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Mobility and activation energy of single-stranded DNA in denaturing cross-linked polyacrylamide slab gels

Zahia Djouadi[a,b,*], Samuele Bottani[a,c], Marie-Alix Duval[a,b], Rainer Siebert[a], Hervé Tricoire[a,d], Luc Valentin[a,e]

[a]*Groupe Interfaces Physique Biologie, Institut de Physique Nucléaire, 91406 Orsay Cedex, France*
[b]*Université d'Evry Val d'Essonne, Boulevard François Mitterrand, 91025 Evry Cedex, France*
[c]*Université Denis Diderot Paris 7, LPTHE, 2 Place Jussieu, 75251 Paris Cedex, France*
[d]*Université Denis Diderot Paris 7, IJM, 2 Place Jussieu, 75251 Paris Cedex, France*
[e]*Université Denis Diderot Paris 7, GMPIB, 2 Place Jussieu, 75251 Paris Cedex, France*

Abstract

An original apparatus based on laser-induced fluorescence detection is presented. One lane migration combined to four equidistant detection points allows the study of the dynamics of DNA bands during electrophoresis. We focus this article on the study of the mobility of DNA sequencing fragments as a function of temperature; mobility is determined in 4% T, 5% C and 4.3% T, 5% C cross-linked polyacrylamide gels at an electric field of 45 V/cm [T=(g acrylamide+g *N,N′*-methylenebisacrylamide)/100 ml solution; C=g *N,N′*-methylenebisacrylamide/% T]. Activation energy has been investigated under these experimental conditions with a temperature varying from 25 to 50°C. The activation energy for migration through the cross-linked polyacrylamide gel decreases with fragment length under our experimental conditions and it varies along the migration.

Keywords: Polyacrylamide gels; Electrophoretic mobility; Activation energy; DNA

1. Introduction

The Genome Project, which aims to read the whole DNA base sequence of human and other organisms has led to the investigation of new electrophoretic separation techniques in order to improve the experiment's speed and throughput. In fact, since the development of methods for rapid DNA sequence analysis in the mid-1970s [1,2] and the synthesis of fluorescent oligonucleotides, the feasibility of automation has been obtained [3–7].

The principal advantages of these methods are the use of fluorescent labels instead of radioactive ones and the possibility to load the sequencing reactions for the four bases in each single lane of migration. In the typical sequencing roots, the detection is performed by scanning the end of the gel and the reading of the sequence information is ensured by base-calling process improving therefore the throughput of DNA sequencing data. In the last decade the DNA separation speeds have been increased with the introduction of the capillary gel electrophoresis (CGE) techniques [8–10]. The high surface-to-volume ratio of the capillaries facilitates heat transfer thus permitting much larger electric fields to be employed for fast separations.

However, despite the great amount of effort devoted to optimize the performances of separation,

*Corresponding author. Groupe Interfaces Physique Biologie, Institut de Physique Nucléaire, 91406 Orsay Cedex, France.
E-mail address: djouadi@ipno.in2p3.fr (Z. Djouadi).

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band broadening still hinders the DNA electrophoresis reading lengths.

The principal limitations of DNA sequencing by electrophoresis depends on both the saturating mobility of DNA fragments [11] and DNA band broadening. If the main factors contributing to this effect have been identified as well as in slab gels and capillary electrophoresis [12], their relative contributions are still not well understood and the first relevant published results for slab gels [13,14] gave contradictory results. In order to study the different contributions to the band broadening we have developed an apparatus dedicated to the study of the evolution of band width during migration. The mechanisms of migration of macromolecules in porous media can also be studied dynamically with this detection system. The aim of this paper is to present some surprising results showing that sample velocities are not constant during migration. This is observed under all used experimental conditions (temperature, gel thickness and concentration). Furthermore in terms of activation energy the temperature dependency of mobility shows different behaviours from those reported previously by Lu et al. [15] in cross-linked polyacrylamide gels in capillary electrophoresis. On the other hand, our results are similar to those found in denaturing non-cross-linked polyacrylamide gels [16]. More recently, activation energy has been investigated in ultra-thin-layer agarose slab gel electrophoresis for the double-stranded (ds) DNA molecules [17] and in composite agarose–linear polymer matrices [18]. While activation energy increases with increasing dsDNA fragments in agarose gels [17] and in agarose–HEC (hydroxyethylcellulose) or agarose–PEO (polyethylene oxide) composite matrices [18], it decreases with increasing fragment lengths in agarose–LPA (linear polyacrylamide) composite matrix [18]. The same group has also studied the activation energy of proteins in composite matrices [19].

