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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HIGH-MOLECULAR-WEIGHT NUCLEIC ACIDS ON THE MACROPOROUS ION EXCHANGER, NUCLEOGEN

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SUMMARY

The application of Nucleogen*, a series of novel silica gel-based anion exchangers of different pore size in the range 60–4000 Å, to analytical and large-scale high-performance liquid chromatography of a wide variety of nucleic acids is described. The influence of pore size, pH, temperature, eluting salts, bivalent metal ions, denaturing reagents and flow-rate on resolution, purity and capacity was studied. High resolution was obtained for oligonucleotides up to a chainlength of 37, natural RNAs from tRNA up to phage RNA (molecular weight 1,000,000), double-stranded RNA and DNA fragments, and supercoiled plasmids from a crude cell lysate. Recovery was over 95%; the isolated nucleic acids were pure according to gel electrophoresis, physico-chemical criteria, and activity in enzymatic tests.

INTRODUCTION

In the last decade high-performance liquid chromatography (HPLC) has become an important technique in biochemistry and molecular biology. With modified silica gels of small particle size, the resolution, sensitivity and speed could be improved remarkably compared to the organic resins previously used. On reversed-phase silica gel (C_{18} or C_8) nucleosides, nucleotides[1,2] and short oligonucleotides[3,4] were analyzed or highly purified within several minutes. Oligonucleotides were also separated on silica gel-based weak anion exchangers[5–10]. Up to now, the molecular weights of nucleic acids which could be separated by HPLC were below 10,000; recovery was not always satisfactory. In the range of higher molecular weights, separation with HPLC was reported only for peptides and proteins; this was achieved by the use of reversed-phase silica gel or weak anion or cation exchangers[11,12].

Nucleic acids of high molecular weight have been separated with high resolution on RPC-5[13], a polychlorotrifluoroethylene coated with a tetraalkylammonium salt (Adogen 464). This material was used successfully for the preparation of tRNAs[14], oligonucleotides[15,16] and DNA fragments[16]. Because RPC-5 cannot be

* Nucleogen is a trademark of Macherey-Nagel, Düren, F.R.G.

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used at high pressure, long elution times and large peak volumes are unavoidable. Furthermore, the resolution decreases after several chromatograms due to stripping of the Adogen 464, an effect which is particularly unfavourable at ionic strengths below 0.2 *M*. Small amounts of organic solvents destroy the coating[16].

A novel type of liquid–liquid chromatography with an aqueous two-phase system of polyethyleneglycol–dextran yields high resolution for high-molecular-weight nucleic acids[17]. It is, however, more time-consuming and more difficult to handle than a routine HPLC technique.

From the description of the presently available techniques, it is evident that an appropriate HPLC resin for application to high-molecular-weight nucleic acids is not available. Such a resin should combine high resolution with high capacity and recovery. Furthermore, it should be chemically stable and should be applicable in a broad range of pH, salt and organic solvents without bleeding of the functional groups. Resistance to high flow-rates in order to achieve fast analysis or short preparation times is desirable.

The present work describes the application of the novel HPLC ion exchanger, Nucleogen, which fulfils most of the requirements mentioned above. Silica gel of optimized pore size was modified to function as a weak anion exchanger. Its application to the large-scale preparation of viroid RNA was described in a preliminary publication[18]. Here, we give several new examples of application, *viz.* oligonucleotides, cellular and viral RNAs, poly(A) · poly(U) strands, DNA fragments and plasmids.

MATERIALS AND METHODS

Chemicals and buffers

All chemicals and solvents were of reagent grade from commercial sources. Buffers were made from high-purity water (Milli-Q-System, Millipore, Neu Isenburg, F.R.G.) and filtered through a 1-μm filter.

Nucleic acids

RNA extracts from tomato plants containing tRNA, 5SRNA, 7SRNA, and viroid RNA from the potato spindle tuber viroid (PSTV) were prepared as described previously[18]. Double-stranded CARNA5 and single-stranded CARNA5[19] were a gift from Dr. J. M. Kaper and the plasmid pBR 322 was prepared according to Holmes and Quigley[20]. MS2RNA, poly(A) and poly(U) were obtained from Boehringer (Mannheim, F.R.G.). Oligo$(A)_n$ was prepared by limited potassium hydroxide hydrolysis of poly(A). Poly(A) · poly(U) strands of limited size were prepared by extensive sonication of poly(A) · poly(U) in 100 m*M* sodium chloride, 10 m*M* Tris–hydrochloric acid, pH 6.8.

Sample preparation

All nucleic acids were precipitated with ethanol and dissolved in 10 m*M* Tris–HCl, pH 7. Oligonucleotides were lyophilised and dissolved in 10 m*M* Tris–HCl, pH 7. Before injection of the samples, they were adjusted to the intial buffer conditions and incubated for 5 min at 37°C.

HPLC equipment

HPLC was performed on an LC 850 liquid chromatograph, equipped with a variable-wavelength UV detector and thermostatted column compartment (DuPont Instruments, Bad Nauheim, F.R.G.). Samples were injected with a loop injector (Rheodyne, Berkeley, CA, U.S.A.). Sample loops of 100 μl, 1 ml and 2.5 ml were used. For some applications, particularly for the fractionations of high-molecular-weight single-stranded RNAs, release of bivalent metal ions from the stainless-steel HPLC equipment and corrosion of the equipment caused by the EDTA and potassium chloride had to be avoided. For this purpose all wetted stainless-steel parts of the HPLC equipment were electroplated with a 20-μm layer of gold (Degussa, AG, Schwäbisch-Gmünd, F.R.G.), the stainless-steel capillary tubings were replaced by heavy-wall PTFE tubing (Nacom Ind., Tustis, U.S.A.) with a maximum operating pressure of 70 bar, and only glass or PTFE columns were used. Adjustable 30 cm × 12.7 mm I.D. glass columns (maximum pressure 20 bar) were purchased from Chromatronix (Berkeley, CA, U.S.A.) or Latek GmbH (Heidelberg, F.R.G.).

