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CAPILLARY ZONE ELECTROPHORESIS OF OLIGONUCLEOTIDES

FACTORS AFFECTING SEPARATION

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SUMMARY

The influence of various parameters affecting separation of oligonucleotides by capillary zone electrophoresis has been examined. The effects of pH, ionic strength, and various additives, including highly charged cations such as spermine, were studied. Using polycytidines as model compounds, it was demonstrated that pH in the range of 5–8 and ionic strength in the range of 20–200 mmol/l do not influence the separation of oligonucleotides substantially. However, with the addition of spermine to the background electrolyte, migration order was inverted as the effective mobilities of the larger oligonucleotides were greatly decreased. With the addition of spermine and sodium dodecyl sulfate, the separation of these model oligonucleotides was also significantly affected. The best separation of a homologous series of polycytidines was obtained with a background electrolyte containing 60 mmol/l histidine, 30 mmol/l glutamic acid, 50 mmol/l sodium dodecyl sulfate and 3 mmol/l spermine.

INTRODUCTION

Fast, efficient separations of small quantities of oligonucleotides are highly important in different fields of biology. Attention has been focused on the separation of oligonucleotides by migration in an electric field since the beginnings of capillary isotachophoresis[1–5]. A substantial advantage of this method has been the concentration of individual substances into their isotachophoretic zones by the self-sharpening effect, which allows efficient separations even in cases when samples contain larger amounts of major components.

Because large nucleic acids show only small differences in effective mobilities in free solution, their electrophoretic separation is best performed in a size-exclusion medium. Polyacrylamide[6] or agarose[7] gels have been traditionally most popular, and

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thus, gel electrophoresis has now become the classical method for the separation of nucleic acids[8–10]. However, due to recent advances in capillary zone electrophoresis[11–14], it is reasonable to extend this latter technique to the separation of oligonucleotides and DNA restriction fragments[15–17].

In general terms, electrophoretic separation is based on differences in the effective mobilities between separated compounds. Effective mobilities can thus be used to define resolution in capillary zone electrophoresis[18]. There are several ways of influencing effective mobilities of various solutes in electrophoresis: pH changes[19], complex-forming equilibria[20], ion association[21], introduction of a micellar pseudostationary phase[22], interaction with non-charged compounds such as crown-ethers or cyclodextrins[23] and "sieving" effects[24] have been most frequently used.

The present study has been aimed at developing an improved understanding of the various factors which may be deemed important in the separation of oligonucleotides. It is also demonstrated how the optimization of these factors can improve practical separations.

EXPERIMENTAL

All reagents were purchased from Sigma (St. Louis, MO, U.S.A.) with the exception of the cytidine homopolymers, which were obtained from Amoco Technology Company. Solutions of the background electrolytes were purified through a 0.2-μm filter.

Capillaries were prepared from fused-silica tubing, 50 μm I.D., from Polymicro Technologies (Phoenix, AZ, U.S.A.). Depending on the type of experiment, column lengths between 50 and 80 cm, corresponding to separation lengths between 30 and 60 cm, were used. In experiments where the background electrolyte did not contain a detergent, capillaries were coated with 3% linear polyacrylamide after silanization with γ-methacryloxypropyltrimethoxysilane, according to the procedure by Hjertén[25].

A system similar to that reported by Jorgenson and Lukacs[11] was constructed. The high-voltage power supply (0–30 kV) was purchased from Spellman High-Voltage Electronics Corporation (Plainview, NY, U.S.A.), while UV detection at 254 nm was accomplished with a Jasco Uvidec-100-IV (Tokyo, Japan) detector utilizing an on-column flow cell prepared from fused-silica tubing.

RESULTS AND DISCUSSION

Effect of pH on nucleoside phosphate separations

Optimization of pH with respect to the background electrolyte represents the most frequent way of finding suitable separation conditions with electromigration methods. From the data published on isotachophoresis of the mono-, di- and triphosphates of adenosine, guanosine, cytidine and uridine[1,2], it follows that the optimum pH for their separation is approximately 4. To ensure the highest buffering capacity of the background electrolyte, it is reasonable to use a co-ion and counter-ion which have close pK_a. Shown in Fig. 1 is an electropherogram of the nucleoside phosphates with a background electrolyte consisting of γ-aminobutyric acid (pK_a = 4.0) and glutamic acid (pK_a = 4.2). In this case, the separation was performed in a capillary

