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APPLICATION OF A WEAKLY BASIC DIMETHYLAMINO-MODIFIED SILICA ION EXCHANGER TO THE SEPARATION OF OLIGONUCLEOTIDES

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SUMMARY

LiChrosorb RP-8, RP-18 and Diol as well as a newly synthesized basic dimethylamino-modified silica ion-exchanger (DMA-silica) were applied for the separation of adenylic acid, cytidylic acid and uridylic acid oligoribonucleotides. On LiChrosorb RP-8 and RP-18, respectively, in aqueous buffered eluents (K_2HPO_4–H_3PO_4), the retention of oligonucleotides was increased with decreasing number of nucleotide units in the solute, *i.e.*, with increasing hydrophobic character. The elution behaviour of oligonucleotides on LiChrosorb Diol followed the same order but took place according to a size-exclusion mechanism. The retention of oligonucleotides on DMA-silica is assumed to be based on electrostatic interactions between the charged solutes and the ionic surface sites of DMA. This is evidenced by (i) the exponential decrease of k' of the solute with increasing salt concentration of buffer, and (ii) the increase of log k' of the solute with the number of nucleotide units, *i.e.*, negative charges in the oligoribonucleotide solute. The relative contribution of ionic and molecular interactions to total retention of the solutes is discussed.

INTRODUCTION

Considerable interest has centred on the separation and analysis of mono-, oligo- and polynucleotides since these are the components of deoxyribonucleic acids (DNAs) and ribonucleic acids (RNAs)[1] respectively. The separation of mononucleotides by high-performance liquid chromatography on reversed phase as well as on ion-exchange packings was reported in a series of papers[2–11]. Oligonucleotides are usually chromatographed on alkylammonium ion-exchangers based on polysaccharides, but this is very time-consuming[12–18]. The analysis time could be drastically reduced on columns packed with pellicular weak anion exchangers and a concentration gradient [19,20], *e.g.*, uridylic acid oligonucleotides up to the heptadeca compound were eluted within 70 min[20].

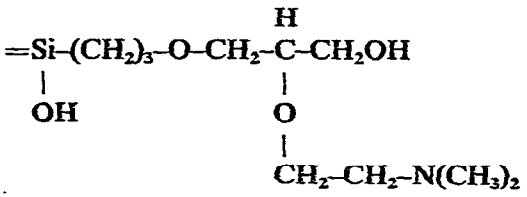
In this work a totally porous microparticulate silica ion exchanger with bonded

dimethylamino groups is applied to the separation of oligoadenylic acid (oligo(A)), oligocytidylic acid (oligo(C)) and oligouridylic acids (oligo(U)) ribonucleotides. In order to examine the retention mechanism of oligonucleotides in more detail, two other chemically bonded microparticulate silicas, LiChrosorb RP-18 and Diol, were employed as packings.

EXPERIMENTAL

Packings

LiChrosorb Si 100 (particle diameter 5 μm; E. Merck, Darmstadt, G.F.R.) was modified with (1,2-eopxy-3-propylpropoxy) trimethoxysilane purchased from Dynamit Nobel, Troisdorf, G.F.R.[21]. The product was treated with reagent grade N,N-dimethylethanolamine (E. Merck). The final packing designated as DMA-silica is believed to exhibit the surface structure:



The surface concentration of the functional groups is 2.0 μmol/m^2. The theoretical exchange capacity derived from the nitrogen content was calculated to be 0.78 mequiv./g. LiChrosorb Diol, RP-8 and RP-18 (all 5 μm) were commercial products from E. Merck.

Chromatographic studies

The chromatograph was a Hewlett-Packard Model 1010 A fitted with a UV photometer of 254 nm wavelength. Columns were 300 × 4 mm I.D. The eluent was a phosphate buffer consisting of K_2HPO_4 and H_3PO_4 adjusted to pH = 7.0. The phosphate concentration, HPO_4^{2-}, was varied from 0.01 to 0.27 *M*. The dimer up to the pentamer of oligo(U) were purchased from Boehringer, Ingelheim, G.F.R. All other oligoribonucleotides used in this study were synthesized by Lipecky and Gassen.