For analytical use, 5 cm × 6.2 mm I.D. stainless-steel LiChromatubes with 5-μm frits were purchased from Chrompack GmbH (Berlin, F.R.G.), or heavy-wall 1/2" PTFE tubes (5 cm × 6.2 mm I.D. max. pressure 65 bar) (Nacom, Tustis, U.S.A.) and gold-plated column fittings (Valco, Houston, U.S.A.) were used.

Nucleogen ion exchanger

Nucleogen is a silica gel-based anion exchanger with a hydrophilic surface and is available with different pore sizes (Macherey-Nagel, Düren, F.R.G.). The ion-exchange group coupled to the silica surface is shown in Fig. 1. As well as the dimethylamino group (DMA, Fig. 1), diethylamine (DEAE) was also used. The pore sizes of the silica gel were 60 Å, 100 Å, 300 Å, 500 Å, 1000 Å and 4000 Å. The particle size was 10 μm. For the reader's convenience, Nucleogen is written with its functional group and its pore size in Å, *e.g.* Nucleogen-DMA-500. The whole series of pore sizes and the different sizes of the columns (*vide supra*) were used only for the research purpose of this work; not all of them are commercially available.

Gel electrophoresis

Five percent polyacrylamide slab gels were prepared as previously described[18]. The gels were stained with silver as described for proteins[21]. This method is extremely sensitive, allowing the detection of amounts of nucleic acid as low as 500 pg.

Analytical ultracentrifugation

Sedimentation coefficients were determined in an analytical ultracentrifuge Spinco model E as described recently[22].

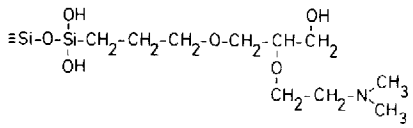


Fig. 1. Dimethylamino-silica gel.

RESULTS AND DISCUSSION

Chromatographic resolution and its dependence upon different parameters

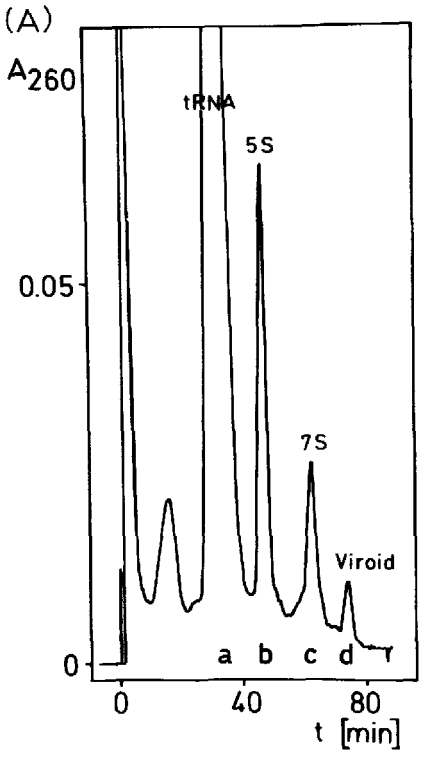
The original purpose for the application of this HPLC technique was the isolation of viroids, which are the infectious agents of several plant diseases and consist only of a circular single-stranded RNA of 120,000 daltons (*cf.* review, ref. 23). The success in viroid isolation with HPLC should encourage its subsequent application to other purification problems. Therefore, the first experiments were carried out on an extract of natural RNA from viroid-infected plants. Those extracts contain mainly transfer RNA (MW 25,000), 5SRNA (MW 35,000), 7SRNA (MW 100,000), ribosomal and messenger RNA (MW 1,000,000), and, in addition, viroid RNA.

In Fig. 2 the elution profile of the RNA extract chromatographed on Nucleogen-DMA-500 is shown. Four nucleic acids were almost completely separated within 80 min. Messenger RNA and ribosomal RNA are eluted at later times and are not shown in the profile. The 7SRNA (slot c) and PSTV (slot d) fractions contained small contaminations of tRNA, 5SRNA, and 7SRNA. In preparative experiments the contaminations were even more serious (*cf.* slots e and f). In further experiments, the chromatographic parameters were systematically varied in order to optimize the resolution or minimize the cross-contamination, respectively. Those runs were done on an analytical scale, and the optimum conditions then transferred and checked for preparative runs.

Effect of urea and EDTA. The contamination of the viroid peak (Fig. 2) with tRNA, 5SRNA and 7SRNA was detected even after chromatography was repeated. This might be due to intermolecular RNA–RNA interactions which are caused by Watson–Crick base pairing between looped regions and salt bridges from bivalent metal ions between the phosphate backbones of two nucleic acid molecules. Those interactions have previously been shown experimentally (*e.g.* ref. 24). Intermolecular interactions favour tailing of the tRNA and 5SRNA into the peaks of the RNA species eluted later. They can be prevented if HPLC is performed in the presence of 5 *M* urea and 0.1 m*M* EDTA. Under these conditions, even a preparative-scale HPLC of 30 mg RNA extract resulted in peaks of superior purity as shown in Fig. 3A and B. Slots h and i may be compared directly to slot f of Fig. 2B because both fractions stem from preparative chromatograms with identical flow-rate and gradient slope but were obtained in the presence (Fig. 3B h and i) or in the absence (Fig, 2B f) of urea and EDTA. On an analytical scale, the elution may be carried out in as little as 25 min without loss of resolution (see Fig. 3C).

The presence of EDTA and potassium chloride increases the corrosion of the equipment and the release of bivalent metal ions. Therefore, tubings were replaced by PTFE tubings, steel columns by glass or PTFE columns and all wetted stainless-steel parts were inactivated by gold. Excellent chemical stability of pumps, valves and mixing chambers was achieved by electroplating these components with a layer of 20-μm gold. It should be mentioned that electroplating of stainless-steel parts was necessary only because pumps and mixing chambers made from inert material were not commercially available. In the meantime, however, several companies have produced instruments with the parts conamed made from ceramics, Kel-F, glass etc. Our modification described above may still be useful for older instruments.

