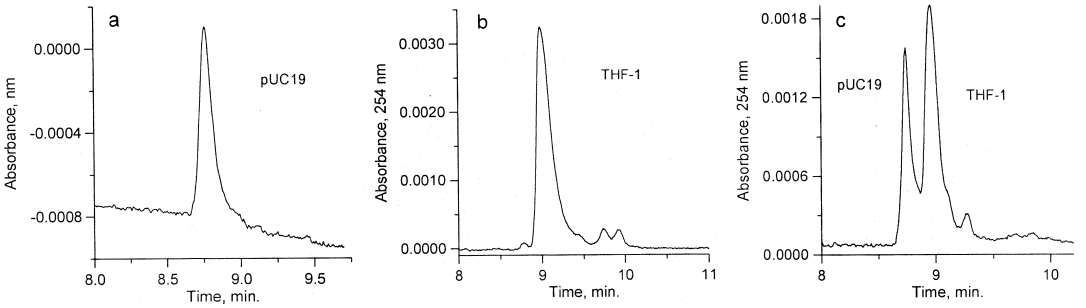


Fig. 3. Elution profiles observed for (a) linear pUC19; (b) the unsubstituted parent fragment THF-1; and (c) the mixture of the two DNAs. In all cases, $E = 150$ V/cm.

mobility marker, linear pUC19, the unsubstituted 118-mer, THF-1, and their mixture are shown in Fig. 3. The two small peaks to the right of the THF-1 major peak (Fig. 3b) probably represent primer dimers, which would be expected to migrate more slowly in free solution than the 118-bp parent fragment [18]. The electropherogram of the mixture of THF-1 and linear pUC19 (Fig. 3c) is the sum of the two individual spectra, indicating that no interactions occurred between the two DNAs during the electrophoretic separation.

Typical electropherograms observed for THF fragments containing three, six and nine cationic phosphoramidate internucleoside residues, with and without linear pUC19, are illustrated in Fig. 4. Although all of the THF derivatives contained some impurities, it can clearly be seen that the mobilities of the various derivatives decreased progressively with the increasing number of positively charged phosphoramidate linkages (Fig. 4a, c and e). Similar results were observed when the solutions contained linear pUC19 as a mobility marker; the mobility differences between linear pUC19 and the THF derivatives gradually increased with the increasing number of cationic phosphoramidate linkages (Fig. 4b, d and f).

The efficiency of the separations illustrated in Figs. 3 and 4 ranged from 15 000–50 000 theoretical plates for the THF derivatives to 64 000–97 000 theoretical plates for linear pUC19. These values are approximately an order of magnitude smaller than observed for alkylphenols [20] and amino acid derivatives [21] in open tube capillaries. However, relatively low electric fields (3.7–5.5 kV) were used in the present study to be sure of good temperature control across the width of the capillary; separation efficiencies are known to decrease with decreasing voltage [19,21].

The mobility differences, $\Delta\mu$, observed for the various THF derivatives and the normalized mobilities calculated from Eq. (3) are compiled in Table 2. Sample THF-3 is not included in this table because the sample was too impure to determine the mobility accurately. The normalized mobilities of the other samples are plotted as a function of the number of cationic phosphoramidate linkages, $N$, in Fig. 5. The free solution mobilities of THF derivatives containing three or six phosphoramidate residues decreased linearly with the number of modified linkages added to the DNA. However, the mobility decrease began to level off when nine cationic phosphoramidate linkages were added. The free solution mobilities of the various THF derivatives also depended on the arrangement of the cationic internucleoside linkages at the 5′- and 3′-ends of the DNA. As a result of these combined effects, the free solution mobility of THF-6, which contained three cationic linkages at the 5′-end and six cationic linkages at the 3′-end, was very similar to the mobility of THF-7, which had six cationic linkages at the 5′-end, even though the two derivatives contained different numbers of cationic phosphoramidate linkages.