Abbreviations are used to designate the oligonucleotides[22]. The bases are abbreviated as adenine (A), uracil (U) and cytosine (C). The corresponding nucleosides are adenosine, uridine and cytidine, respectively. A nucleotide is written as Np where Np means the nucleotide unit comprising the base, the pentose and the phosphate group, *e.g.*, $(Up)_4U$ means a pentanucleotide made up of four Up units and a terminating uridine.

RESULTS AND DISCUSSION

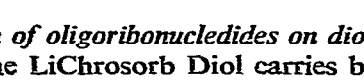
Retention of Oligoribonucleotides on reversed-phase packing

Since the oligonucleotides are strong acids, the proton of the phosphate bridge is dissociated at pH 7.0. It is expected that the compounds will be retarded weakly on reversed-phase packings in an acetonitrile–water eluent owing to their pronounced

hydrophilic character. Indeed, on LiChrosorb RP-18 and with acetonitrile–water (80:20 to 20:80) the uridine was found to be retained slightly whereas the oligoribonucleotides (oligo(U)) were eluted before uridine as the reference substance. No appreciable effect on retention was observed when the water in acetonitrile–water (20:80) was replaced by a solution of K_2HPO_4 and H_3PO_4 (pH 7.0) and the salt concentration was varied from 0.05 to 0.25 *M*. In purely aqueous buffer solutions (0.01 *M* K_2HPO_4–H_3PO_4, pH 7.0) the capacity factor of oligo(C) decreased with increasing number of nucleotide units in the range between CpC and $(Cp)_{12}C$. This elution behaviour can be explained by the fact that the polar character of the solute increases with the number of nucleotide units. Under these conditions the capacity factor range was 0–2. Longer retention might be achieved by increasing the salt concentration but such studies were not done. In conclusion, oligoribonucleotides can be separated on reversed-phase packings in the elution order of increasing hydrophobic character.

Retention of oligoribonucledides on diol packing

The LiChrosorb Diol carries bonded groups of type:



As eluents, phosphate buffer solutions of pH 7.0 were employed containing various HPO_4^{2-} concentrations. Again, the chromatographic studies were performed with the oligo(U) series as solutes. At a given HPO_4^{2-} concentration the solutes are eluted in the sequence:

$$(Up)_4U < (Up)_3U < (Up)_2U < UpU < \text{uridine}$$

Increasing the salt concentration from 0.01 to 0.23 *M* increases retention for a given oligo(U), but all solutes are still eluted before t_0, indicating a type of size-exclusion mechanism (see. Fig. 1). Because of the limited separation range of the packing only $(Up)_2U$, UpU and uridine could be resolved completely.

Retention of oligoribonucleotides on DMA-silica

When conditioned with buffer solution the DMA-silica is a basic anion exchanger. Since the pK_b value of the base cation is around 9–11 the *terminating* dimethylamino group is protonated at pH $\leqslant$ 7.0. A simple calculation shows that a high population of silanol groups still exists at the modified surface, as well as organofunctional groups. The surface concentration, α_{OH}, of hydroxyl groups on native silica before reaction is *ca.* 8.0 μmol/m^2. The resulting surface concentration of the organic moiety is calculted to be *ca.* 2.0 μmol/m^2. Assuming a bifunctional reaction of the modifier, $2 \cdot 2.0 = 4.0$ μmol/m^2 of hydroxyl groups undergoes reaction and $8.0 - 4.0 = 4.0$ μmol/m^2 does not react. In addition, one methoxy group per modifier molecule remains unreacted and is converted, after bonding through hydrolysis, into a silanol group. Its concentration also equals 2.0 μmol/m^2, so that total concentration of 6 μmol/m^2 of hydroxyl groups is expected to be present at the modified surface.

