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Review

Size-dependent chromatographic separation of nucleic acids

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ABSTRACT

Chromatographic procedures currently used for the size-dependent fractionation of nucleic acids are reviewed. First, an attempt is made to clarify the concept of "size" of nucleic acids and then various aspects of the chromatography of nucleic acids are considered. It is emphasized that consideration of the dynamic three-dimensional structure of large polynucleotides in a rapidly flowing eluent is essential for both the better understanding of mechanism and the development of sophisticated procedures. Of the practical chromatographic techniques that are not based on true size fractionation, ion-exchange chromatography on non-porous column packings appears to be the most efficient. Other methods, such as hydrophobic interaction, are unlikely to become popular. As for truly size-dependent modes, there are gel permeation and slalom chromatography. Although media with extremely large pores become available, the efficiency of gel permeation is still low as a practical separation procedure for large nucleic acid molecules. Its best use seems to be in the field of physicochemical research into nucleic acids in solution. The newly discovered slalom chromatography is based on a principle completely different from all other chromatographic modes. It enables the efficient separation of large double-stranded DNA fragments of 5–50 kilo base pairs by discriminating their length. It has proved not only to be useful as a tool for nucleic acid research but also to have great significance in other fields, *e.g.* the hydrodynamics of polymer solutions, the search for new chromatographic modes, etc.

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LIST OF ABBREVIATIONS

bp	Base-pair
HPLC	High-performance liquid chromatography
kbp	Kilo base-pair
PCR	Polymerase chain reaction
RRT	Relative retention time

1. INTRODUCTION

A peculiar aspect of the size-dependent chromatographic separation of nucleic acids is that this goal is attainable even if the separation mode used is not based on size difference. In contrast to other biological macromolecules, some properties of nucleic acids, *e.g.* electrostatic or hydrophobic ones, change proportionally with the degree of polymerization because only four monomer units with similar properties are sequentially connected. Therefore, any chromatographic mode that can respond to a difference in one of these properties can be used for size-dependent separation, especially when a target substance is in a mixture of homopolynucleotides. Thus, chromatographic modes so far used are divided into two groups: purely size-dependent modes, and modes that eventually result in size-dependent separation. Gel permeation chromatography has long been the only true size-dependent mode, but recently a new size-dependent mode, slalom chromatography, was discovered. Other modes, such as ion-exchange or hydrophobic chromatography, are not truly size-dependent, though they have been used.

In this review, which takes a practical viewpoint, chromatographic procedures currently available for size-dependent separation of nucleic acids are discussed regardless of their basis. Several recent reviews have covered some similar subjects [1–6], so this one will discuss mainly the recent literature on high-performance liquid chromatography (HPLC).

2. SIZE-DEPENDENT SEPARATION OF NUCLEIC ACIDS

2.1. Purpose of size-dependent separation of nucleic acids

Size-dependent separation of polynucleotides is the first step of not only the majority of basic biosciences but also a variety of applied sciences. In many applications, chromatography is versatile as a research tool for both preparation and analysis, because as soon as a separation process is complete, valuable information is also obtained, on such aspects as quantity, quality and

composition. In nucleic acid research, the loading capacity of columns is not so important because if only a very small amount of DNA is collected, it can be easily multiplied either in bacterial cells or by the polymerase chain reaction (PCR) technique. Even RNA can be multiplied, although this is somewhat complicated. Therefore, analytical-scale chromatography often serves the purpose of preparation.

Size-dependent separation is very useful as a tool for biochemical research because it enables both pure target substances and information on their molecular masses to be obtained at the same time. Therefore, the more sophisticated the chromatographic mode, the higher the quality of the information obtained. For example, affinity chromatography of a protein will provide information on its binding site [7,8], and gel permeation chromatography of a protein will provide a value for its molecular mass. This is why size-dependent chromatographic separation is important from both preparative and analytical viewpoints. Size-dependent separation of nucleic acids is also needed in a variety of applied fields, but these are outside the scope of this review.

2.2. What does "the size of nucleic acids" mean?

The term "size" can have a variety of meanings. In biochemistry, it is generally used to mean either molecular mass or degree of polymerization of macromolecules, rather than more fundamental physical parameters such as the volume occupied by a macromolecule or the length of a macromolecule. The size of polynucleotides has been exclusively expressed by the degree of polymerization, *e.g.* number of base pairs or kilobase pairs (abbreviated as bp or kbp) in the case of double-stranded DNA. However, any chromatographic method that enables size-dependent separation is based on or related to one of these different meanings of size. Therefore, it is necessary to define what the term "size" means when used in a particular situation.

Polynucleotides take different shapes depending on their degree of polymerization. A small double-stranded DNA fragment, *e.g.* 20 bp (M_r *ca.* 13 000; dimensions 2 × 6.4 nm), has a rigid and globular shape. On gel permeation chromatography, such a molecule behaves like a protein molecule of similar molecular mass. Therefore, the size of DNA molecules of less than 20 bp can be considered to be similar to that of protein molecules.

Large double-stranded DNA fragments are fibrous, and it is becoming difficult to treat them as globular molecules. A fragment with *ca.* 100 bp (M_r *ca.* 70 000; dimensions 2 × 34 nm) should be treated as a rod with a certain degree of elasticity. Physical length seems the most appropriate way to expess the size of such a molecule. However, there is no chromatographic technique that can distinguish DNA molecules on the basis of physical length.

Fragments larger than 1000 bp (length 340 nm; M_r *ca.* 700 000) can no longer be regarded as a rigid rod. A long double-stranded DNA molecule has a kink at every *ca.* 50 nm (150 bp) and consequently forms a random coil, which is entropically the most favourable shape [9,10]. For example, bacteriophage T_4 DNA, 166 kbp (extended length 50 μm; M_r $1.1 \cdot 10^8$) was found to form a random coil with a diameter of *ca.* 2 μm in its most contracted state. The random coil is very flexible, and its shape changes without a break between the contracted and slightly extended forms (rotational ellipsoid, rod, etc.) by an external force provided by Brownian motion of water molecules. Therefore, it is difficult to define the size in terms of the volume of the molecule. The speed of the intramolecular movement (expansion and contraction) can be as fast as *ca.* 15 μm/s, though the migration velocity of the whole molecule (centre of gravity) is only 1 μm/s. As discussed above, the shapes of double-stranded DNAs change apparently continuously according to their degree of polymerization, and consequently the meaning of size also changes.

The situation becomes more complicated when a DNA solution flows through a narrow channel, such as the spaces between column packing particles. A DNA molecule is stretched by the velocity gradient of the flowing solvent (laminar flow). As the flow-rate increases, the shape of the DNA

changes sequentially from a random coil, to a rotational ellipsoid, to a thick thread and finally to a thin filament [9,10]. When the molecule is fully extended, the size can be defined by the most straightforward physical term, *i.e.* length. As discussed later, slalom chromatography enabled such an approach for the first time.