The mobilities observed for the various THF derivatives in free solution and in agarose gels are compared in Fig. 6. To compensate for the differences in mobility observed in free solution and in agarose gels, the fractional decrease in mobility, $\theta$, was calculated for each THF derivative from Eq. (6):

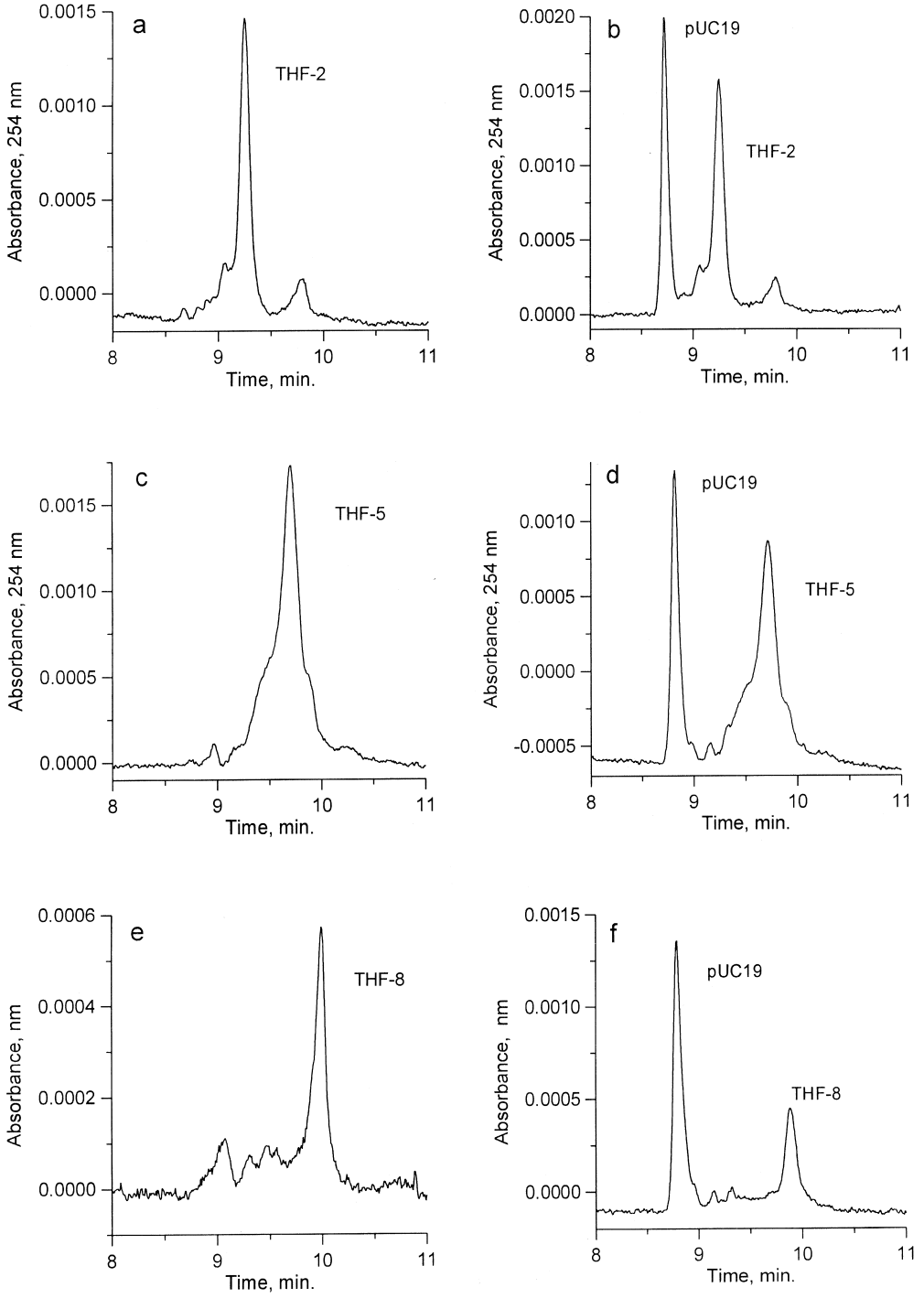


Fig. 4. Comparison of elution profiles observed for phosphoramidate-substituted THF fragments. (a) THF-2 (three cationic linkages); (b) linear pUC19 plus THF-2; (c) THF-3 (six cationic linkages, three at each end); (d) linear pUC19 plus THF-3; (e) THF-8 (nine cationic linkages, six at the 5′-end); (f), linear pUC19 plus THF-8. In all cases, $E = 150$ V/cm.