In aqueous solution the silanol groups act as a weak acidic cation exchanger,

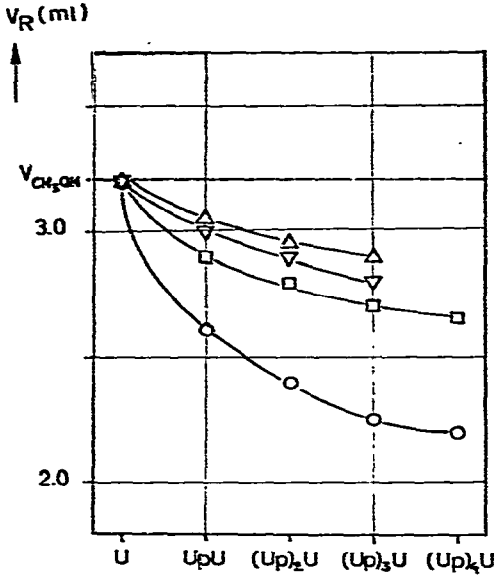


Fig. 1. Retention volume of uridine and oligouridylic acids on a LiChrosorb Diol column as function of the number of nucleotide units at various HPO_4^{2-} concentrations of the phosphate buffer. Conditions: column, 300 × 4 mm I.D.; packing, LiChrosorb Diol, 5 μm; eluent, K_2HPO_4–H_3PO_4 (pH 7.0). ○, 0.01 *M*; □, 0.06 *M*; ▽, 0.11 *M*; △, 0.23 *M*; detector, UV at 254 nm.

releasing a proton and forming a negatively charged siloxane group. Several models were proposed to explain the deprotonation properties of silanol groups at the silica surface[23]. By applying different methods such as infrared spectroscopy, titration, etc. the pK_a of silanol groups was estimated to be 7.1 ± 0.5[24–26]. This value relates to unmodified silica and the question arises as to what extent the pK_a is influenced by bonding organo functional groups at the silica surface. Considering the newly formed hydroxyl groups bonded at the silicon atoms introduced by the modifier, the adjacent propyl group behaving as a weak electron donor is able to induce a charge to the silicon atom. This charge accumulation reduces the polarization of the dipole of the hydroxyl group and hence may decrease the pK_a of his type of hydroxyl group. On the other hand, the unreacted hydroxyl groups, like those at native silica, should show a more pronounced acidity due to $(d–p)_\pi$ conjugation effects with neighbouring siloxane bonds[27]. For comparison, the pK_a of monosilicic acid is estimated to be *ca.* 9.9 (ref. 28). Since the DMA-silica columns is run at pH 7.0 it is believed that no more than half of the hydroxyl groups (6 μmol/m^2) are deprotonated.

It is of interest to consider the effect of these negative surface chafges on the retention behaviour in addition to that of the positive dimethylammonium groups. As already discussed by Knox and Pryde[29] for short-chain aminopropyl silica, a neutralization of both groups may occur, provided that they are mobile enough to be attached closely. This effect seems to be highly improbable in our case since the organic chain should possess a high degree of rotational mobility. Assuming a linear extended organic chain in DMA-silica, the distance between the positively charged terminating dimethylammonium group and the negatively charged SiO^- group fixed to the silica surface may be calculated to be *ca.* 1.5 nm, which is fairly large. Because

of the favourable steric position of the dimethylammonium group, it is considered to be most active in interactions with charged solutes, particularly when they are as large as the oligoribonucleotides.

Discussing an ion-exchange mechanism for retention of ribonucleotide acids on DMA-silica, we have again a special situation because the nucleotides are polyvalent ions or polyelectrolytes. In addition, as for RNA, the oligoribonucleotides exhibit a primary structure and a type of secondary structure[1]. The primary structure is represented by the nucleotide backbone comprising the phosphate bridge and the pentose with the attached base. The secondary structure is determined by the spatial arrangement of the nucleotide chain, which may form either an ordered helix with base stacking or a disordered random coil. The conformation of oligoribonucleotides has been examined in several papers[30–32]. Oligo(U) are reported to have a low tendency to stack and hence show no ordered structure at room temperature[30,31], while oligo(A) form mainly helices[32]. In the helix structure the sugar phosphate backbones are pointed outwards with one negative charge per phosphate, while the stacked bases form a hydrophobic core[33]. According to the number of nucleotide units, the oligoribonucleotide acids show a high negative charge which is compensated by surrounded positive conter ions. Thus, it appears that electrostatic interaction with the ionic functional groups is possible through multisite attachment. The strength of interaction seems to be dependent not only on the nominal total charge of the oligoribonucleotide but also on the secondary structure of the acid.