2. Experimental

2.1. Instrumentation and experiments

The primary consideration in the development of the instrument was the multi-distance-detection possibility of the same band at different stages of migration. Therefore we have built an apparatus based on LIF (laser-induced fluorescence detection) with four 111-mm-spaced detection points. The two glass plates (66 cm×23 cm×3 mm) are temperature regulated. The focused laser ensures a longitudinal resolution of 25 μm. A complete description of the instrument will be given in a forthcoming article.

Before each experiment a pre-run of 2 h is performed at the chosen temperature for the run and a voltage of 3000 V (45 V/cm) establishing a constant electric current. During the pre-run the current falls to about 50% from its initial value. Fig. 1 shows typical electropherograms obtained at each detector position. In this case, the run was performed with an electric field of 45 V/cm and a temperature of 45°C. These plots represent the amplitude of the signal of fluorescence emitted by the fluorescein labelled fragments versus migration time in the four detectors located at 21.1, 32.2, 43.3 and 54.4 cm from the sample injection point. Notice the improvement of the band resolution with the increasing migration distance clearly seen for instance on the first two peaks corresponding to the doublet 147–148. As can be seen from these plots, the peaks emerge from a baseline which is due to some composite fluorescence light, like the glass plates and the gel contributions ones. Each peak can be observed in the four different positions in the gel, retention time and peak width can be easily extracted if the peaks emerge properly from this baseline.

2.2. Chemicals

A T base terminating M13mp18 is synthesized in a thermocycling machine (Progene Tech). The single-stranded (ss) M13mp18+ template (200 μg/ml) and the fluorescent labelled –40 Primer (3 pmol/μl) are from Pharmacia. The Amersham Life Sciences kit containing deoxynucleotide triphosphates, dideoxynucleotide triphosphatase, Thermosequenase DNA polymerase is used.

Separations are performed in polyacrylamide slab gels 4% or 4.3% T, 5% C, close to conventional sequencing gel concentrations [T=(g acrylamide+g *N,N′*-methylenebisacrylamide)/100 ml solution; C= g *N,N′*-methylenebisacrylamide/% T]. Acrylamide, 8 *M* urea, TBE buffer, pH 8.3 (100 m*M* Tris, 90 m*M*

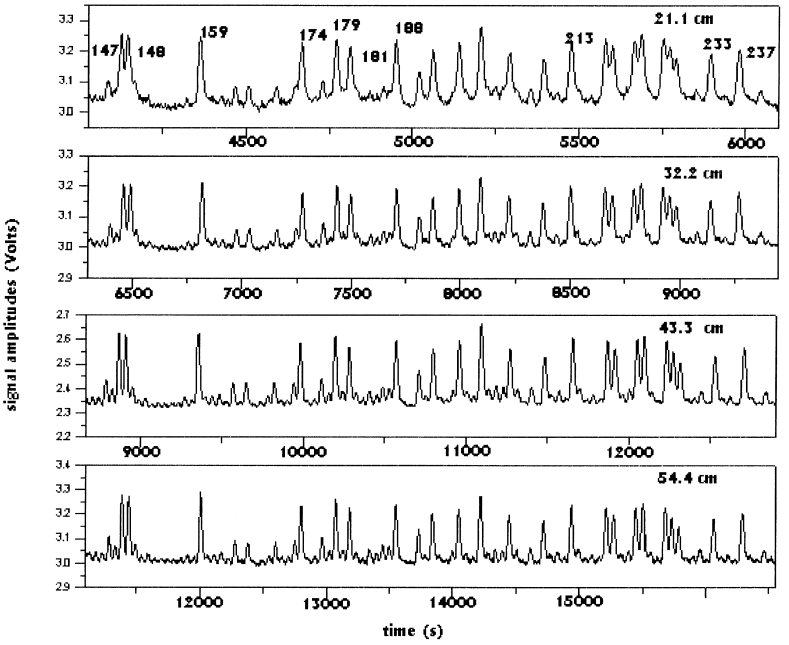

Fig. 1. Example of spectra obtained with a migrating voltage of 45 V/cm and a temperature of 45°C at each detection position located at 21.1, 32.2, 43.3 and 54.4 cm from the sample injection point. Horizontal time axes are scaled to show the same fragment region from 147 to 237 bases.