Table 2
Normalized electrophoretic mobility of THF fragments[a]

| Sample | Modification pattern[b] | $\Delta\mu \cdot 10^4$ (cm$^2$ V$^{-1}$ s$^{-1}$) | $\mu_{THF} \cdot 10^4$ (cm$^2$ V$^{-1}$ s$^{-1}$) |
|---|---|---|---|
| THF1 | 0, 0 | 0.0894±0.0016 | 3.680 |
| THF2 | 0, 3 | 0.218±0.001 | 3.551 |
| THF4 | 3, 0 | 0.215±0.001 | 3.554 |
| THF5 | 3, 3 | 0.348±0.001 | 3.421 |
| THF6 | 3, 6 | 0.394±0.003 | 3.375 |
| THF7 | 6, 0 | 0.382±0.002 | 3.387 |
| THF8 | 6, 3 | 0.419±0.002 | 3.350 |

[a] Normalized by subtracting $\Delta\mu$ from $\langle\mu_{pUC19}\rangle$ according to Eq. (3).
[b] Number of cationic phosphoramidate linkages at the 5′- and 3′-ends, respectively.

$$\theta = [\mu(0) - \mu(x)]/\mu(0) \quad (6)$$

where $\mu(0)$ is the mobility of THF-1, the parent fragment with no phosphoramidate linkages and $\mu(x)$ is the mobility of modified derivatives THF-2 to THF-8. The values of $\theta$ in free solution were calculated from the mobilities in Table 2; the mobilities used to calculate $\theta$ in 3.0% agarose gels were taken from Fig. 2. The fractional decrease in mobility expected for the various THF derivatives was calculated from the reduced net negative charge of each derivative and is indicated by the straight line in Fig. 6.

The fractional decrease in mobility observed for the THF derivatives in free solution was close to the theoretically expected value when three and six cationic phosphoramidate linkages were added to the DNA, as shown by the close juxtaposition of the circles in Fig. 6 with the theoretical line. A smaller fractional decrease in mobility was observed for THF

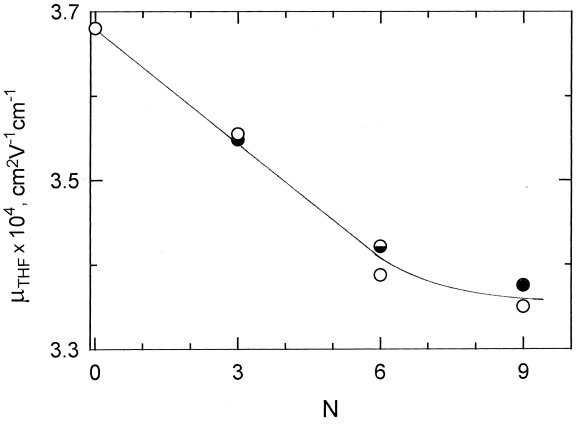


Fig. 5. Dependence of the normalized mobility of the THF fragments, $\mu_{THF}$, on the number of cationic phosphoramidate linkages in the molecule, *N*. The normalized values of $\mu_{THF}$ were calculated from Eq. (3). The open symbols correspond to derivatives with more cationic linkages at the 5′-end; the closed symbols correspond to derivatives with more cationic linkages at the 3′-end; the half-filled symbol corresponds to the derivative with an equal number of positive charges at each end. The drawn curve is meant to guide the eye.

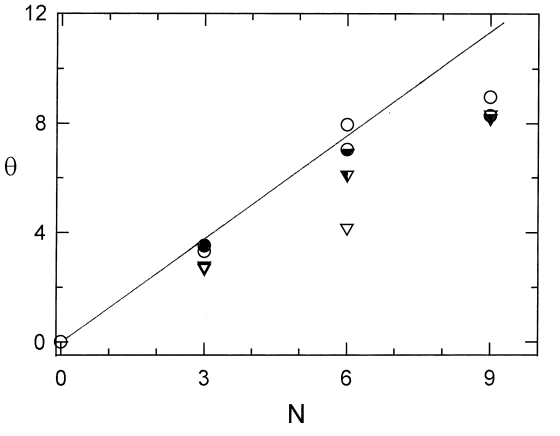


Fig. 6. Dependence of the fractional decrease in mobility, $\theta$, on the number of cationic phosphoramidate linkages in the molecule, *N*, observed in free solution (○) and in 3% agarose gels (▽). Values of $\theta$ were calculated from Eq. (6). The open symbols corresponds to derivatives more cationic linkages at the 5′-end; the closed symbols correspond to derivatives with more cationic linkages at the 3′-end; the half-filled symbols corresponds to the derivative with an equal number of positive charges at each end. The solid line corresponds to the fractional decrease in mobility expected from the reduced negative charge of the various derivatives.

derivatives containing nine cationic linkages, in agreement with the results illustrated in Fig. 5. In agarose gels, the fractional decrease in mobility of THF derivatives containing three and nine cationic linkages was very similar to that observed in free solution (compare the circles and upside-down triangles in Fig. 6). However, the fractional decrease in mobility of THF derivatives containing six phosphoramidate linkages was significantly larger in free solution than in 3.0% agarose gels. Moreover, in free solution, THF-7, with six cationic linkages at the 5′-end of the molecule, migrated more slowly than THF-5, which contained three cationic linkages at each end. The opposite behavior was observed in agarose gels: THF-5, the symmetrically substituted derivative, migrated faster than THF-7, which had all the cationic linkages at the 5′-end.