The fundamental aspects of the ion exchange of polyelectrolytes such as type of interactions, thermodynamic equilibria, effect of temperature, etc. are reviewed by Feitelson[34] and Marinsky[35]. Based on the work of Bernardi[36–40] on the elution behaviour of DNA and RNA fragments, oligonucleotides and nucleoside polyphosphates on hydroxyapatite columns, Kawasaki[41–53] made extensive calculations on the chromatographic behaviour of macromolecules with rigid structures on hydroxyapatite columns. In the first two papers[41,42] he developed the theory for the chromatography of macromolecules on hydroxyapatite both under static and dynamic conditions. In the following publications[43–53] he discusses special cases.

In order to determine the type of retention mechanism, the concentration of phosphate eluent, C, was varied and the capacity factors (k') of solutes were measured. In the case of an ion-exchange mechanism the capacity factor of a solute falls exponentially with increasing C[54]. The corresponding plots for oligo(U) and oligo(A), respectively, given in Figs. 2 and 3, show the expected behaviour. It should be emphasized that the k' values of the respective nucleosides (uridine and adenine) are scarcely influenced by the salt concentration. By plotting the logarithm of k' *vs.* the number of nucleotide units for oligo(U) and oligo(A), respectively, at constant salt concentration, straight lines are obtained (Figs. 4 and 5). As expected, the logarithm of k' increases linearly with the number of negative charges in oligoribonucleotides. As shown in Fig. 6, this relationship also holds for long-chain oligoribonucleotides, *e.g.*, up to the heptadeca oligomer of oligo(A). The results obtained reveal that an ion-exchange is predominant on DMA-silica.

Bernardi[37], in his study of nucleic acid retention on hydroxyapatite columns, also suggested an ion-exchange mechanism between the phosphate groups of polynucleotides and the calcium ions of the packing with no direct intervention of the base and the sugar residue. In other papers[36,38–40] he concluded the following elution

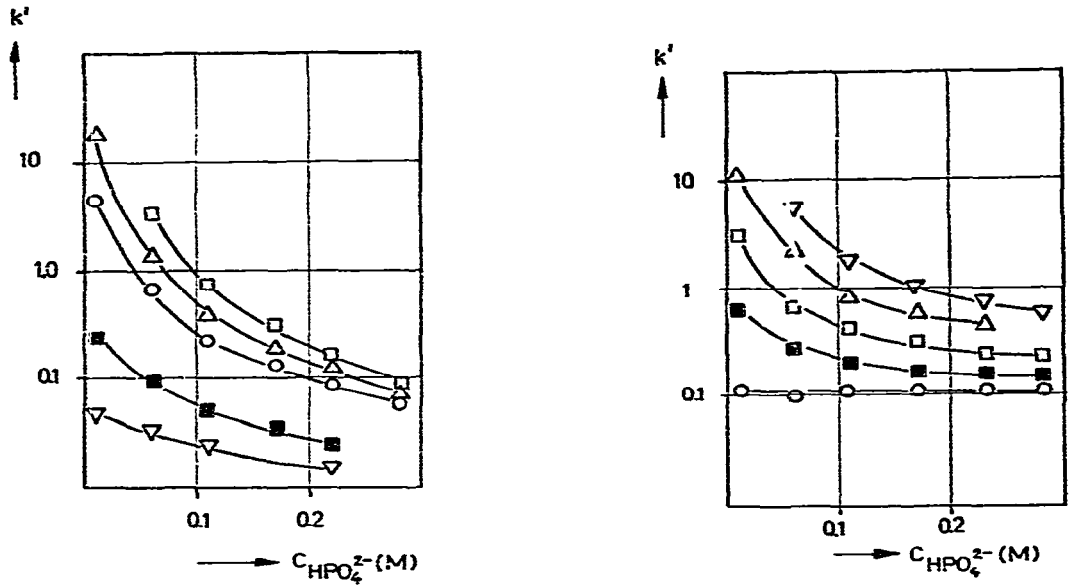


Fig. 2. Dependence of k' of oligo(U) on the HPO_4^{2-} concentration of the eluent. Conditions: column, 300 × 4 mm I.D.; packing, DMA-silica (5 μm); eluent, phosphate buffer (pH 7.0); flow-rate, 1.0 ml/min; detector, UV at 254 nm. Solutes: ▽, uridine; ■, UpU; ○, $(Up)_3U$; △, $(Up)_4U$; □, $(Up)_5U$.