boric acid and 1.0 m*M* EDTA), *N*,*N*,*N*′,*N*′-tetramethylethylenediame (TEMED) and APS (ammonium persulfate) were purchased from Gibco BRL.

3. Data analysis and discussion

The first application of our instrument shows original results on the mobility of given bands at the four different detection points as well as the temperature dependence studied in terms of activation energy. For this study we have chosen only the isolated peaks in the range of 53 to 720 bases. After identifying each peak, we extract retention time corresponding to the position of its maximum.

3.1. Determination of the instantaneous velocities and study of the mobility

The measured migration times of various fragments at the different detectors are first used to determine the band velocity. Knowing the distance d between two consecutive detectors ($d=111$ mm), one can easily determine the average migration velocity between the detection points i and j by the relation:

$$\langle V_{ij}\rangle = \frac{d}{t_j - t_i} \tag{1}$$

where $i=1$, 2 or 3 and $j=i+1$.

The average velocity $\langle V_{ij}\rangle$ can also be written as:

$$\langle V_{ij}\rangle = \frac{1}{t_j - t_i}\int_{ti}^{tj} V(t)\mathrm{d}t \tag{2}$$

where $V(t)$ is assumed to be a polynomial function and is determined from the three equations obtained by Eq. (1). After determining the velocity at each detector position, one can calculate the electrophoretic mobility for each fragment by the relation:

$$\mu = \frac{V}{E} \tag{3}$$

Fig. 2 shows the mobility versus the inverse of the fragment length for the longest fragments (204 to 617 bases) where the linearity is ensured (correlation coefficient $r^2 > 0.999$). The mobility of shorter frag-

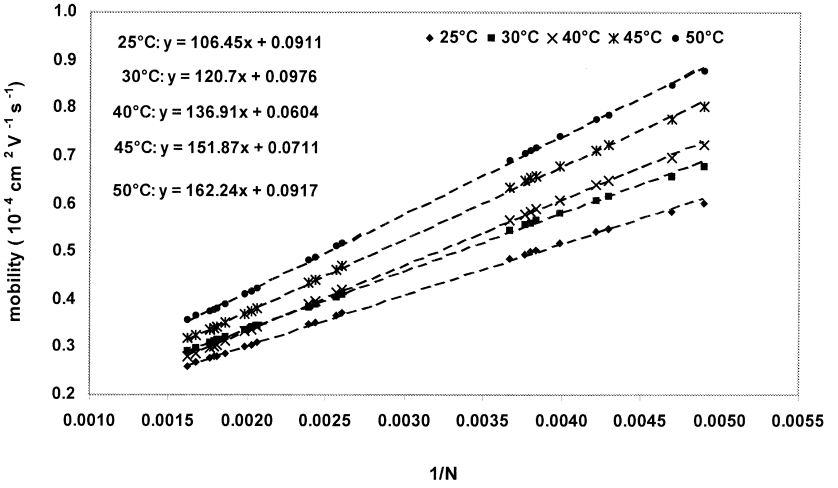

Fig. 2. Mobility at 54.4 cm of migration versus the inverse of fragment length for an electric field of 45 V/cm. The gel temperature varies from 25 to 50°C. Dashed lines give the linear fits of our raw data (symbols). Linear equations are given for each studied temperature.