Effect of pH, temperature, eluting salt and flow-rate. A pH value of 8.0 is the



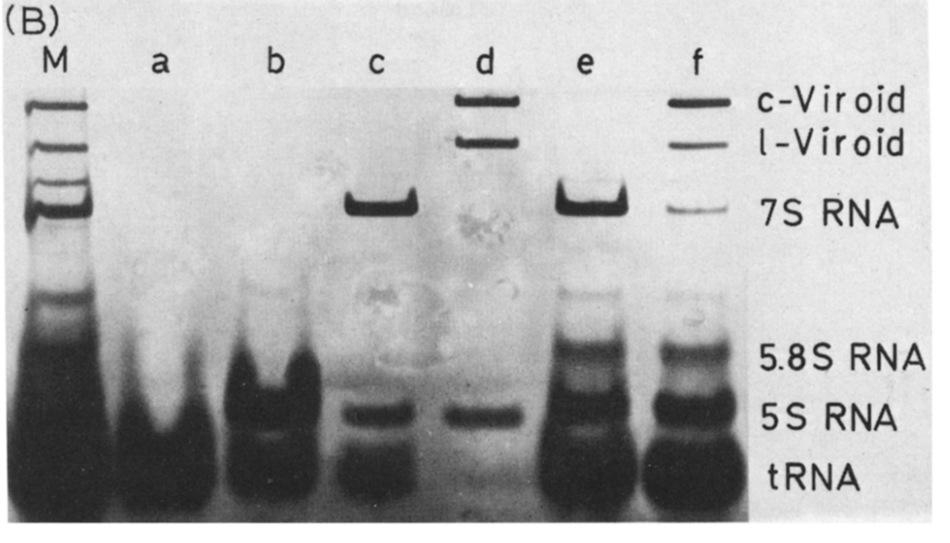


Fig. 2. (A) HPLC of a mixture of tRNA, 5S RNA, 7S RNA, and viroid RNA without urea and EDTA on a 5 cm × 6.2 mm I.D. Nucleogen-DM-500 column. Gradient 250 m*M*–1 *M* KCl in 100 min, 20 m*M* potassium phosphate, pH 6.6, flow-rate 2.5 ml/min, 26 bar, 22°C. (B) 5% polyacrylamide gel electrophoresis[18] of the peak fractions from 2A as indicated in the chromatogram; the unfractionated sample is in slot M. Because of the denaturing conditions in the electrophoresis the circular and linear forms of viroids are separated into two bands. Slots e and f are the 7S RNA and viroid RNA fractions from a preparative HPLC of 15 mg crude RNA extract on a 15 cm × 1/2" I.D. Nucleogen-DEAE-500 column. Gradient as in Fig. 2A in 400 min, flow-rate 3 ml/min (chromatogram not shown).

upper limit for the chemical stability of the silica matrix. In the pH range 4.5–8.0, the best results for ribonucleic acids and DNA fragments were obtained at pH 6.5–7.0 (data not shown). Decreasing the pH below 6.5 increased the eluting ionic strength and resulted in a small loss of resolution for high-molecular-weight nucleic acids. At higher pH values the peaks were eluted at slightly lower ionic strength and closer together. Oligonucleotides (see below) were not well resolved at pH 6.5, but decreasing the pH to 5.5 yielded good resolution (Fig. 6).

Plasmids, (see below) were eluted at very high ionic strength (*ca.* 1.5 *M* potassium chloride) at pH 6.5. In order to decrease the ionic strength, the pH value was increased to pH 7.0 without loss of resolution (Fig. 10).