In contrast to double-stranded DNA, single-stranded polynucleotides such as RNAs have completely different features. Formation of intramolecular base pairs makes their conformation very complicated. tRNAs, which are small (*ca.* 80 nucleotides, M_r *ca.* 25 000) and have a large proportion of intramolecular base-pairs, can be regarded as compact molecules similar to protein molecules. Because they are small and rigid, their shape will be scarcely affected by laminar flow. However, only limited knowledge on the physical structure is available for larger RNAs, such as ribosomal RNAs. Therefore, it is difficult to discuss the size of these RNAs except in terms of the degree of polymerization. The size and structure of mRNAs are much more difficult to generalize: the flow will significantly affect their shape (dimensions or apparent physical size).

Flow makes the chromatography of polynucleotides different from that of proteins; the conformation of most proteins does not change under the flow-rates usually applied in HPLC, and the effect of the flow-rate has been discussed only from the viewpoints of equilibrium and mass transfer. However, in the case of polynucleotides, consideration from the viewpoint of hydrodynamics is essential, especially for gel permeation chromatography. The flow-rate should be kept low in order to ensure not only that equilibrium is maintained but also that the dimensions of the random coil are not changed. This precaution becomes increasingly important with the development of HPLC. Any change in the shape of polynucleotides caused by flow should also affect the physical contact between column packing particles and the target polynucleotides, even in the case of other chromatographic modes such as ion-exchange and hydrophobic chromatography. The binding strength may change when the shape of a polynucleotide changes from random coil to extended form. Careful analysis of the flow-rate dependency may provide information on the dynamic properties of nucleic acids, *e.g.* elasticity, plasticity and topology, etc.

2.3. DNA and RNA

Whether a target polynucleotide is double-stranded or single-stranded has a critical importance for the determination of its chromatographic behaviour. Both the chemical and physical properties of the former are much simpler than those of the latter. Every base in double-stranded DNA molecules forms a complementary base pair and therefore is chemically neutralized and physically less exposed. The influence of the base composition and nucleotide sequence on the chromatographic separation is relatively small. Intramolecular and intermolecular interactions are also negligible. Therefore, these molecules can be considered as a simple filament (except closed-circular DNAs, which form supercoil).

On the other hand, most RNA molecules are generally single-stranded, and a number of internal base pairs are formed. This results in the formation of a variety of three-dimensional structures, and the shapes of RNA molecules will depend greatly on their primary structure. If intramolecular complementarity is high, the molecule is likely to form a more compact and rigid structure. If this is not the case, the molecule will form a much looser structure, which always fluctuates between a number of statistically possible, unstable conformations. In the latter case, the shape should be more susceptible to changes of flow-rate. These situations should make it possible to distinguish RNA molecules with the same size but different overall conformations. If the separation of RNA on a purely size-dependent basis is desired, the secondary structure should be destroyed by a suitable agent, such as urea.

2.4. Base composition

For size-dependent separation, it is desirable

to minimize the effect of base composition. Because neither gel permeation chromatography nor slalom chromatography depends in principle on interactions with the column packing, separation by these methods is expected to be independent of the chemical nature of the target molecules.

Size-dependent separation of nucleic acids is also possible by chromatography based on interaction with the column packing, such as ion-exchange chromatography and hydrophobic chromatography. However, separation by these modes is sometimes influenced by the base composition.

2.5. *Chromatographic modes currently applicable for size-dependent separation of nucleic acids*

Table 1 summarizes the chromatographic modes that have been used for the size separation of nucleic acids. Generally speaking, separation of DNA has been studied more in detail, probably because of the availability of appropriate sets of DNA molecules with different sizes, and easier interpretation of results. Size-dependent separation of RNA seems to be limited to either homopolymers, such as oligoadenylic acids, or some RNAs with a definite size, such as ribosomal RNAs.

Each chromatographic mode has its own optimal range. It seems difficult to separate large double-stranded DNA molecules by gel permeation chromatography. Practically, DNAs of several kilo base pairs seem to be the upper limit for the presently available packings. Even if we use media for high-performance gel permeation, the time required for one run is long. The usefulness of gel permeation therefore seems to be in analysis rather than preparation. Among the available chromatographic supports, newly developed ion-exchange columns (non-porous) seem to be useful because a wide range of DNA fragments can be separated in a short time [11]. Slalom chromatography, which uses a completely new principle, is very promising [12]: it can separate double-stranded DNAs ranging from 5 to 50 kbp by using isocratic eluent in a short time. Hydroxyapatite (mix-mode), two-phase partition, etc., are more suitable to distinguish the chemical nature of polynucleotides.

As to the resolution efficiency, all currently available chromatographic procedures are apparently inferior to gel electrophoresis. For example, gel electrophoresis applied to DNA sequence analysis can resolve fragments of *ca.* 500 bases differing in only one nucleotide. A wide range of double-stranded DNA fragments and even topoisomers of a closed-circular DNA can be separated by Agarose gel electrophoresis. Though separation of DNA molecules larger than 100 kbp is not possible by any chromatographic mode, it was achieved by pulse-field gel electrophoresis. However, gel electrophoresis has its drawbacks, *e.g.* it takes longer for both separation and recovery, and the recovered sample is usually contaminated with impurities contained in the Agarose. Because chromatography and electrophoresis are complementary to each other

TABLE 1

COMPARISON OF CHROMATOGRAPHIC MODES APPLICABLE TO SIZE FRACTIONATION OF NUCLEIC ACIDS

Mode	Size range		Speed	Resolution
	RNA	Double-stranded DNA (kbp)		
Ion-exchange	–	<25	High	High
Hydrophobic	<28S	< 3	Low	High
Gel-permeation	<16S	< 6	Low	Low
Slalom	–	5–50	High	Medium

from various viewpoints, efforts to improve chromatographic methods should be continued.

3. SIZE-DEPENDENT SEPARATION OF NUCLEIC ACIDS BY ION-EXCHANGE CHROMATOGRAPHY

Separation of nucleic acids by ion-exchange chromatography is mainly based on electrostatic interactions between the phosphate groups of the former and the positively charged groups of the ion exchanger. At extreme pH, secondary interactions due to the ionization of nucleic acid bases become significant.

Fractionation of synthetic homopolymers of relatively small sizes has been effectively achieved by ion-exchange chromatography. Macroporous supports for HPLC having DEAE groups, such as TSKgel IEX DEAE (silica-based) [13] and Nucleogen (silica-based) [14], have been used. Adsorbed oligonucleotides were eluted by a salt gradient in the order of the degree of polymerization. Non-porous particles of very small diameter (2–3 μm), which have been developed recently, minimize the time required for mass transfer of target molecules into intrapore spaces and significantly improve the resolution [15]. Oligonucleotides of up to 20 bp were effectively separated according to their size in less than 7 min by using a small column (35 mm × 4.6 mm I.D.). A merit of polymer particles is their stability at alkaline pH. The effect of base composition was rather small at pH 8.5–9.5. When a salt gradient was used, the retention times of oligonucleotides were affected by their base composition. The use of a sodium perchlorate gradient was found to reduce this effect.