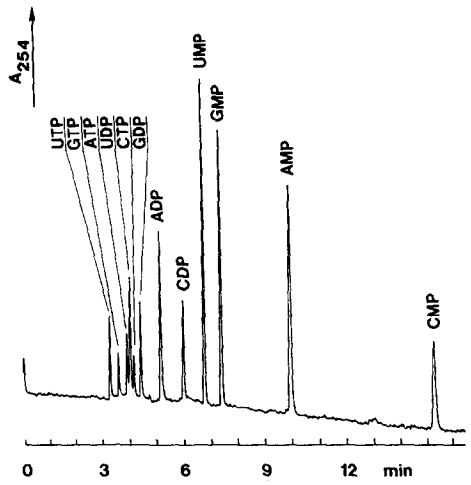

Fig. 1. Separation of nucleoside phosphates by capillary electrophoresis. Background electrolyte: 40 mmol/l glutamic acid/γ-aminobutyric acid. Capillary: 50 μm I.D., coated with 3% linear polyacrylamide: total length, 60 cm; separation length, 40 cm. Voltage: 27 kV. Current: 13 μA.

coated with linear polyacrylamide. The migration order of the individual nucleotides agrees with results obtained by isotachophoresis[1,2]: triphosphates migrate faster than diphosphates, which migrate faster than monophosphates. Furthermore, in all three structural groups, the migration order of a given derivative is: uridyl-, guanidyl-, adenosyl- and cytidylphosphate. The one exception, uridyldiphosphate, is, however, in agreement with published data[1,2,26], and occurs because cytidylphosphates have somewhat higher pK_a values than the other structural groups.

Effect of pH and ionic strength on polycytidine separations

Polycytidine oligonucleotides (monomer–decamer, marked in the figures as 1–10) were chosen as a model mixture for the study of migration behavior. At pH values below 7, the electroosmotic flow was reduced in untreated capillaries to the extent of overcoming electromigration of shorter oligomers which migrated, to the cathode, while the longer nucleotides migrated to the anode. It was found necessary, then, to eliminate the electroosmotic flow by coating the capillary with linear polyacrylamide. This also negated effects caused by a low reproducibility of electroosmotic flow (determined from the migration time of uncharged compounds such as phenol or acetone), which fluctuated in the range of 2.0–4.5% when the capillary was alternately rinsed with 1 *M* NaOH and water for 1-min intervals each. Migration times of the cytidine oligonucleotides are shown in Table I. In comparison with the untreated capillary, reproducibility was improved substantially with the use of a coated capillary.

The effect of pH on the separation of longer oligonucleotides is shown in Fig. 2. Shorter oligomers are not plotted because they are separated easily. As a criterion of the separation ability, "selectivity", p, as defined by Giddings[18] was used. Resolution velocity, *i.e.* resolution generated per unit time (R/t), could not be used because incomplete separation of the higher oligonucleotides precludes peak width determina-

TABLE I

REPRODUCIBILITY OF RETENTION TIMES OF MODEL OLIGOCYTIDINES (n = 6)

Background electrolyte: 60 mmol/l histidine, 30 mmol/l glutamic acid. For additional experimental conditions, see Fig. 2.

Compound	*Mean value of retention time (s)*	*Standard deviation (s)*	*Relative standard deviation (%)*
Decamer	403.8	1.06	0.264
Nonamer	408.4	1.17	0.286
Octamer	414.6	1.37	0.330
Heptamer	422.5	1.26	0.298
Hexamer	433.8	1.07	0.246
Pentamer	452.3	1.11	0.244
Tetramer	485.8	1.34	0.277
Trimer	568.7	1.89	0.332

tion. As shown in Fig. 2, in the pH range of 5.1–8.1, the separation of oligomers was not substantially influenced. The general approach of using a pH at which the components are only partially ionized was unsuccessful, since at pH 3.5 only lower nucleotides migrated through the detector, while the higher ones were probably precipitated. Reverse migration is not likely, because, at the given pH, these oligonucleotides do not possess a positive charge. At pH 5 the octamer, nonamer and decamer migrated together as one unresolved peak. At pH 8.1, reproducibility of the migration times was reduced, while the magnitudes of individual oligomer migration times in subsequent runs were increased as a result of an increased electroosmotic flow. This appears to be the result of cleaving the bonded silane from silica. For the above reasons, pH 6 was chosen for these separations.