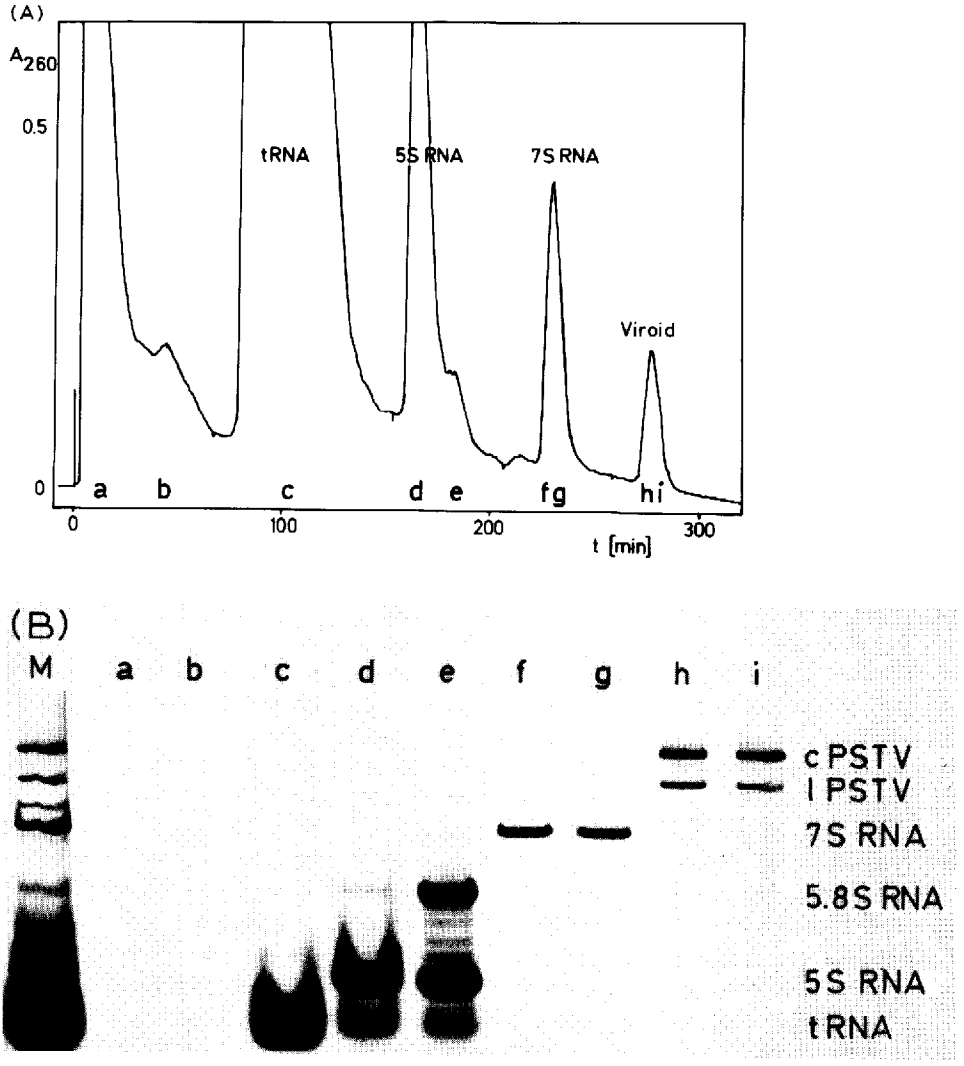


Fig. 3.

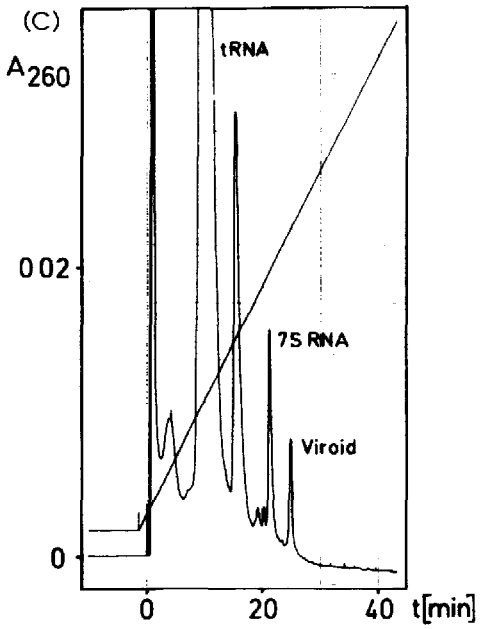


Fig. 3. (A) Preparative HPLC of 30 mg crude RNA extract from viroid-infected plants in the presence of 5 *M* urea and 0.1 m*M* EDTA on a 15 cm × 1/2 in. I.D. DMA-500 PTFE column. Gradient 250 m*M*–1 *M* KCl in 400 min, 20 m*M* potassium phosphate, pH 6.6, flow-rate 3 ml/min, 21 bar, 22°C. (B) 5% polyacrylamide gel electrophoresis of the peak fractions from A as indicated in the chromatogram; the unfractionated sample is in slot M. (C) Analytical HPLC of 20 μg of crude RNA extract on a Nucleogen-DMA-4000 (5 cm × 3 mm I.D. PTFE) column. Gradient 250 m*M*–1 *M* KCl in 45 min, flow-rate 1 ml/min, 42 bar, 24°C, other conditions as in Fig. 3A.

Temperature increase from room temperature to 60°C did not affect the resolution, and the elution was shifted only slightly to higher ionic strength (20 m*M*/10°C).

HPLC of macromolecules is less sensitive to the flow-rate than HPLC of low-molecular-weight substances. No significant differences in the resolution could be observed if the linear velocity ($u = L/t_0$) was between 1 cm/min and 5 cm/min. The disadvantage of high flow-rates is the dilution of the eluted samples and consequently a decrease of the recording sensitivity.

Substantial differences were observed if different salts were used in the eluent. The replacement of potassium chloride by potassium phosphate or sodium acetate resulted in a loss of resolution (data not shown). Sodium chloride and ammonium sulphate showed a similar resolution to potassium chloride, with slight peak broadening. Optimal resolution was observed using potassium chloride and 20–50 m*M* potassium phosphate as a buffer. Buffers like Tris · HCl, sodium acetate, sodium phosphate, and potassium phosphate are without influence on the resolution.

Effect of pore size and functional group. Fig. 4A–E shows the elution profiles of an RNA mixture of tRNA, 5SRNA, 7SRNA and viroid RNA obtained with HPLC on Nucleogen-DMA of different pore sizes. Scarcely any resolution was achieved on Nucleogen-DMA of 100-Å pore size (Fig. 4A). This can be explained by

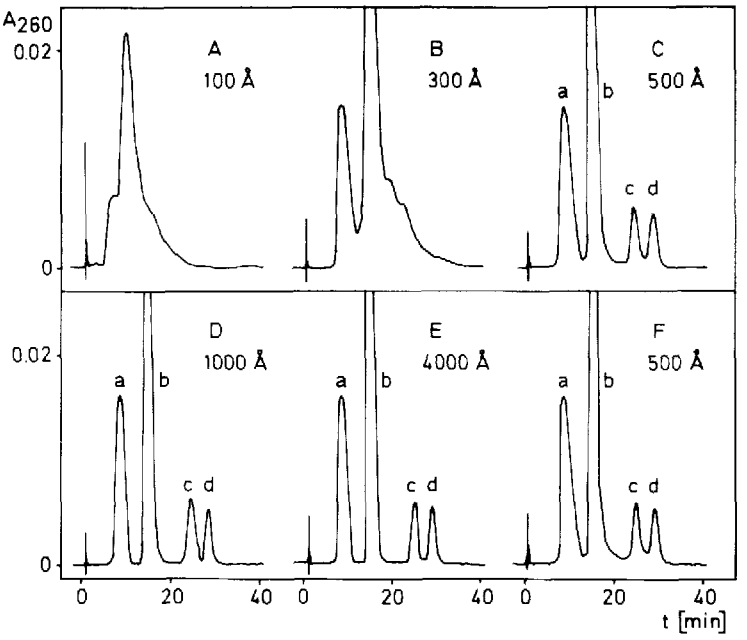


Fig. 4. Influence of the pore size on the resolution of high-molecular-weight RNA. A mixture of tRNA (a), 5S RNA (b), 7S RNA (c), viroid RNA (d) were chromatographed on a 5 cm × 6.2 mm I.D. Nucleogen-DMA (A–E) or DEAE (F) column. The pore sizes are indicated in the figure. Gradient 400 m*M*–1 *M* KCl in 50 min, 20 m*M* potassium phosphate, pH 6.7, 5 *M* urea, flow-rate 1 ml/min, 21 bar, other conditions as in Fig. 3A.

the fact that the nucleic acids are unable to penetrate well into the pores and to interact with the exchange groups in an optimal mode. On Nucleogen-DMA of 300 Å pore size, tRNA and 5SRNA are able to penetrate into the pores and are separated (Fig. 4B), whereas 7SRNA and PSTV are not. Further increase of the pore size allows penetration of all the nucleic acids and results in the resolution of all RNA components of the mixture (Fig. 4C–E).