The separation of double-stranded DNA fragments larger than several hundred base pairs by ion-exchange chromatography on macroporous supports has been difficult. Most DNA fragments cannot permeate the pores of the ion exchanger and this resulted in too low an operational capacity. Gel-permeation chromatographic experiments have already shown that the maximum size of a double-stranded DNA fragment that can permeate pores of 100-nm diameter is *ca.* 800 bp (corresponding to extended length of 270 nm) [16]. These experiments were carried out at very low flow-rates, and consequently the DNA fragments should have been in a random coil state. However, at the flow-rates usually applied for HPLC (*e.g.* 1 ml/min), DNA fragments should take a stretched form. The end-to-end distance depends on the velocity gradient of eluent generated in the spaces between the packing particles, *e.g.* a 1000-bp fragment will be stretched to a maximum of 340 nm, a value far greater than the pore size. This makes it more difficult for the fragments to permeate the pores and interact with intrapore ionizing groups. Though attempts have been made by using Nucleogen [17], DEAE 5-PW (polymer-based) [18], Mono Q (Agarose) [18], Mono P and Mono Q [19], ion-exchange chromatography of DNA fragments remains inefficient because of poor resolution and slow speed.

This situation was markedly improved by the development of non-porous, small-size ion exchangers. Reports on the use of this type of medium have appeared, *e.g.* Gen-Pak FAX (DEAE, 2.5 μm) [20,21], TSKgel DEAE-NPR [11,22], an original product [23] and Shodex IEC QA-620N (2.5 μm) [24]. It is now possible to fractionate DNA fragments up to *ca.* 20 kbp. DNA fragments no longer need to permeate the pores and they have only to interact with ionizing groups on the surface of packing particles. In the experiment shown in Fig. 1A [11], fragments ranging from 18 to 587 bp were well resolved within 13 min (flow-rate, 1.5 ml/min; slope of NaCl gradient, 8.8 m*M*/min). Separation seemed to depend mainly on the chain length, but was slightly affected by the base composition. A fragment with a high AT content was slightly retarded from the elution volume expected from its chain length. Fig. 1B shows the fractionation of a *Hin*dIII digest of λDNA (flow-rate, 1.0 ml/min; slope of the NaCl gradient, 100 m*M*/min). Though the first peak contained two fragments (2 kbp and 2.3 kbp), the other four fragments, ranging from 4.3 to 23 kbp, were well separated. The presence of salt in the fractionated samples is not so disadvantageous because DNA fragments can be easily precipitated by the addition of ethanol.

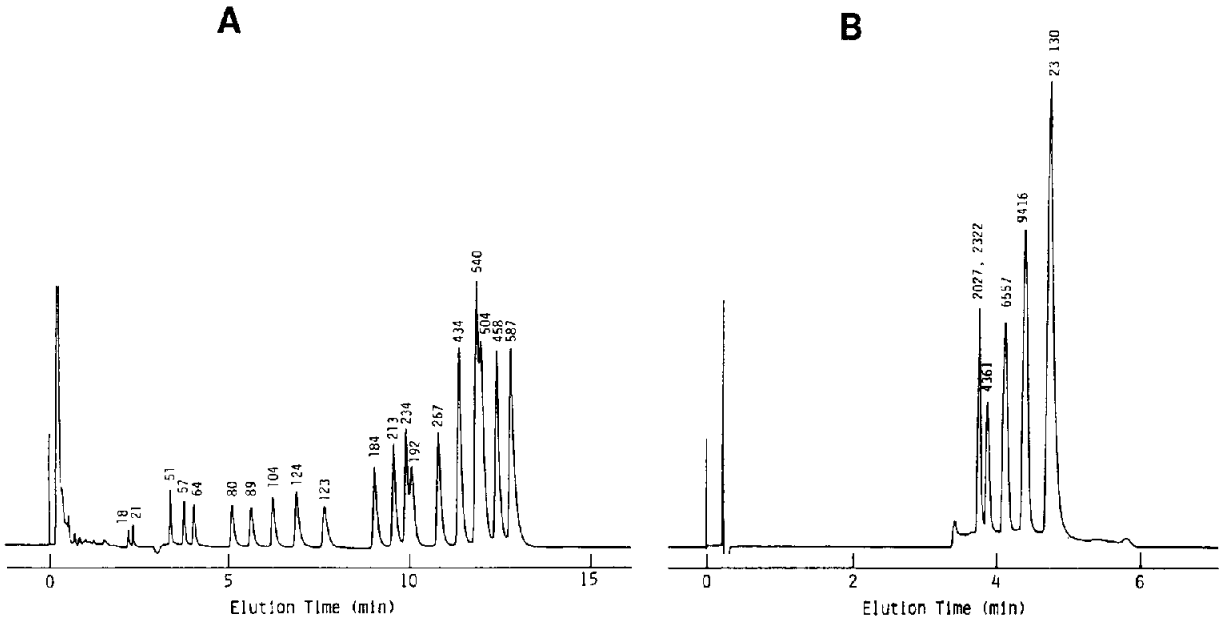

Fig. 1. Size-dependent separation of double-stranded DNA molecules on a non-porous ion-exchange column (TSKgel DEAE-NPR, 35 mm × 4.6 mm I.D.). (A) Chromatogram of *Hae*III digest of pBR322 DNA (4.8 μg). Gradients of NaCl in 20 m*M* Tris–HCl buffer (pH 9.0) at a flow-rate of 1.5 ml/min were applied as follows: a 0.1-min linear gradient from 0.25 to 0.45 *M*, a 2.9-min linear gradient from 0.45 to 0.5 *M*, and a 57-min linear gradient from 0.5 to 1.0 *M*. The numbers on the peaks are chain lengths of the fragments in base pairs. (B) Chromatogram of *Hin*dIII digest of λDNA (2 μg). A 10-min linear gradient from 0.5 to 1.0 *M* NaCl at a flow-rate of 1.0 ml/min was applied. Other conditions are the same as in A. (Reprinted with permission from ref. 11.)

It was necessary to select flow-rates appropriate to the size of the fragments. Fragments less than 1000 bp were well separated at a flow-rate of 1.0 ml/min, whereas good separation of fragments larger than 5 kbp was observed at lower flow-rates, such as 0.3–0.5 ml/min. Though such differences might be attributed to mass transfer problems, it is possible that a change in the shape of DNA fragments affected the interaction with the ion-exchange particles. This needs investigation. The slope of the gradient should also be optimized: a steeper gradient is preferable for the fractionation of larger fragments. The amount of sample that can be used is rather limited, because the capacity of non-porous adsorbents is generally not so high. In the case of TSKgel DEAE-NPR (35 mm × 4.6 mm I.D.), the maximum amount of λDNA-*Hin*dIII that did not affect the resolution was *ca.*10 μg.