Fig. 3. Dependence of k' of oligo(A) on the HPO_4^{2-} concentration of the eluent. Conditions as in Fig. 2. Solutes: ○, adenosine; ■, ApA; □, $(Ap)_2A$; △, $(Ap)_3A$; ▽, $(Ap)_4A$.

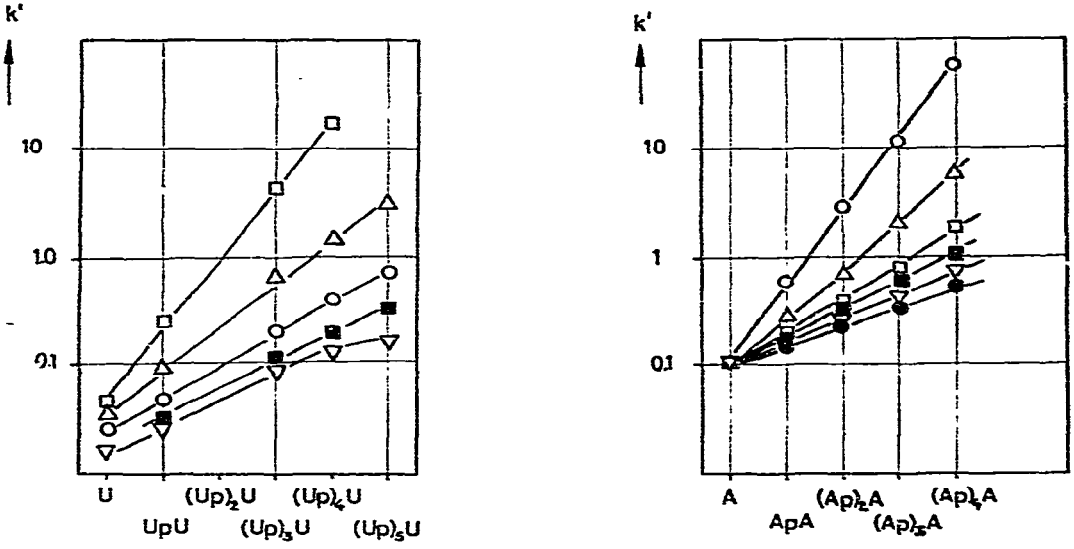


Fig. 4. Dependence of k' of oligo(U) on the number of nucleotide units. Conditions as in Fig. 2. Eluent $C_{HPO_4^{2-}}$: ▽, 0.23 M; ■, 0.17 M; ○, 0.115 M; △, 0.06 M; □, 0.01 M.

Fig. 5. Dependence of k' of oligo(A) on the number of nucleotide units. Conditions as in Fig. 2. Eluent $C_{HPO_4^{2-}}$: ●, 0.28 M; ▽, 0.23 M; ■, 0.17 M; □, 0.115 M; △, 0.06 M; ○, 0.01 M.

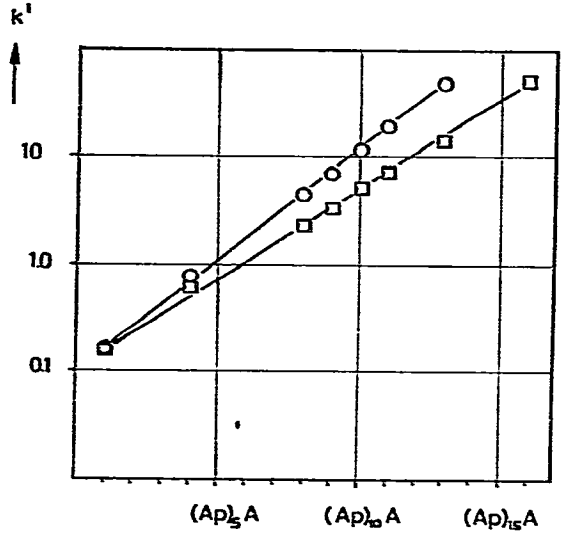


Fig. 6. Dependence of k' of oligo(A) on the number of nucleotide units. Conditions as in Fig. 2 Eluent $C_{HPO_4^{2-}}$: □, 0.28 *M*; ○, 0.23 *M*.

principles: (i) large size polynucleotide solutes require higher phosphate buffer concentrations for elution than smaller ones; and (ii) polynucleotide molecules of given mean molecular weight and regular secondary structure are eluted at higher phosphate buffer concentration than those with the same molecular weight but having random coil structure.