ments is well described by the Ogston model (results not shown) while for the plotted fragment sizes the $1/N$ dependency of the mobility in the reptation regime of the biased reptation model is well verified. Our data are in good agreement with the biased reptation model which describes the mobility μ of longer fragments by the relationship:

$$\mu = \chi\left[\frac{1}{N} + \frac{1}{N^*}\right] \quad (4)$$

where χ is a constant related to the free solution mobility of DNA, N is the fragment length given in base number, and N^* is a constant providing a measure of the onset of biased reptation. N^* can be regarded as the limit in length of fragments that can be resolved in a given run since fragments much longer than N^* will co-migrate with similar mobility. Fig. 3 shows N^* versus temperature of migration determined from Eq. (4) and data of Fig. 2. While biased reptation model predicts a quadratic increase of N^* with temperature [20], we found that it increases dramatically between 35 and 40°C, reaches a maximum for a temperature around 40°C and then decreases as temperature increases. This behaviour has been obtained for the two gel thicknesses. Moreover N^* for 200 μm thick gel is greater than N^* for 270 μm thick gel for all the studied temperatures. This is due to a better dissipation of the heat created inside the gel. From these two observations we deduce that a longer reading is reached at a temperature around 40°C and a gel thickness of 200 μm.

Unexpectedly our different measurements gave non-constant velocities during migration. Fig. 4 shows different velocity profiles observed during experiments with 200 and 270 μm thick gels and a voltage of 3000 V, the variable parameter being the temperature. As can be seen the variations can reach 20%. Referring to a previous study which revealed sample velocity variations in the first and last centimeters of the gel due to a gel edge effect [21], we performed pre-runs with inverted polarity, but the variations of the velocity still remained. More recently [22] the same group explained the velocity gradient observed in the edges of the gel, by the existence of field gradients mostly due to the difference in ion transference numbers at the gel–buffer interface, which should be caused by the high viscosity of the urea solution contained in the gel. Here we point out the fact that the velocity differences observed with our apparatus are along the whole migration lane in the gel and not only on the edges. Some preliminary experiments done with shorter run times (6 h instead of 12 or 14 h) and with permanent buffer renewal exclude effects of ionic depletion between the buffer tanks [23]. The different profiles observed could be caused by irregularities in the polymerisation of the gel which

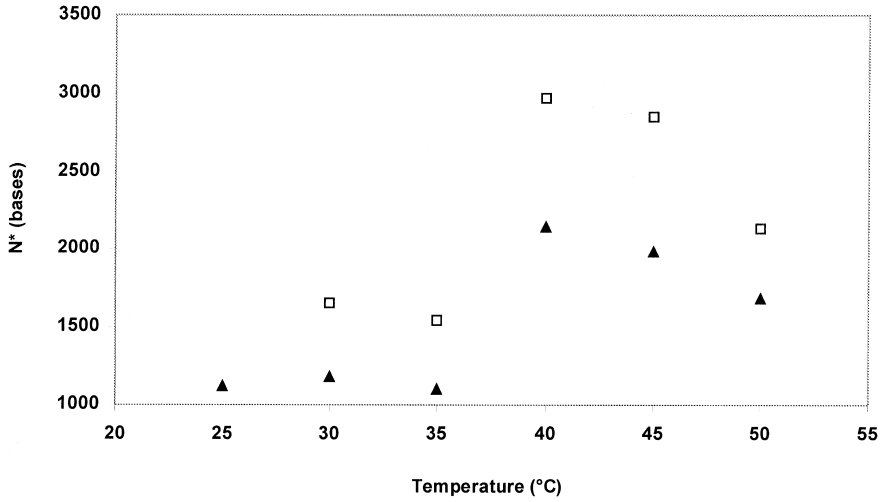

Fig. 3. *N** versus temperature for the two different gels: 4.3% T, 5% C, 270 μm thickness (triangles) and 4% T, 5% C, 200 μm thickness (open squares).

are difficult to control and to reveal. We point out that the experiments were performed with conventional gel and sample preparation practice as well as conventional DNA sequencing conditions. The highlighted mobilities variations must also exist in common sequencing applications.