4. SIZE-DEPENDENT SEPARATION OF NUCLEIC ACIDS BY CHROMATOGRAPHIC MODES INCLUDING HYDROPHOBIC INTERACTION

The hydrophobicity of oligo- and polynucleotides is due to their purine and pyrimidine bases, and this increases according to the degree of polymerization. Though this property has been used as a basis for size-dependent separation, the chromatographic techniques required are somewhat complicated. They generally take advantage of mixed effects of both hydrophobic and ionic interactions, and thus seem to be more suitable for fractionation of tRNA. One of advantages is that volatile eluents, which are removable after fractionation, can be used.

Size-dependent separation of nucleic acids was first tried by using a support with both hydrophobic and ionic nature (mixed-mode chroma-

tography). RPC-5, which is a hydrophobic matrix bearing charged groups (dichlorodifluoropolyethylene bead coated with a quaternary amine with saturated hydrocarbon substituents eight to ten carbons in length), was used for separation of DNA restriction fragments during the 1970s [25,26], though this matrix was initially intended for use in the fractionation of tRNA species. The original RPC-5 resin is no longer available, but substitutes were prepared (*e.g.* 5 μm reversed-phase support coated with tetraalkylamine) [27]. It was reported that oligouridylic acids of less than 25 mer were well resolved by applying a gradient of ammonium acetate, though it took a very long time (several hours). A halogenated hydrocarbon matrix (Voltalef 300LD) with similar properties was also reported to substitute RPC-5 [28]. DNA fragments ranging from 72 to 1353 bp were separately eluted by a linear gradient of NaCl, though several hours were needed to accomplish the elution of all fragments. These matrices do not seem to be practical for preparative purposes.

Use of a hydrophobic support in the presence of amines with hydrophobic groups has been more widley examined. These amines form ion pairs with the phosphate groups, neutralize the negative charges, and increase the hydrophobicity of the target polynucleotides. This mode is called ion-pair reversed-phase chromatography. As amine components, compounds with aliphatic groups, such as tetrabutylammonium salts, are often used. These amine components were found to contribute to the reduction of the effect of the base composition and to facilitate the size-dependent separation. As supports, silica gel bearing octadecyl groups has been used for separation of mRNA (μBondapak C_{18} [29]) or oligonucleotides (LiChrosorb RP-18 [30], TSKgel oligoDNA RP [31]). The size of pores is usually 10–30 nm. Packings with smaller pores did not give good resolution. Packings with octyl groups or phenyl groups, which were found useful in the field of protein purification, do not retain nucleotides. A mixture of a volatile buffer, such as ammonium acetate or ammonium formate, with acetonitrile is generally used as the eluent, and the proportion of the organic component is gradually increased. Elution of oligonucleotides is accelerated at higher temperatures. Though the resolution is inferior to that of ion-exchange columns, the removal of buffer components is easier.

Separation of double-stranded DNA fragments ranging from 10 to 3000 bp was performed by using PepRPC (C_2/C_{18}) (5 μm particle size, silica-based, 10-nm pores) (Fig. 2) [32]. A volatile buffer composed of a triethylammonium salt and gradient of acetonitrile was used. Resolution of larger fragments was greatly influenced by the flow-rate. For fragments larger than 1500 bp, flow-rates below 0.25 ml/min should be used; for fragments of 500–1500 bp, flow-rates could be raised to 0.5 ml/min. The small pore size of 10 nm restricts the permeation of the pores by fragments larger than 100 bp, especially when they are stretched by rapidly flowing eluent. It seems necessary to keep the bulk size as small as possible by reducing flow-rate. The recovery of most of fragments was *ca.* 60%. The influence of the base composition on the elution order, which was observed in the case of ion-exchange chromatography, was not observed, and separation occurred primarily according to size. Therefore, it was less sensitive to base composition. Unfortunately, the time required was still rather long (several hours).

Hydrophobic-interaction chromatography was also used as a substitute for CsCl density gradient centrifugation in the separation of various RNA species. Ion-pair reversed-phase chromatography was used for size-dependent fractionation of mRNA by Nucleosil C_4 columns [33]. Poly-A-enriched rat liver RNA was adsorbed and eluted by a gradient of acetonitrile. In order to destroy the secondary structure, methylmercuric hydroxide was added. This compound inhibits both the formation of base pairs and the action of RNases by attaching to the imino groups of thymidine and guanosine. Fractions of mRNA eluted at different acetonitrile concentrations were translated *in vitro*, and the sizes of the proteins they coded were estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis. From this estimation, mRNAs were shown

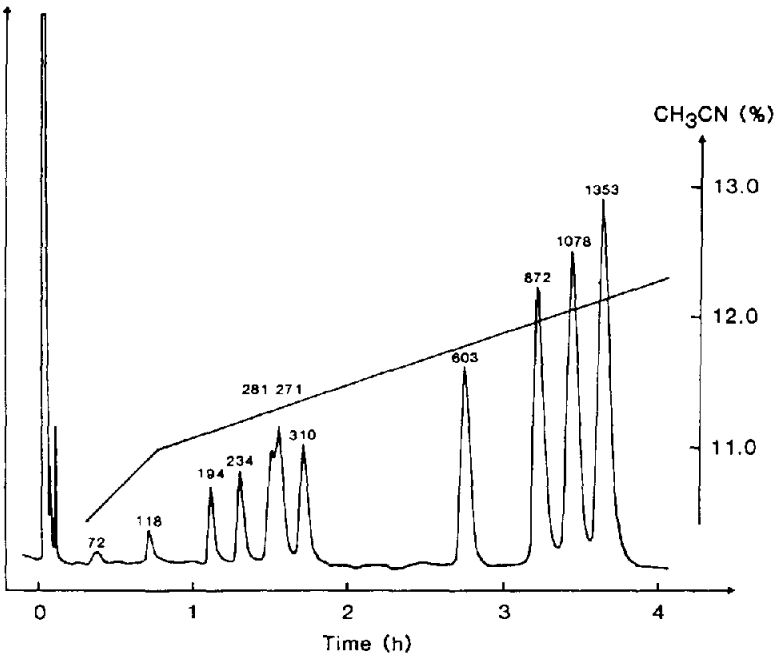

Fig. 2. Separation of *Hae*III digest of ϕX-174 RF DNA (10 μg) by ion-pair reversed-phase chromatography on a PepRPC (C_2/C_{18}) HR5/5 column (50 mm × 5 mm I.D., Pharmacia). Buffer, 50 m*M* triethylammonium acetate (pH 6.5); flow-rate, 0.4 ml/min; gradient, 10.0–11.0% acetonitrile in 50 min and 11.0–12.0% in 180 min. The numbers on the peaks are chain lengths of the fragments in base pairs. (Reprinted with permission from ref. 32.)

to be distributed in the eluate according to the order of molecular mass. Because single-stranded polynucleotides were used, it was essential to destroy the secondary structure in order to make the fractionation only size-dependent. Ribosomal RNAs were also separated by the order of their degree of polymerization under similar conditions: *ca.* 90 min were required to complete this experiment.