A further distinction between ionic (electrostatic) and molecular (adsorption) interactions in the retention of solutes seems to be possible applying a concept proposed by Latt and Sober[55] and Record *et al.*[56]. Latt and Sober[55] measured the association constants, K_{obs}, for a series of oligolysines interacting with polyribonucleotides. In this case, in contrast to our system, the negatively charged polyribonucleotide is taken as matrix and the positively charged oligolysine solute is the counterpart. Graphs of log K_{obs} *vs.* the negative logarithm of the sodium concentration of the solution gave a family of straight lines constrained to intersect at a common point. The slope of the lines was found to correspond with the number of ion pairs formed between the oligolysine and the polyribonucleotide. In addition, the linear extrapolation to a standard state of 1 *M* salt concentration yields the non-electrostatic component of the free binding energy.

Figs.7 and 8 present the analogous function of the logarithm of k' *vs.* $\log 1/C_{HPO_4^{2-}}$ for oligo(U) and oligo(A), respectively. Adapting the concept of Record *et al.*[56] to our conditions, it is assumed that k' of the solute is proportional to the association constant for the equilibrium between the DMA-silica ion-exchanger and the oligoribonucleotide solute. For oligo(U) the k' values extrapolated to salt concentration of 1.0 *M*, are in the order of $1 \cdot 10^{-2}$, thus indicating a negligibly small contribution of molecular interactions to the total interactions according to the model of Record *et al.*[56]. For oligo(A), however, of this salt concentration the straight lines intersect the abscissa at $k' \approx 0.1$ which is one order of magnitude higher than for oligo(U).

The results are in fairly close agreement with some previous findings. It can

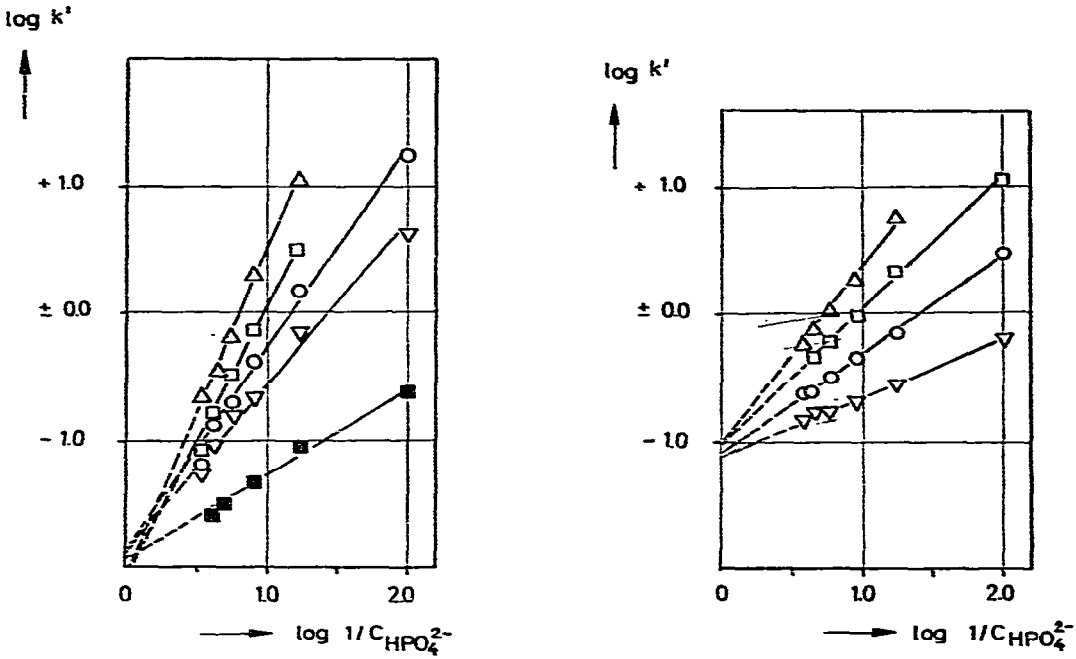


Fig. 7. log k' of oligo(U) *vs.* log $1/C_{HPO_4^{2-}}$. Conditions as in Fig. 2. Solutes: ■, UpU; ▽, $(Up)_3U$; ○, $(Up)_4U$; □, $(Up)_5U$; △, $(Up)_6U$.