## 4. Discussion

The free solution mobility of a series of THF derivatives containing zero, three, six, or nine cationic phosphoramidate internucleoside linkages has been investigated, with and without linear pUC19 added to the solution as a mobility marker. Since the electropherograms of the mixtures were always the sum of the individual spectra, the two DNAs did not interact during the electrophoretic separation.

The free solution electrophoretic mobility of the various THF derivatives decreased with the increasing number of cationic phosphoramidate linkages in each molecule, as shown in Figs. 3–5 and Table 2. Since large DNA molecules are free draining in electrophoresis, the free solution mobility is determined primarily by the charge/mass ratio [18,22,23]. The mass of each of the THF derivatives was approximately constant, since each derivative contained 109–118 bp linked by negatively charged phosphodiester bonds and 9–0 unpaired bases linked by cationic phosphoramidate residues. However, the net charge of each of the THF derivatives was strongly dependent on the number of modified linkages, because two negatively charged phosphodiester bonds were lost and one positively charged phosphoramidate residue gained by the addition of each modified internucleoside linkage.

The fractional decrease in mobility expected from the decrease in net negative charge is indicated by the straight line in Fig. 6. In free solution, the expected fractional decrease in mobility is observed for THF derivatives containing three and six cationic phosphoramidate linkages. However, the fractional decrease in mobility is smaller than expected for THF derivatives containing nine cationic linkages (three at one end and six at the other). It is possible that the conformation of DNA molecules containing nine cationic phosphoramidate linkages differs from that of derivatives with fewer modified linkages, decreasing the fractional mobility decrement. Alternatively, the decrease in net charge of the phosphoramidate derivatives may be less than 3 charge units per substitution (loss of two negatively charged residues and gain of one positive residue) if neighboring phosphoramidate residues interact electrostatically with each other, reducing their effective p$K_a$ values. If this type of interaction were to occur, the p$K_a$ values of the phosphoramidate residues would become progressively more acidic with each substitution, resulting in a smaller positive contribution to the net charge. However, this explanation seems unlikely, since THF-2, with three positive charges at the 3′-end, and THF-7, with six positive charges at the 5′-end, migrated with the expected fractional mobility decrements (Fig. 6). However, THF-8, with six positive charges at the 5′-end and three positive charges at the 3′-end (i.e., the combined cationic phosphoramidate linkages of THF-2 and THF-7), migrated with a fractional mobility decrement that was smaller than expected (Fig. 6). Further studies with more highly substituted THF derivatives are needed to determine why the fractional mobilities of DNA fragments with cationic phosphoramidate linkages at the 3′- and 5′-ends appear to approach a plateau at high degrees of modification.

Fig. 6 also compares the fractional decrease in mobility observed for the THF derivatives in free solution (circles) and in agarose gels (upside-down triangles). Equivalent fractional decreases in mobility are observed in free solution and agarose gels for derivatives containing three and nine cationic phosphoramidate residues. Hence, agarose gels serve as a good reporter of the free solution mobility of these derivatives, as expected because DNA mobilities in agarose gels are independent of sequence [24,25]. However, significant differences between the frac-

tional mobility decrements are observed for THF derivatives containing six cationic residues. Not only are the fractional decreases in mobility higher in free solution than in 3.0% agarose gels (Fig. 6), but the fractional mobility decrements depend on the arrangement of the modified linkages in the THF derivatives. In free solution, THF-7, which has six cationic phosphoramidates at the 5′-end of the molecule (open circle), migrates faster than THF-5, which has three cationic linkages at each end (half-filled circle). The opposite behavior is observed in agarose gels: THF-5 (half-filled upside-down triangle) migrates faster than THF-7 (open upside-down triangle). The mobility differences observed for THF-7 and THF-5 in free solution may reflect sequence-dependent differences in counterion condensation at the 5′- and 3′-ends of the DNA molecules and/or the lack of sequence symmetry at the two ends of the parent DNA fragment. The opposite behavior observed in agarose gels suggests that interactions of the positively charged dangling ends of the THF derivatives with the negatively charged agarose gel fibers [26] may affect the observed mobilities. Further studies will be needed to differentiate between these possibilities.

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