Some other adsorbents, such as controlled-pore glass (CPG-10) [34], have been used for the fractionation of various RNAs. First, RNA was adsorbed under a high concentration of salt, which reinforces the hydrophobic interactions, and eluted by lowering the salt concentration. This preliminary experiment suggested that rRNA and 5S RNA were fractionated according to their sizes. A similar approach was used with CL-Sepharose 4B [35]. A sample containing DNA and RNA was applied to the column in the presence of 2.5 *M* NaCl. Although DNA was not retained, tRNA was retarded and rRNAs and mRNAs were adsorbed. The adsorbed RNAs were eluted by decreasing the concentration of NaCl, and 18S and 28S RNAs were eluted at 0.7 *M* and 0.1 *M* NaCl, respectively. mRNAs were distributed in both the 18S and 28S rRNA fractions. These reports show that hydrophobic chromatography can be used as a substitute for density gradient centrifugation. However, it seems unlikely to become a popular procedure.

5. SIZE-DEPENDENT SEPARATION OF NUCLEIC ACIDS BY GEL-PERMEATION CHROMATOGRAPHY

5.1. Limitations of gel-permeation chromatography

Gel-permeation (size-exclusion) chromatography is the only chromatographic method that makes it possible to separate biomolecules on the basis of true size difference. However, it has been neglected by most nucleic acid researchers because of several severe limitations, which are described below.

First, the size of the nucleic acids of interest was usually too large to be applicable to a gel-permeation column. Because the upper size limit is inevitably determined by the pore size of the packing particles, it seems extremely difficult to overcome this limitation. Adequate mechanical stability and a large pore size are a contradictory requirements in the manufacture of packing materials for high-performance chromatography. For example, it is not realistic to produce 10-μm highly porous particles having 1-μm pores that can resist pressures up to 100 kg/cm^2, unless some extremely hard material, such as diamond, becomes available. Even pores as large as 1 μm are not sufficiently large, because they do not allow the permeation of DNA fragments larger than several kilo base pairs. This upper limit will be significantly lower if DNA fragments are stretched by flow. Therefore, the flow-rate should be kept low in order not to stretch the DNA chains.

Second, the resolution attainable by gel permeation chromatography is usually inferior to that of other modes, such as ion-exchange and reversed-phase. This is due to the limited volume in which separation occurs. All molecules are eluted within a narrow range of elution volumes, corresponding to the intrapore volume (total permeable volume), *i.e.* the difference between the column volume and the void volume. Such a situation is completely opposite to that in other modes, in which target molecules are retarded beyond the column volume. This becomes more serious for packings for HPLC, because the intrapore volume relative to the column bed volume is generally much smaller in comparison with the hydrophilic gels, such as cross-linked dextran and Agarose, used for low-pressure chromatography. If one desires to improve the resolution, use of a longer column is probably the only solution.

Third, it is difficult to speed up the operation because it is essential to maintain an equilibrium state of distribution of the target molecules between two phases, *i.e.* stationary phase retained in intrapore spaces and the mobile phase. This becomes more and more serious when the targets are substances with large mass and small diffusion constant, such as DNA molecules. Therefore, it will be impossible to reduce the time required for operation below a certain limit.

In addition to these fundamental drawbacks, there is a powerful alternative method in the field of nucleic acid research, namely gel electrophoresis. If resolution is the major concern, gel-permeation chromatography cannot match gel electrophoresis. The risk of damage to DNA molecules also makes researchers hesitate to use chromatographic procedures.

Because these limitations tend to discourage researchers, it seems unlikely that gel-permeation chromatography will survive as a high-resolution size-dependent procedure for nucleic acid research. However, this does not mean that this technique is entirely useless. If the number of nucleic acid species in a sample is small and the size differences between them are large, gel permeation is a useful and practical preparative procedure as a substitute for CsCl density gradient centrifugation. Moreover, there are few other methods that can provide information on the mass or size of nucleic acid molecules on a physicochemical basis. The best way to make use of gel permeation will be found in analytical fields. Simple experiments using an appropriate column will make it possible to detect changes in three-dimensional structure and other physicochemical properties.

5.2. Behaviour of polynucleotides during gel permeation

The mechanism of the separation of polynucleotides, especially large ones, has not been studied in detail because of the complexity of their shape and the unavailability of a suitable gel matrix. As discussed before, the overall shape of DNA molecules should be considered at least three levels. Smaller DNA fragments (less than *ca.* 20 bp) can be regarded as rigid and globular molecules, whereas fragments larger than 1000 bp take a random coil form. Fragments of intermediate size are considered to be flexible and elastic rods. No distinct boundary exists between these different states, and transition occurs gradually.

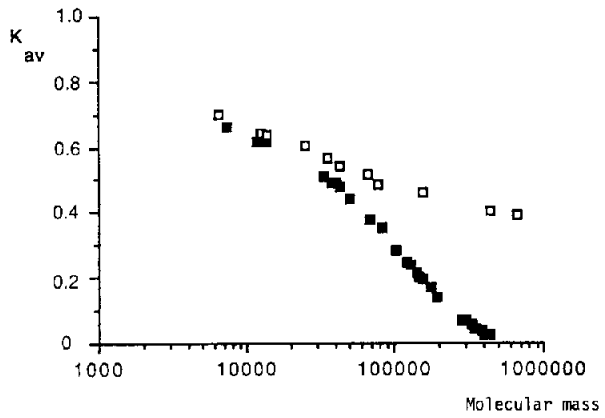

Fig. 3. Relationship between elution volume and molecular mass of proteins (□) and DNA fragments (■) on a gel-permeation column (Superose 6, 106 mm × 10 mm I.D., Pharmacia). Buffer, 0.02 *M* Tris HCl (pH 7.6) containing 0.15 *M* NaCl; flow-rate, 0.35 ml/min; $K_{av} = (V_e - V_0)/(V_c - V_0)$, where V_e is the elution volume, V_0 is the void volume and V_c is the geometrical column volume. (Reprinted with permission from ref. 16.)