Comparison of HPLC on Nucleogen-DMA (*cf.* Fig. 4C) and on Nucleogen-DEAE (Fig. 4F) shows no difference between the two functional groups.

The dependence of the resolution upon the pore size was also investigated with small nucleic acids such as oligonucleotides. In Fig. 5 the HPLC of a mixture of oligoadenylic acids on Nucleogen-DMA with pore sizes of 100 Å, 500 Å, and 4000 Å is depicted. Nucleogen with pores of 100 Å or 500 Å showed good resolution whereas with pores of 4000 Å the resolution of oligonucleotides was lower. It must be concluded that there is an upper as well as a lower limit in pore size if optimal resolution is required.

As an additional example the plasmid pBR 322 was purified from *E. coli* cell lysate. It is not well resolved in pores of 1000 Å (data not shown), but could be resolved very well on Nucleogen-DMA with 4000 Å pore size as described in detail below.

We assume that for an optimal interaction the surface of the macromolecule

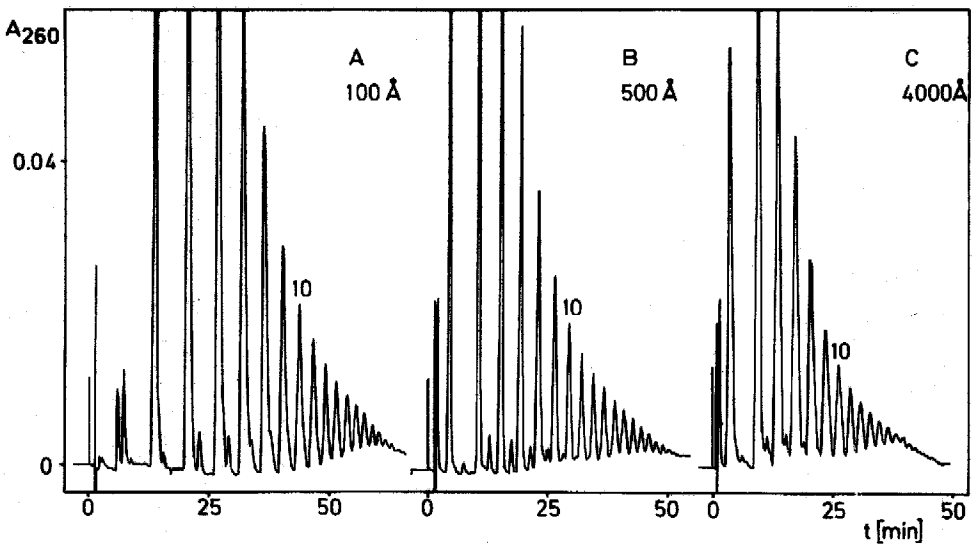


Fig. 5. Influence of the pore size on the resolution of oligonucleotides. A mixture of oligo(A)$_n$ was chromatographed on 5 cm × 6.2 mm I.D. Nucleogen-DMA columns. The pore sizes are indicated in the figure. Gradients 0–1 *M* KCl in 100 min, 10 m*M* potassium phosphate, pH 5.5, 2.0 ml/min, 24 bar, 22°C.

has to match the curvature of the pores. Consequently, stretched-out molecules such as viroids, double-stranded DNA fragments, plasmids etc. interact favourably with very large pores. Speaking in structural terms, not only do the macromolecules have to fit into the pore diameter with their maximal extension, but also their persistence length has to match the inner curvature of the pores. This also explains why oligonucleotides interact better with smaller pores; their somewhat globular structure interacts with more charges of the resin in smaller pores than in larger.

Application to other nucleic acid separation problems

Oligonucleotides. The purification of synthetic oligonucleotides is a common problem in molecular biology and gene technology. Fig. 6 shows the chromatogram of a series of homoribooligonucleotides on Nucleogen-DMA-60 within 120 min. Resolution was achieved up to a chain length of 37 nucleotides. It may be expected that even higher chain lengths can be resolved if a shallower gradient was applied. Deoxyoligonucleotides have been separated with the same resolution (data not shown).

Natural high-molecular-weight RNAs. High-molecular-weight RNAs, such as messenger RNAs and viral RNAs, have to be isolated for many studies in gene expression and related purposes. As is evident from the fractionation of the crude RNA in Fig. 3, the best resolution can be expected in the presence of 5 *M* urea and 0.1 m*M* EDTA. Fig. 7 shows the HPLC of an artificial mixture of different RNAs. Baseline resolution was observed for all RNAs present in the mixture. The structures of these RNAs are quite different: tRNA, 5S RNA, 7S RNA, ssCARNA5 and MS2RNA are single-stranded RNAs with a different degree of a tertiary globular structure whereas dsCARNA5 is a homogeneous double-stranded RNA and viroid