Fig. 5 shows an example of the velocity at 54.4 cm for each fragment length determined (i) the standard way as the mean over the migration distance and (ii) as an estimate of the instantaneous velocity around the detection position. The relative differences between the two curves show that quan-

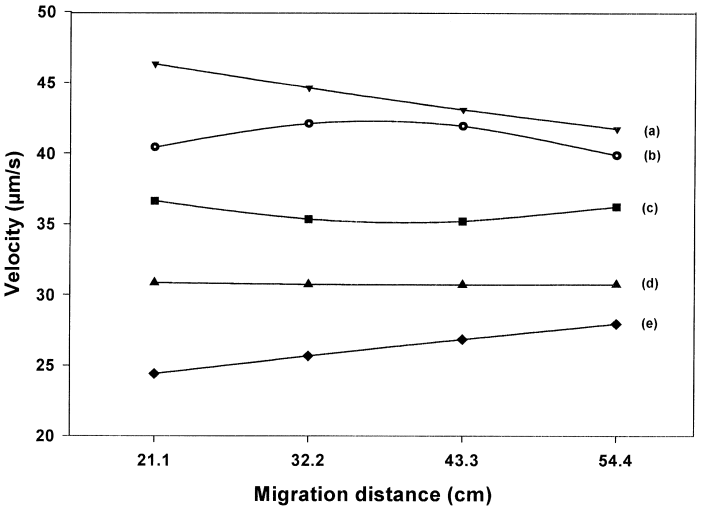

Fig. 4. Examples of the velocity profiles found under our different experimental conditions. The separations were performed at an electric field of 45 V/cm. These plots are obtained for the 237 fragment length. For a given run the same velocity behaviour is obtained for each fragment length. (a) 4% T, 5% C, 200 μm thickness and 50°C. (b) 4.3% T, 5% C, 200 μm and 45°C. (c) 4% T, 5% C, 200 μm and 35°C. (d) 4.3% T, 5% C, 270 μm and 45°C. (e) 4.3% T, 5% C, 270 μm and 35°C.

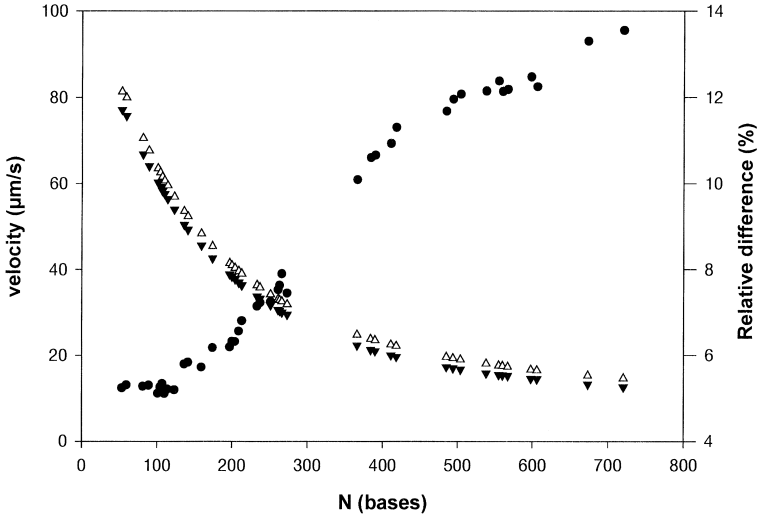

Fig. 5. Comparison between the velocities at 54.4 cm for each fragment length determined the standard way as the mean over the migration distance (opened triangles up) and as an estimate of the instantaneous velocity around the detection position (full triangles down). The full circles represent the relative difference between the two determinations. The temperature of migration is 40°C, the gel is 4% T, 5% C and 200 μm thick.

titative deductions based on the mean velocity must be considered with caution.

3.2. Activation energy

In the study of the influence of the temperature on the migration we have to take into account the previously discussed mobility variations. The temperature dependency of the mobility may be expressed in terms of activation energy E_a for a DNA fragment to pass through a gel pore:

$$\ln \mu = \ln\left(\frac{Q}{\xi}\right) - \frac{E_a}{RT} \quad (5)$$

This relation is determined from the Arrhenius

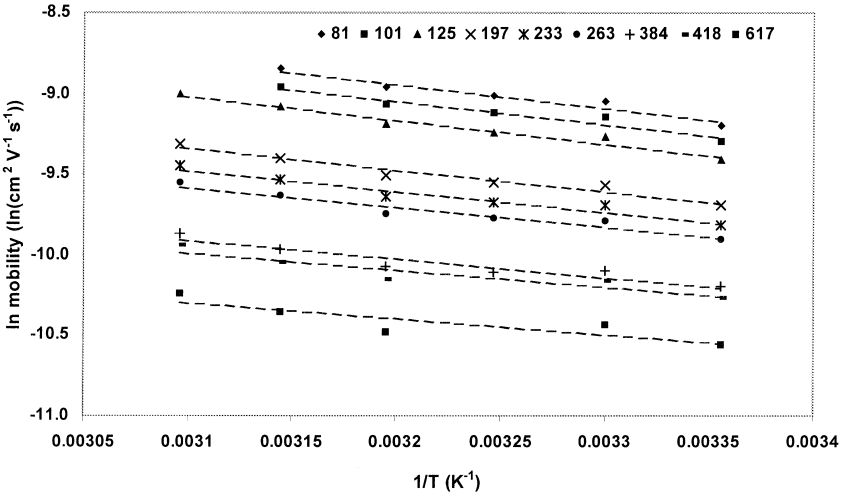

Fig. 6. Logarithm of mobility at 54.4 cm of migration versus the inverse of absolute temperature for some fragments indicated.

equation [15,16]. Q is the charge on the DNA fragment, ξ is a constant, R is the universal gas constant and T the absolute temperature. E_a can be regarded as the energy required by the fragment to overcome the obstacles created by the polymer separating network. Since the mobility is not constant in the gel it is clear from Eq. (5) that it is not possible to assign a single value for E_a along migration. In Fig. 6 we report the variation of the logarithm of mobility versus the inverse of absolute temperature at the migration distance of 53 cm for some fragments. It can be seen that linearity is found ($0.76<r^2<0.95$) suggesting a good agreement with Eq. (5). This latter behaviour has been obtained at each detection position (curves not shown). We have determined the activation energy for each detection position as proposed by Eq. (5) from the slopes of the fitting lines of these curves. Fig. 7 gives the activation energy versus fragment length for different distances of migration. While the magnitude of E_a is