Fig. 8. log k' of oligo(A) *vs.* log $1/C_{HPO_4^{2-}}$. Conditions as in Fig. 2. Solutes: ▽, ApA; ○, $(Ap)_2A$; □, $(Ap)_3A$; △, $(Ap)_4A$.

be easily seen in Fig. 3 that the k' value of adenosine remains generally unaffected by the salt concentration and also equals 0.1, as shown in Fig. 8. For uridine (see Fig. 2) a slight dependence of k' on the salt concentration is observed and k' approximates values between 0.02 and 0.04 which corresponds to that extrapolated at $C = 1\ M$ in Fig. 7.

The curves in Figs. 7 and 8 can be described by

$$\log k' = a \log 1/c_{HPO_4^{2-}} + \log b$$

where a is the slope of the function and b the ordinate is a measure of the contribution of molecular interaction to total retention. It appears that the slope of the straight lines includes the number of negative charges, Z, of the oligoribonucleotides as a variable. For a given nucleotide, Z equals the number of nucleotide units per molecule minus one. For a given series such as oligo(U) the nominal value of a, however, is not equal to Z but differs by a constant factor, p. This factor p is calculated to be 0.42 ± 0.2 for oligo(U) and 0.36 ± 0.2 for oligo(A). The constancy of p within a given series of oligoribonucleotides shows that k' is directly proportional to the total nominal charge of the solute. The observed fact that the negative charge is not equal to Z but remarkably lower can be explained by a protection of the charge of the respective oligoribonucleotide and/or the fixed ionic sites of the DMA-silica. The factor p, termed the protection factor, is smaller for oligo(A) than for oligo(U). A plausible explanation for this

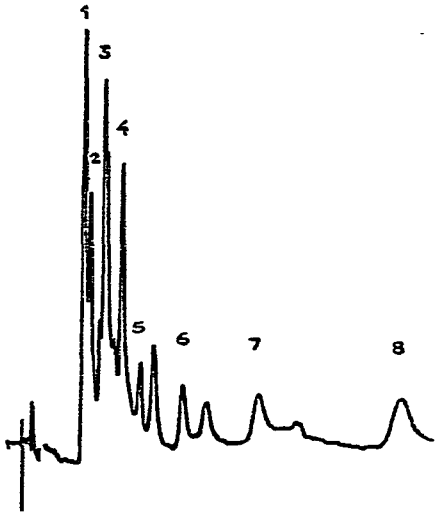


Fig. 9. Separation of a homologous series of oligo(U). Conditions as in Fig. 2, except $C_{HPO_4^{2-}}$ = 0.115 *M* and chart speed 300 mm/h. Compounds: 1 = uridine, UpU; 2 = $(Up)_2U$; 3 = $(Up)_3U$; 4 = $(Up)_4U$; 5 = $(Up)_5U$; 6 = $(Up)_6U$; 7 = $(Up)_7U$; 8 = $(Up)_8U$.

difference may be that the base adenine in oligo(A) is larger than uracil in oligo(U) and therefore leads to a higher protection.

In conclusion, at a given phosphate buffer concentration we found less retention for oligo(U) than for oligo(A) on DMA-silica for the same number of nucleotide units. An analogous pattern was observed by Bernardi[40] for poly(U) and poly(A) on hydroxyapatite columns.

Separation of oligoribonucleotides on DMA-silica

As demonstrated in Fig. 9, separation of oligo(U) can be completed within 20 min under isocratic conditions. The method enables the biochemist, when synthesizing oligonucleotides, to monitor rapidly the composition of a reaction mixture and to derive some kinetic parameters.

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