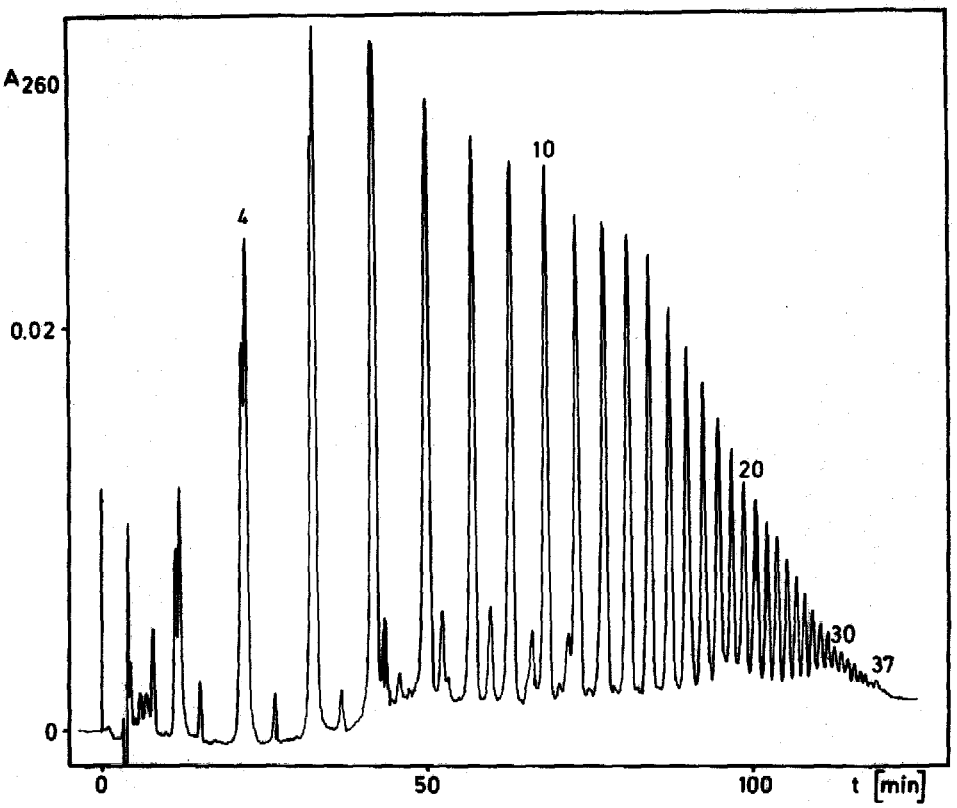


Fig. 6. HPLC of 150 μg of oligo $(rA)_n$ on a 7.5 cm × 6.2 mm I.D. Nucleogen-DMA-60, column gradient 0–1000 m*M* KCl in 200 min, other conditions as in Fig. 5. Chain lengths are indicated in the figure. Satelite peaks at low chain lengths are from incomplete removal of the 2'- or 3'-phosphates by alkaline phosphatase.

RNA a single-stranded RNA with a quasi-double helical structure. This example clearly demonstrates the versatility of the resin used in this work.

Poly(A) · poly(U). Poly(A) · poly(U) double-strands, which have often been used as model nucleic acids in biophysical studies, had been fractionated on hydroxyapatite[25] but were obtained only as inhomogeneous fractions. Therefore, sonicated poly(A) · poly(U) was fractionated on Nucleogen-DMA-1000. The broad peak in Fig. 8A was collected in several pools and rechromatographed under the same conditions (Fig. 8B). The rechromatography and the homogeneous boundaries in sedimentation experiments indicated a narrow size distribution of the pooled fractions. The *s* values of the fractions are listed in Table I.

Double-stranded DNA fragments. Purified DNA restriction fragments are often used in biophysical studies and in cloning and hybridization experiments. The fractionation of those fragments ranging from 43 to 880 base pairs on Nucleogen-DMA-500 is shown in Fig. 9. Because the four fragments with 430 base pairs have a different G:C content[26] it was concluded that the elution behaviour depends upon the length of the fragments only, and is not influenced by the G:C content. It was

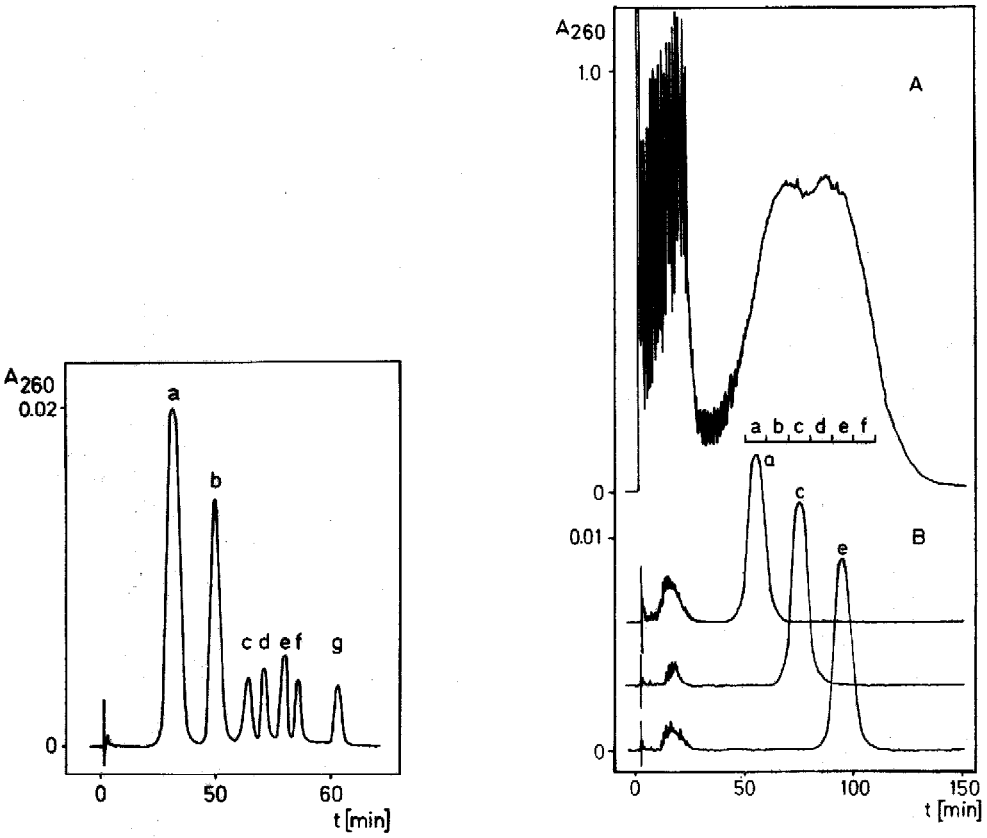


Fig. 7. HPLC of a mixture of viral and cellular RNA on a 5 cm × 12.7 mm I.D. Nucleogen-DMA-1000 glass column. Gradient 250 m*M*–1.5 *M* KCl in 150 min, 20 m*M* potassium phosphate, pH 6.6, 5 *M* urea, 0.1 m*M* EDTA, flow-rate 2 ml/min, 19 bar, 22°C. The peaks indicated in the figure are: tRNA (a), 5S RNA (b), 7S RNA (c), single-stranded CARNA5 (d), viroid RNA (e), double-stranded CARNA5 (f), and MS2 RNA (g).