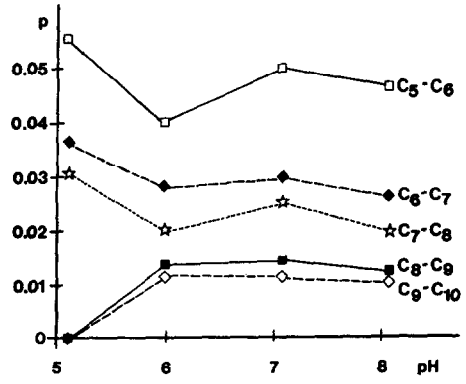

Fig. 2. Separation of polycytidines: influence of background electrolyte pH on selectivity, p. Capillary: 50 μm I.D., coated with linear polyacrylamide, total length, 50 cm; separation length, 35 cm. Voltage: 15 kV. Background electrolyte: 80 mmol/l glutamic acid + 160 mmol/l counter-ion (urotropine at pH 5.1, histidine at pH 6.0, imidazole at pH 7.1, Tris at pH 8.1).

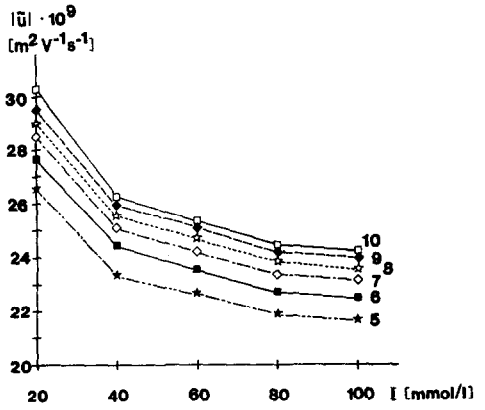

Fig. 3. Effect of background electrolyte ionic strength (I) on the absolute values of effective mobility ($\bar{u}$) of polycytidines. Background electrolyte: 2 × I mmol/l histidine, I mmol/l glutamic acid. Other conditions as in Fig. 2.

The effect of ionic strength was then investigated using a simple buffer lacking any additives in the background electrolyte. As expected, the effective mobilities of the oligomers were reduced as the ionic strength of the background electrolyte was increased (Fig. 3). However, within the range of experimental error, the selectivity of the separation was not dependent on the ionic strength (Fig. 4).

Effect of buffer additives on polycytidine separations

The effect of certain buffer additives was also examined. A possible utilization of complex-forming equilibria was considered, since these have been successfully used in isotachophoresis[20]. However, when the background electrolyte known to be useful in the separation of polythymidines[15] was used, poorly shaped peaks appeared in the electrophorerogram.

Because of the large charge on longer oligonucleotides, more emphasis was placed on interionic interactions with the use of highly charged cations. Diethylami-

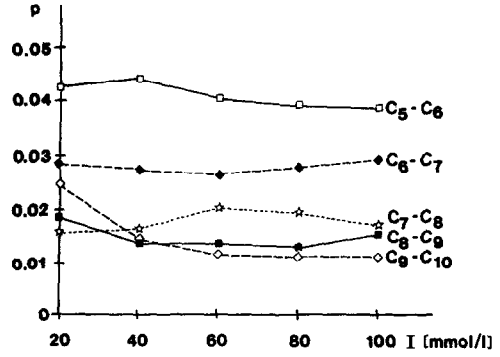

Fig. 4. Influence of background electrolyte ionic strength on selectivity of polycytidines. Background electrolyte: 2 × I mmol/l histidine, I mmol/l glutamic acid. Other conditions as in Fig. 2.

noethyl-dextran (DEAE-Dextran) initially appeared promising when shorter oligomers were separated; however, it proved unsuitable for the separation of longer oligomers because of excessive ionic attraction. Chain lengths of the hexamer and longer polycytidines either precipitated or comigrated in a complex with DEAE-Dextran as a cation.