The dependence of the relative retention volume of DNA fragments and proteins on their molecular mass was examined by using Superose 6 [16]. It was found that fragments up to 1000 bp (corresponding to M_r 660 000) were eluted in order of size, and fragments of 540, 234 and 123 bp were almost completely separated in 45 min. When calibration curves for both DNAs and proteins were compared, the curve for DNA fragments with M_r less than 12 000 (18 bp) was similar to that for proteins, suggesting that they behaved as globular molecules. Larger DNA fragments eluted much earlier than proteins of corresponding molecular mass, and the resultant curve deviated from that for proteins and became much steeper, suggesting that they no longer behaved as globular molecules but as rod-like molecules (Fig. 3). DNA fragments longer than several hundred base pairs cannot be regarded as stretched rods because they form random coils, and the calibration curve will again change. However, unfortunately, the fractionation range of Superose 6 was too small, and this phenomenon could not be experimentally verified. A similar size dependency was observed with TSKgel G5000PW [36]. Sephacryl S-500 showed a similar calibration curve for DNAs and proteins [16].

There have been attempts to create a universal calibration curve that can correlate the retention volumes of substances to only one parameter that is not affected by their shape [37,38]. Values that have been used to describe the size of macromolecules, such as that defined by diffusion (Stoke's radius) and X-ray scattering or light scattering (radius of gyration) were not appropriate, and the hydrodynamic equivalent radius, which is the product of intrinsic viscosity and molecular mass, was found to be best suited for this purpose [38].

5.3. Application to DNA fractionation

The number of chromatographic media available for efficient gel permeation of double-stranded DNA fragments is small. Because macroporous particles with extremely large pores are required, usable packings are limited to those made from silica (TSKgel SW series) [39], polymer (TSKgel PW series) [36,40] and Agarose. TSKgel G3000SW proved to be able to separate double-stranded DNA fragments of less than 100 bp [39]. To achieve good resolution, it was necessary to use two columns (600 mm × 75 mm I.D.) in tandem, and one run usually took *ca.* 1 h at flow-rates of 0.3–1.0 ml/min. Lower flow-rates generally resulted in better resolution. Elution was delayed as the ionic strength of the eluate was increased. This suggests that DNA molecules took a more compact conformation at higher ionic strength because electric repulsion became weaker.

Development of a TSKgel-G-PW series with extremely large pores, such as G5000PW [36] and G-DNA-PW [40], made it possible to fractionate relatively large double-stranded DNA molecules. G5000PW could resolve a 1000-bp fragment from a 1857-bp fragment. The M_r exclusion limit of this column was estimated at *ca.* 10^6 for double-stranded DNA (*ca.* 1500 bp) and *ca.* $5 \cdot 10^6$ for RNA (16 kbp). This suggests that the conformation of double-stranded DNAs is less compact than that of RNA. The medium presently available that allows permeation of the largest DNA fragments is TSKgel G-DNA-PW, with an as-

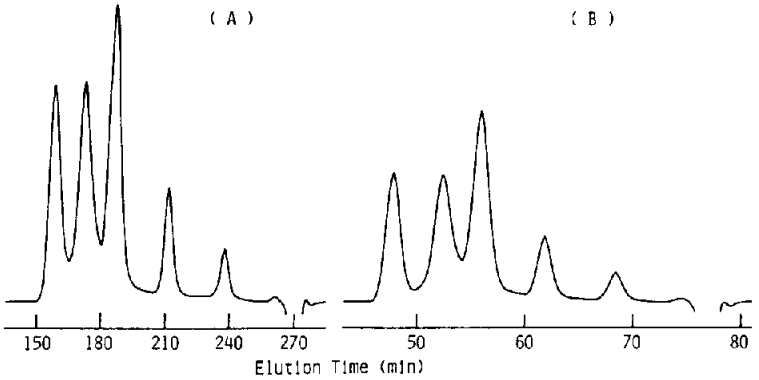

Fig. 4. Gel-permeation chromatography of restriction fragments derived from pBR322 DNA (9.7 μg) on four TSKgel DNA-PW columns (300 mm × 7.8 mm I.D.) in tandem. Buffer, 0.1 *M* Tris–HCl (pH 7.5) containing 0.3 *M* NaCl and 1 m*M* EDTA; flow-rates, 0.15 ml/min (A) and 0.5 ml/min (B). The sizes of the fragments contained in each peak were 4362, 1857, 1060 + 928, 383, 121 and 13 bp, respectively, in the order of elution. (Reprinted with permission from ref. 40.)

sumed pore diameter of 200–300 nm [40]. A column system of four TSKgel G-DNA-PW columns (300 mm × 7.8 mm I.D.) connected in series was used to separate fragments larger than 2 kbp. Fig. 4 shows chromatograms for *Eco*RI-cleaved pBR322 DNA. A flow-rate of 0.15 ml/min was applied, and one run took 4 h. Of six fragments expected, those of 1060 and 928 bp could not be separated. A calibration curve suggested that the exclusion limit is *ca.* 7 kbp. The authors observed a slightly better resolution between 4.4- and 1.9-bp peaks at a higher flow-rate (0.5 ml/min). Though they gave no explanation for this phenomenon, there should have been some hydrodynamic effect. The higher the flow-rate, the more extended the fragments, and consequently it becomes more difficult for the largest fragment (4.4 kbp) to permeate the pores. Therefore, the elution volume of the 4.4-kbp fragment should have been nearer the void volume, whereas the smaller fragments could easily permeate the pores. Though this experiment suggested that this system is of great promise, the cost of four columns is too high for average research laboratories. It is necessary to devise an efficient recycling system to cut down the expense.

Agarose-based media, Superose 6 and Sephacryl S-500, were found to allow the permeation of double-stranded DNA of up to several hundred base pairs, though the resolution between 400 and 500 bp was not satisfactory [16]. Most of the reported experiments on the fractionation of double-stranded DNA fragments have been limited to evaluation of new media by using model polynucleotides, and the number of examples of practical use is rather small. Table 2 summarizes the properties of several gel permeation columns.

There have been reports on the practical use of gel-permeation media for the rough fractionation of restriction fragments, *e.g.* that of bacterial DNA (up to *ca.* 12 kbp) and of a plasmid (2.7 kb) by using Sephacryl S-1000 [41]. Fractionation of *Hae*III digests of pBR 322 by Superose 6 showed that fragments of less than 100 bp were relatively well separated, though the resolution of fragments larger than 200 bp was unsatisfactory [42]. Fractogel TSK HW75 [43], Sephacryl S-1000 [44] and Superose 6 [45] were used for large-scale purification of plasmid DNA without CsCl gradient centrifugation. Ultrogel A2 (2% Agarose) and Bio-Gel A150m (1% Agarose) were used for the separation of double-stranded DNA fragments, and it was found that complete one-step separation of fragments that differ in size by a factor of 2 was possible [46].