Fig. 8. (A) HPLC of sonicated poly(A) · poly(U) on Nucleogen-DMA-4000 (5 cm × 1/2″ *I.D.* glass). Gradient 0–1 *M* KCl in 200 min, 20 m*M* potassium phosphate, pH 6.6, flow-rate 2 ml/min, 16 bar, 22°C. (B) The fractions from A were pooled (a, c, e) and rechromatographed under the same conditions.

observed that DNA fragments were eluted at higher ionic strength than double-stranded RNA of the same size. For example, double-stranded viral RNA of 335 base pairs (dsCARNA5) was eluted at 0.9 *M* potassium chloride whereas 350-base-pair DNA was eluted at 1.1 *M* potassium chloride. As concluded from later experiments, for this separation problem a larger pore size should be more favourable.

Plasmids. Although plasmids are used in many laboratories and are produced on a industrial scale, their large-scale preparation is still time-consuming and expensive. Therefore, an isolation procedure based on HPLC would be most desirable. Particularly helpful would be a procedure which yields plasmids in their native supercoiled form. In order to test Nucleogen in this context, a crude cell lysate was chromatographed on Nucleogen-DMA-4000 in the absence of urea (Fig. 10). Cellular

TABLE I

SEDIMENTATION COEFFICIENTS OF CRUDE AND FRACTIONATED POLY(A) · POLY(U)

cf. Fig. 8.

RNA	$S_{20,w}$ *Svedberg units*
crude poly(A) · poly(U)	10–20
fraction a	2.3
fraction b	3.5
fraction c	4.4
fraction d	5.0
fraction e	6.0

RNA and DNA were eluted first, and the plasmid pBR 322 was eluted well resolved from the cellular RNA and DNA near 1.3 *M* potassium chloride. The effective capacity of the column could be increased substantially if the sample was applied to the column at 1.0 *M* potassium chloride. Under these conditions cellular RNAs and DNAs were not absorbed, and the capacity of the column could be exploited completely for the plasmid adsorption. Gel electrophoretic analysis demonstrated that the plasmid peak consisted of the 95% pure supercoiled form. If crude cell lysates

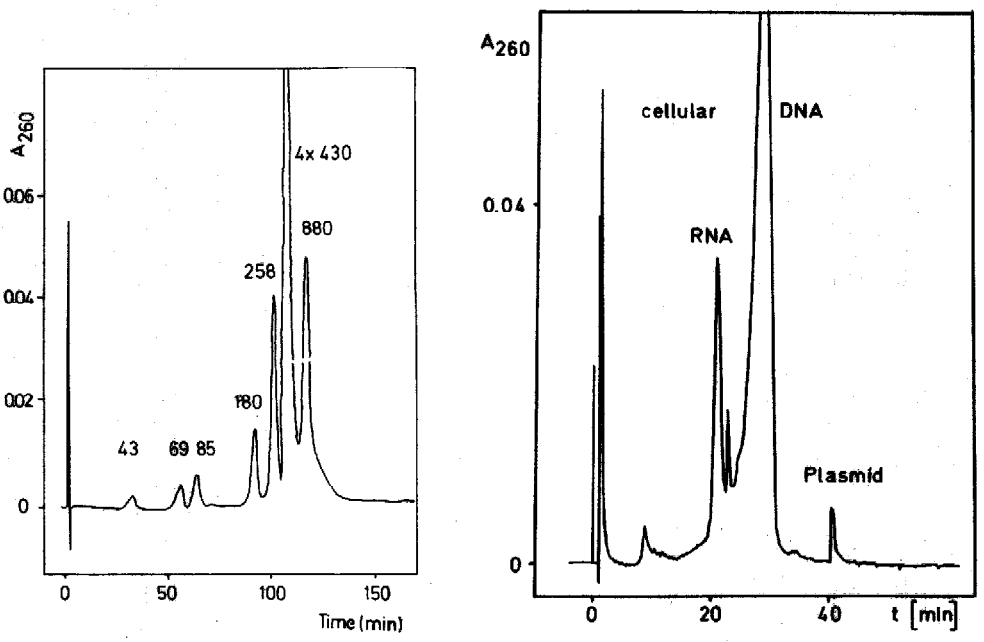


Fig. 9. HPLC of mixture of DNA fragments on a 5 cm × 6.2 mm I.D. Nucleogen-DMA-500 column. Gradient 0–2 *M* KCl in 200 min, 20 m*M* potassium phosphate, pH 6.6, 5 *M* urea, flow-rate 1 ml/min, 19 bar, 22°C. The number of base pairs of the fragments is indicated in the figure.

Fig. 10. HPLC of a crude *E. coli* cell lysate containing the plasmid pBR 322 on Nucleogen-DMA-4000 7.5 cm × 6.2 mm I.D.). Gradient 0–1.5 *M* KCl in 50 min, 20 m*M* potassium phosphate, pH 6.9, flow-rate 2 ml/min, 35 bar, 22°C.

were used containing less plasmid in its supercoiled form, rechromatography of the plasmid peak in the presence of 5 *M* urea resulted in a clear separation of the supercoiled and linear forms (Fig. 11). It may be expected that the procedure described here will serve in future as a routine procedure for plasmid preparation.