Spermine, whose migration behavior has been extensively studied by isotachophoresis[21], was then selected as a candidate for an appropriate additive to the background electrolyte. When completely ionized, at pH 6 and below, the spermine molecule carries a charge of +4. Additionally, spermine is known to associate with native DNA to neutralize its tremendous negative charge. The influence of spermine on the effective mobilities of oligonucleotides is shown in Fig. 5. In this experiment, 200 mmol/l histidine/morpholinethanesulfonic acid (MES) was used as the buffer. From this, it can be seen that the order of effective mobilities was inverted near a spermine concentration of 1 mmol/l. The mobility values are then further reduced with increasing concentrations of spermine, although the effect appears to attenuate. It should be noted that these mobility changes are also dependent on the total concentration of the buffer (counter-ion). When a lower concentration of buffer is used, the observed mobility inversion occurs with lower concentrations of spermine. It thus seems that the reduction of mobilities is dependent more on the concentration ratio of spermine to the counter-ion than on the absolute concentration of spermine itself.

The separations of model oligonucleotides with and without spermine in the background electrolyte are compared in Fig. 6a and b. Reversal of the migration order and improvement in separation are obvious. Moreover, when a simple buffer lacking spermine was used as the background electrolyte, as in Fig. 6b, two unidentified, irreproducible peaks frequently appeared just prior to the pentamer peak.

Effect of sodium dodecyl sulfate on polycytidine separations

Separation of oligonucleotides in the presence of a detergent was investigated next. With the most popular anionic detergent, sodium dodecyl sulfate (SDS), no substantial changes in separation were observed in the concentration range of 20–60

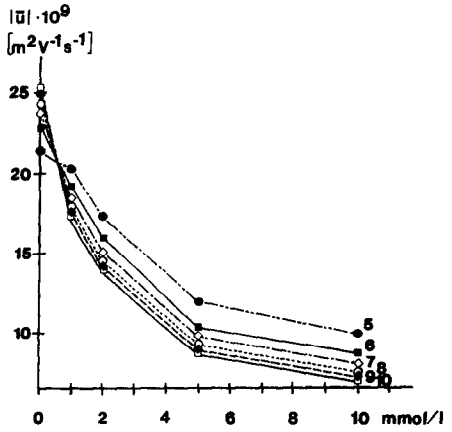

Fig. 5. Effect of spermine addition to the background electrolyte on the effective mobilities of polycytidines. Background electrolyte: 200 mmol/l histidine/MES. Other conditions as in Fig. 2.

mmol/l SDS. Arbitrarily, then, a 50 mmol/l SDS solution was used in the remaining experiments. The presence of SDS, however, did ensure a sufficient level of electroosmotic flow so that subsequent experiments could be performed in uncoated capillaries. The use of uncoated capillaries is advantageous, since the difficult-to-separate