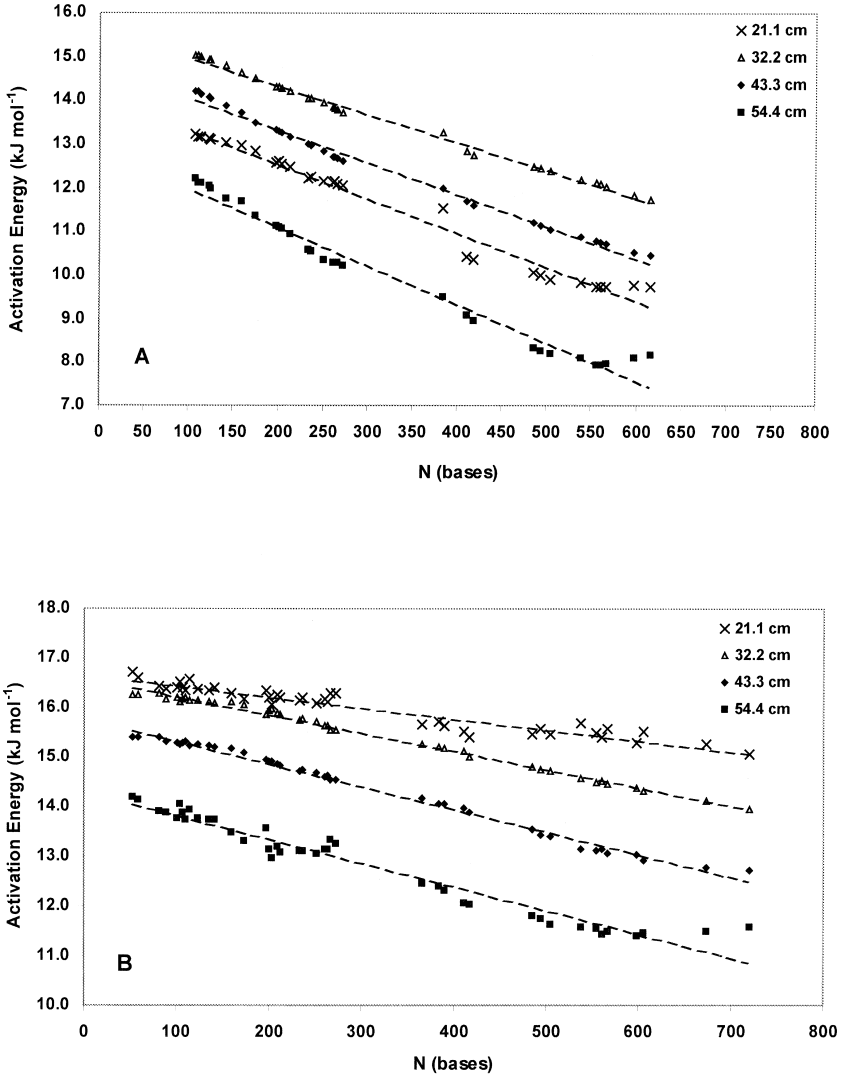

Fig. 7. Activation energy against fragment length determined for the four different migration distances. (A) 4.3% T, 5% C, 270 μm thick gel. (B) 4% T, 5% C, 200 μm thick gel.

similar to the activation energy reported by Lu et al. [15] in capillaries and cross-linked polyacrylamide gels the behaviour of our data with fragment length is completely different. Notice that they performed their separations in 4% T LongRanger gels at an electric field of 300 V/cm.

On the other hand similarly to Fang et al. [16], who worked with 5% T, 0% C non-cross-linked polyacrylamide gels in capillaries at an electric field of 150 V/cm, we found a decrease of E_a with fragment length. It means that the small fragment lengths require more energy to pass through the obstacles formed by our polymer network and the larger ones have less difficulty to pass. This could be due to the difference in conformation of the fragments, small fragments migrate in a random coil structure they require more energy to pass through a given pore than longer fragments which squeeze through it in an elongated configuration.

Finally we present in Fig. 8 the activation energy at 54.4 cm determined with the standard mean mobility and with the instantaneous mobility estimated with our instrument. Although the global variation is the same, the absolute values are up to 18% different. As expected from the discussion on velocity, this reflects the caution necessary when dealing with mobility values.

4. Conclusion

We report the first results obtained with a new detector built for band enlargement studies of different ssDNA fragment lengths in cross-linked polyacrylamide gels. We observe a non-constancy of the velocity in the gel, confirming that it is meaningless to treat with mean velocities or mobilities. Consequently we find unique results about activation energy: not only does it decrease with increasing fragment length but it depends on the migration distance, leading us to suggest that E_a is perhaps a not so well defined observable.

Because of its four detection points our apparatus offers the unique feature to characterise the history of migration over a whole run. In a forthcoming article we will exploit this for the study of band broadening during migration.

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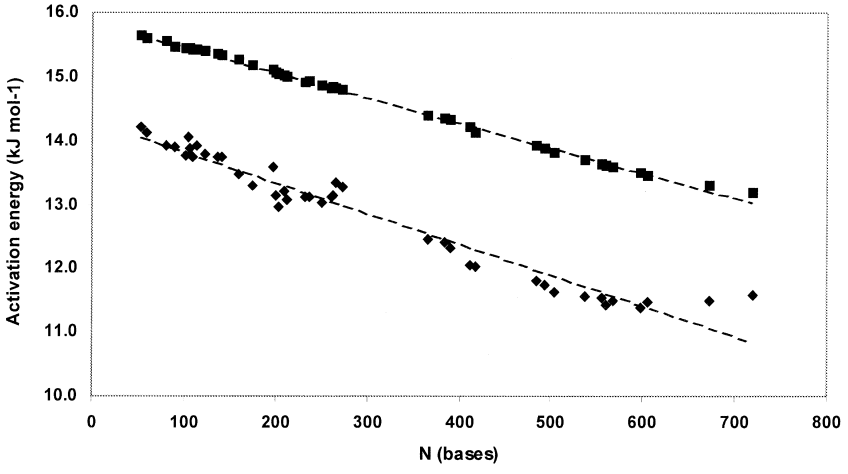

Fig. 8. Activation energy against fragment length using the velocity (and thus mobility) determined the standard way (squares) and our way (diamonds).

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