General characteristics of the HPLC resin and the purified samples

The following description of general features of the HPLC technique developed for this work may be helpful for planning further experiments.

Capacity. The capacities of the Nucleogen resin were determined with tRNA as being representative of large nucleic acids. A 5 cm × 6.2 mm I.D. column was filled with a known amount of Nucleogen and the packing density was determined by weighing the residual silica gel.

The capacity for nucleic acids was dynamically determined by injecting a concentrated tRNA sample (20 mg/ml) at 20 m*M* potassium phosphate, pH 6.8, until the column was overloaded. The column was washed with the low-salt buffer and eluted with a stepwise gradient of 1 *M* potassium chloride, 20 m*M* potassium phosphate, pH 6.8. The eluted nucleic acid was collected and the amount was determined spectrophotometrically. Table II shows the capacities and some physical properties of the Nucleogen resins.

The low capacity of Nucleogen-100 is due to the inaccessibility of the 100-Å pores for the tRNA as mentioned above. The functional groups on the surface of the silica particles provide only a low capacity. The pore size of the DMA-300 is big enough to admit tRNA. Therefore, the capacity increases drastically from 4.8 to 55 mg/g. The resins with pores of 500 Å, 1000 Å, and 4000 Å show decreasing capacity for tRNA due to their lower surface area. If the capacity is given in mg tRNA/m^2 resin surface, it increases strongly with increasing pore size if penetration is facilitated, and is nearly constant for larger pores.

According to our experience, optimum resolution was achieved when not more than 40% of one maximum capacity was utilised, see Table II.

Purity of the isolated nucleic acids. The purity of the species of nucleic acid isolated was tested according to the following criteria. Although an extremely sensitive staining method was used, gel electrophoresis of the fraction of the purified

TABLE II

MAXIMUM CAPACITIES OF THE CHROMATOGRAPHIC RESINS DETERMINED BY tRNA ADSORPTION

Nucleogen	*Pore size (Å)*	*Surface area (m^2/g)*	*Capacity (mg tRNA/g)*	*Capacity (mg tRNA/m^2)*
DMA-60	60	300	not determined	
DMA-100	100	250	4.8	0.02
DMA-300	300	250	55	0.22
DMA-500	500	50	17.5	0.35
DMA-1000	1000	20	7.4	0.37
DMA-4000	4000	6	2.3	0.38
DEAE-500	500	50	16.0	0.32
DEAE-1000	1000	20	7.6	0.38

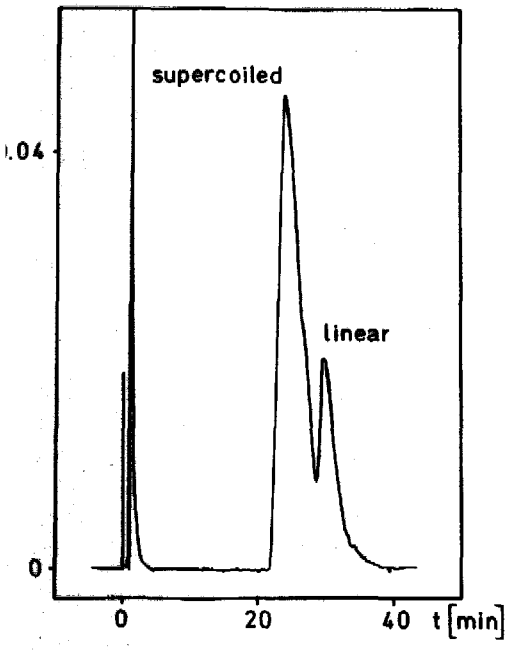


Fig. 11. HPLC of 20 μg supercoiled and 15 μg linear plasmid pBR 322 on Nucleogen-DMA-4000 (7.5 cm × 6.2 mm I.D.). Gradient 900 m*M*–1.3 *M* KCl in 50 min, 5 *M* urea, 30 m*M* potassium phosphate, pH 6.9, flow-rate 2 ml/min, 35 bar, 22°C.

species produced only a single band, even if 100 ng nucleic acid were applied to a gel slot which allowed the detection of less than 1 ng. Physico-chemical results such as absorption spectra, thermal denaturation curves, and the sedimentation profile in the analytical ultracentrifuge agreed exactly with those of the homogeneous nucleic acid species. Furthermore, the isolated nucleic acids show their full activity as substrates or templates in enzymatic reactions. This was tested with different RNases, DNA restriction enzymes, phosphokinases and reverse transcriptase. The enzymatic criteria are particularly important for studies in molecular biology, because samples, which had been eluted from preparative gels, often showed inhibition of enzymatic reactions.

A recent publication describes viroid purification by gel-permeation HPLC[27]. According to the rigorous criteria for purity used above, pure viroid was obtained only after rechromatography.

Recovery. The recovery of different nucleic acids such as oligonucleotides, tRNA, 5SRNA, viroid RNA, and plasmid was determined by injection of known amounts of nucleic acid, collection of the total peak and spectrophotometrical determination of the amount of the nucleic acid recovered. Recovery was higher than 95% for all the nucleic acids.

Chromatographic stability of the resin. Aging of the column occurs if the silica gel is dissolved in the mobile phase and the macroporous structure is disrupted[28]. This process is favoured at pH values above 8.0, which are, however, never used for nucleic acid separation. Furthermore, the column may be clogged by residual im-

purities in the buffer. The solubility of silica in the mobile phase is decreased substantially by the surface modification, but probably cannot be excluded completely. The HPLC column can be protected against both effects, if a small precolumn (5 cm × 3.2 mm) filled with unmodified silica gel (40–100 μm) is connected between the HPLC pump and the loop injector. With the routine use of a precolumn the lifetime of a column (7.5 cm × 6.2 mm) was more than 200 l buffer. This would correspond to about 1000 consecutive HPLC runs.

One column was always used under strongly denaturing conditions for nucleic acid structures, *viz.* 50% formamide at 60°C. After one month of intensive use (about 50 l buffer) no decrease in resolution was observed.

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