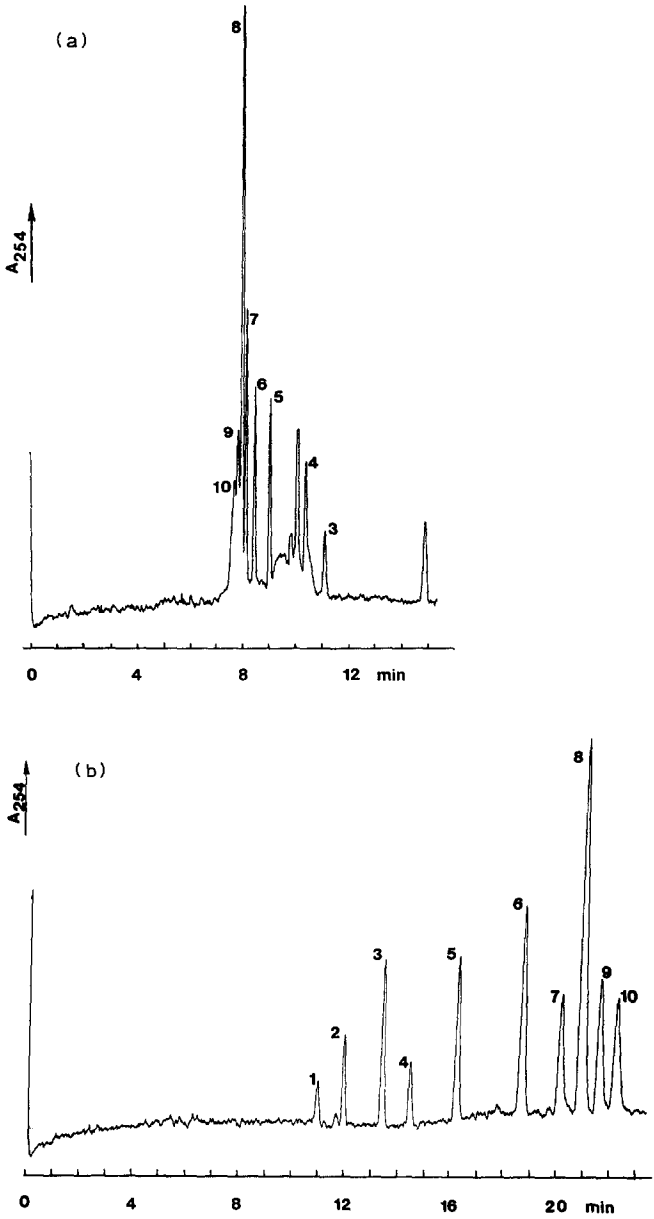

Fig. 6. Separation of polycytidines in the presence/absence of spermine. Background electrolyte: (a) 200 mmol/l histidine/MES, (b) 200 mmol/l histidine/MES–5 mmol/l spermine tetrahydrochloride. Other conditions as in Fig. 2.

higher oligonucleotides enter the detector last, whereas the easily separable lower oligomers appear at the beginning of the electropherogram.

The separation of polycytidines when SDS was added to the background electrolyte, pH 8.1, is shown in Fig. 7a, while the influence of spermine and SDS addition to the background electrolyte is shown in Fig. 7b. The migration order is not reversed as it was in the absence of SDS (Fig. 6a). Clearly, the spermine addition improved the separation, especially for the nonamer–decamer pair which was only partially separated in the absence of spermine. A nearly identical effect was observed at pH 6 (Fig. 8). At pH 4.5, when the charge of oligonucleotides is reduced so that they may be expected to enter micelles more easily, no separation was achieved, and poorly shaped peaks, comparable to those in Figure 6a, appeared in the electropherogram.

The influence of spermine concentration in the presence of SDS on separation of the individual oligonucleotides may be quantitatively expressed as the resolution velocity (resolution generated per unit time) *versus* spermine concentration (Fig. 9). In this case, the optimum concentration of spermine was found to be 3 mmol/l. When the concentration of spermine was increased, the retention times of neutral species were also increased as the result of a reduced electroosmotic flow (reduced zeta potential). However, the retention time of Sudan III, which was used to estimate the migration velocity of micelles[22], was also surprisingly increased with an increasing concentration of spermine, so that the mobilities of dodecyl sulfate micelles (more exactly, their absolute values) were increased, as well. Note that here, the calculation of parti-

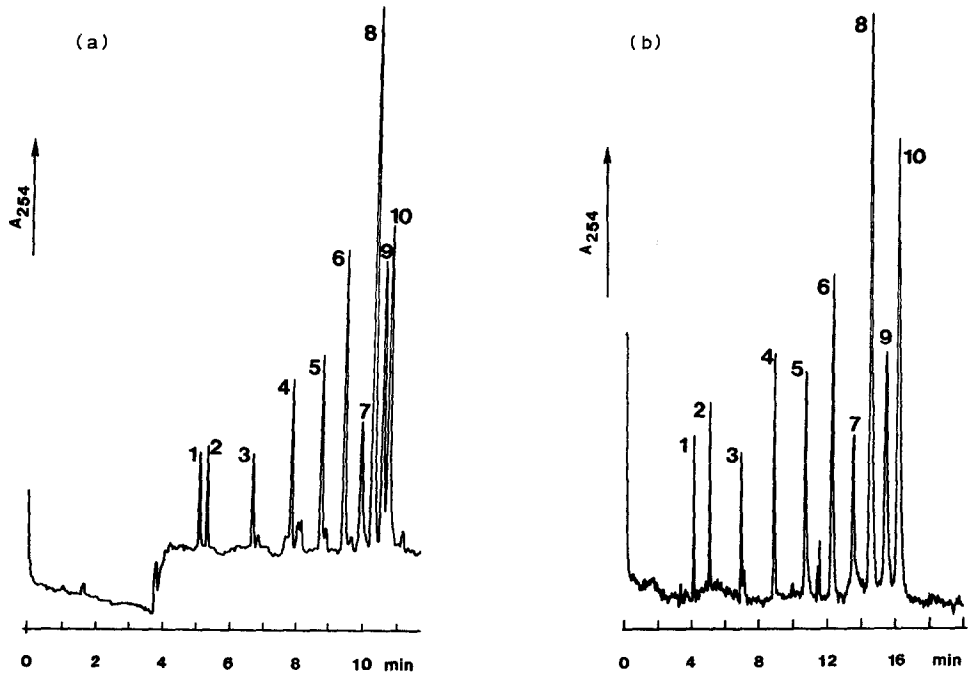

Fig. 7. Separation of polycytidines in the presence of SDS at pH 8.1. Background electrolyte: (a) 60 mmol/l Tris, 30 mmol/l glutamic acid, 50 mmol/l SDS; (b) as (a), with the addition of 3 mmol/l spermine. Capillary: 50 μm I.D., uncoated; total length, 60 cm; separation length, 45 cm. Voltage: 25 kV.