5.4. Application to RNA fractionation

Fractionation of different RNAs by gel-permeation chromatography instead of CsCl density

TABLE 2

HIGH-PERFORMANCE GEL PERMEATION COLUMNS APPLICABLE TO NUCLEIC ACID RESEARCH

Column	Exclusion limit			Ref.
	Double-stranded DNA		RNA (molecular mass)	
	Molecular mass	Bp		
TSKgel G2000SW	50 000	80	70 000	36
TSKgel G3000SW	100 000	150	150 000	36,39
TSKgel G4000SW	300 000	450	1 500 000	36,39
TSKgel G5000PW	1 000 000	1500	5 000 000	36
TSKgel G-DNA-PW	4 600 000	7000	–	40
Superose 6	400 000	600	1 000 000	16,42

gradient centrifugation has been attempted in some laboratories. Components of ribosomal RNA were separated by using TSKgel-G-SW series or PW series [47–49]. Complete separation of large RNAs, *e.g.* 23S and 16S, was usually difficult, whereas smaller RNA species, such as 5S RNA (120 b) and 4S RNA (80 b, tRNA), were well separated. Small nuclear RNAs, *e.g.* 7S, U2, 5.8S, 5S and 4S, were also fractionated [49]. Superose 6 was used for a similar purpose [42], but the separation of ribosomal RNAs was incomplete. mRNAs were also found to be fractionated according to size because the mRNA fraction eluted earlier coded for larger proteins [50]. The addition of urea resulted in the reduction of the mRNA peak widths, probably because aggregation of mRNA was prevented [48]. Although the time required for these experiments was several hours on average, the authors emphasized that the technique is simpler and more economical than CsCl density gradient centrifugation.

6. SIZE-DEPENDENT SEPARATION OF NUCLEIC ACIDS BY SLALOM CHROMATOGRAPHY

6.1. What is slalom chromatography?

The principle of slalom chromatography was recently discovered by Hirabayashi and Kasai [12]. Large double-stranded DNA molecules can be separated according to their sizes by using an ordinary HPLC system and a gel-permeation column, though the order of elution is the opposite of that expected for gel-permeation chromatography: larger fragments are eluted later than smaller ones. Separation patterns significantly depend on the flow-rate and the size of packing particles, but not on the pore size or chemical nature of the packings [12,51,52]. This phenomenon could not be explained by any separation mode previously known. The proposed mechanism, described below, is based not on an equilibrium phenomenon but on a hydrodynamic one.

A column that is closely packed with hard and spherical beads has narrow and tortuous open spaces. When DNA molecules are applied to the column, they are unfolded and extended owing to the laminar flow generated by the solvent passing through the narrow channels. For example, a 10-kbp fragment will become 3.4 μm at maximum. These extended molecules must flex quickly under a fast flow of mobile phase to pass through the openings (Fig. 5). If we use a 30-cm column packed with particles of 10 μm diameter, DNA molecules should turn as many as 36 000 times, because the number of layers of particles reaches 1200 per centimeter of column length. A fragment that has a retention time of 10 min will turn 60 times per second. It is quite possible that the longer the DNA molecule, the more difficulty it

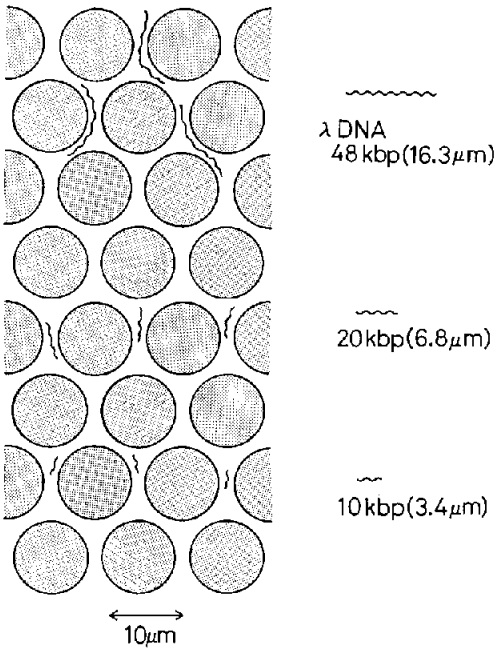

Fig. 5. Illustration of DNA separation in slalom chromatography. DNA fragments are extended by the laminar flow. They turn frequently in passing through the narrow and tortuous openings between closely packed spherical particles (indicated by shaded particles). The larger the fragments, the more difficult it is for them to turn around the particles. If the DNA molecules are extended to the maximum, their length will be comparable with the diameter of the packing particles. The distance between particles is exagggerated. (Reprinted with permission from ref. 52.)

encounters in passing through the openings. Therefore, size-dependent separation in the opposite order to gel permeation should occur. This new mode of separation was named "slalom chromatography", because the proposed model reminds us of a person on skis going down a slope and turning quickly around flags [12]. The results of all the experiments showed that this explanation is satisfactory as a first approximation. Similar observations were made independently by Boyes *et al.* [53].

Important characteristics of slalom chromatography revealed by extensive studies are summarized below.

(1) DNA fragments do not interact with the column packing.

(2) Only the particle size of the packing is important. Smaller particles can resolve smaller DNA fragments and larger particles can resolve larger DNA fragments.

(3) Pore size and the chemical nature of packing particles are not important.

(4) Separation depends largely on the flow-rate: the higher the flow-rate, the more the DNA fragments are retarded. The temperature also has an influence on the separation.

These points will be discussed in the following sections.

6.2. Fundamental characteristics of slalom chromatography

6.2.1. Effect of particle size

One of the most important characteristics of slalom chromatography can be seen in the chromatograms shown in Fig. 6, in which eight DNA fragments ranging from 10 to 38 kbp are separated by two high-performance gel-permeation columns differing in particle size only (exclusion limit for protein, M_r $5 \cdot 10^5$) [12,51]. One was packed with 5-μm particles (Fig. 6A, Asahipak GFT-510) and the other with 9-μm particles (Fig. 6B, Asahipak GS-510). Both columns separated

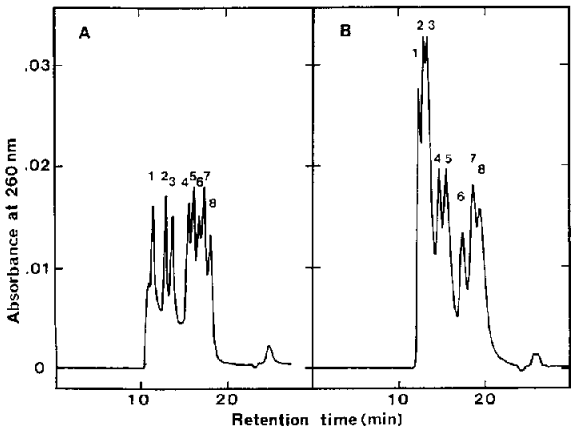

Fig. 6. Chromatography of restriction fragments of λDNA on (A) Asahipak GFT-510 (5 μm) and (B) GS-510 (9 μm). Both columns were the same size (250 mm × 7.6 mm I.D.). A set of DNA fragments was applied. The flow-rate was 0.3 ml/min. Fragment sizes: 1 = 10.09 kbp; 2 = 15.00 kbp; 3 = 17.05 kbp; 4 = 22.63 kbp; 5 = 24.77 kbp; 6 = 29.95 kbp; 7 = 33.50 kbp; 8 = 38.42 kbp. (Reprinted with permission from ref. 51.)

eight fragments in the order of smaller to larger. Although the pore sizes are the same, the column packed with 5-μm particles was superior for the separation of smaller fragments (less than 20 kbp), while larger fragments (greater than 20 kbp) were better separated by the column packed with 9-μm particles. These results apparently indicated that the principle of separation was completely different from that of gel permeation. The fractionation range depends on the particle size of the column packing but not on the pore size.