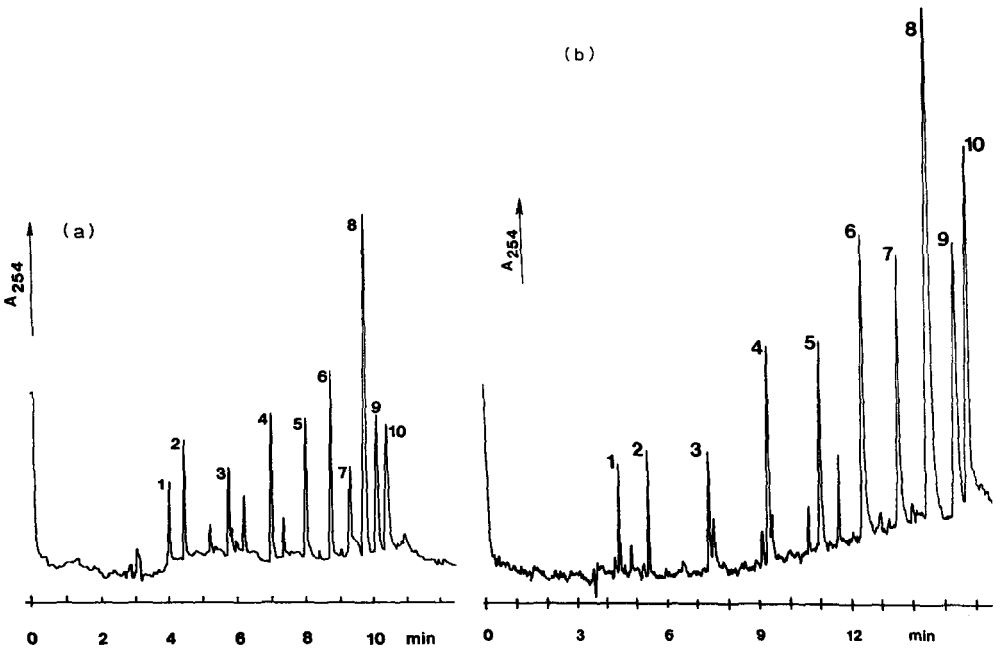

Fig. 8. Separation of polycytidines in the presence of SDS at pH 6. Background electrolyte: (a) 60 mmol/l histidine, 30 mmol/l glutamic acid, 50 mmol/l SDS; (b) as (a), with the addition of 3 mmol/l spermine. Other conditions as in Fig. 7.

tion coefficients is not particularly useful, because the migration times are affected by interactions between the spermine molecules and oligonucleotides in the aqueous phase.

Finally, size discrimination in gel structures is yet another principle which could be utilized to improve the separation of oligonucleotides with capillary electrophore-

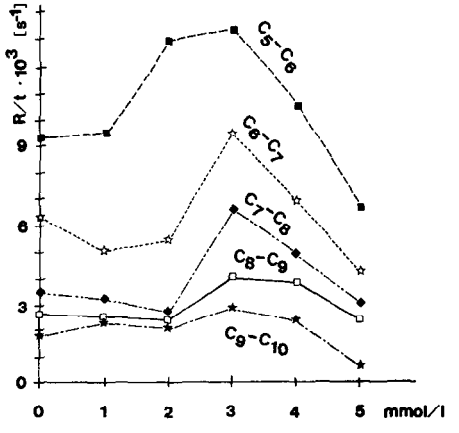

Fig. 9. Effect of spermine addition on the resolution velocity for polycytidines. Background electrolyte: 60 mmol/l histidine, 30 mmol/l glutamic acid, 50 mmol/l SDS. Other conditions as in Fig. 8.

sis, especially in the case of higher homologues. Our preliminary results in this area show that this approach is worth further exploration.

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