Results of more systematic experiments using four columns (average particle diameters 5.0, 9.0, 13.1 and 19.1 μm, Asahipak GS-310 series) are shown in Fig. 7 [52]. The relative retention time (RRT), defined as the ratio of the retention time of a particular fragment to that corresponding to the void fraction, was plotted against the number of base pairs of DNA fragments. Apparently, the four columns have different ranges of resolution: *e.g.* at a flow-rate of 0.6 ml/min (middle curves in Fig. 7A–D), the 5-, 9-, 13- and 19-μm columns could separate DNA fragments larger than 7, 9, 13 and 17 kbp, respectively, from the fragments that appeared at the void volume (RRT = 1.0). In addition, smaller packings showed better resolution for smaller DNA fragments, whereas larger ones were better for larger fragments. High-resolution zones can be assigned as 9–17, 15–30, 23–40 and 35–50 kbp for the 5-, 9-, 13- and 19-μm packings, respectively.

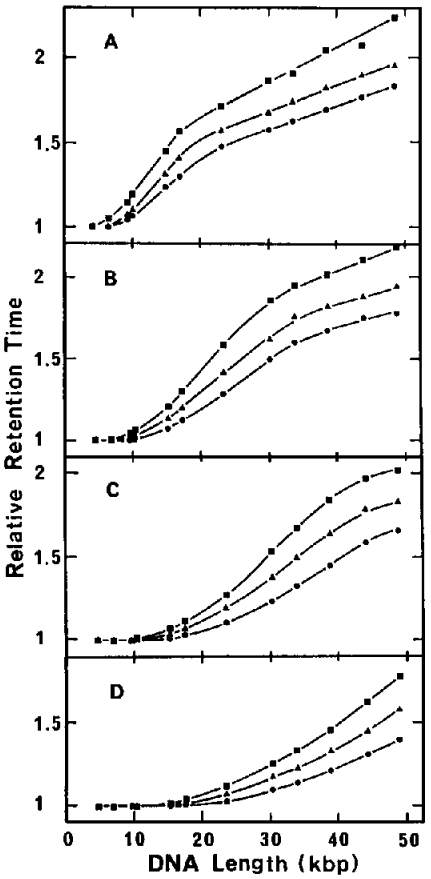

Fig. 7. Dependence of relative retention times of DNA fragments on their lengths. Packings of different particle size were used and different flow-rates were applied. Relative retention time (RRT) was plotted as a function of DNA length (kbp). Asahipak GS-310 columns were used. Particle sizes: (A) 5 μm; (B) 9 μm; (C) 13 μm; (D) 19 μm. Flow-rates: (●) 0.3 ml/min; (▲) 0.6 ml/min; (■) 1.2 ml/min. (Reprinted with permission from ref. 52.)

6.2.2. *Effect of flow-rate*

Results shown in Fig. 7 also reveal that slalom chromatography has a peculiar flow-rate dependency. In other chromatographic modes, it is unlikely that the flow-rate affects the size range of fractionation [52]. A lower flow-rate is usually preferable to obtain good resolution in order to ensure equilibrium between the stationary and mobile phases. However, in slalom chromatography, better resolution is attained at higher flow-rates, and the range of separable sizes is also increased. Application of a higher flow-rate resulted in a significant increase in the RRT of all DNA fragments (Fig. 8). On the other hand, at the lowest flow-rate applied (0.03 ml/min, corresponding to a linear flow-rate of 0.067 cm/min), all DNA fragments were eluted almost in the void fraction (Fig. 8B). At a low flow-rate, all DNA fragments will take random-coil form and thus be eluted without retardation. On the other hand, when a higher flow-rate was applied, DNA molecules were more extended and consequently more retarded.

6.2.3. *Effects of pore size and chemical nature of packings*

Although the phenomenon of slalom chromatography was first observed with packings for gel

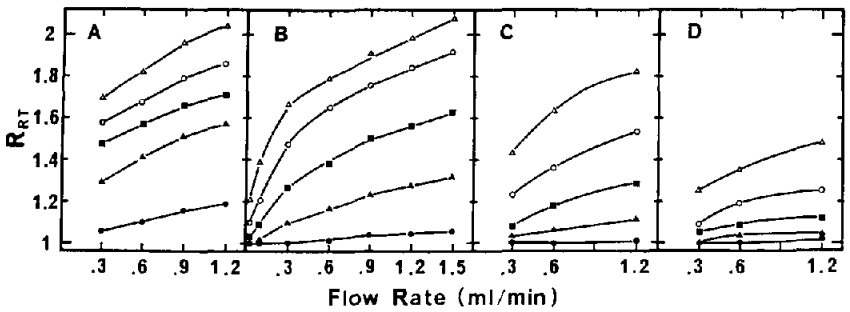

Fig. 8. Relationship between retardation of the fragments and flow-rate. Relative retention time (RRT) was plotted as a function of flow-rate. Asahipak GS-310 columns were used. Particle sizes: (A) 5 μm; (B) 9 μm; (C) 13 μm; (D) 19 μm. Fragments used: (●) 10.09 kbp; (▲) 17.05 kbp; (■) 23.13 kbp; (○) 29.95 kbp; (△) 38.42 kbp. (Reprinted with permission from ref. 52.)

permeation, their pore size was found to have no relation to the resolution. When columns packed with material of the same particle size (9 μm) but differing in pore size (*e.g.* Asahipak GS-220, 310 and 510: exclusion limit for proteins, M_r $3 \cdot 10^3$, $4 \cdot 10^4$ and $3 \cdot 10^5$, respectively) were compared, no significant differences in RRTs for DNA fragments were observed [52]. This suggests that DNA fragments do not permeate the pores.

The chemical nature of column packing was also proved to have no effect on separation. Comparative experiments carried out using silica-based media (TSK G2000SW and TSK G3000SW, 10 μm diameter) gave essentially the same results as those obtained with polymer-based media. Although they have different exclusion limits (for dextran: G2000SW, M_r $1 \cdot 10^5$; G3000SW, M_r 5×10^5), both separation patterns and flow-rate dependency were almost the same, and also very similar to those obtained for the column packed with 9-μm polymer particles [52].

Thus, it is evident that separation depends on the particle size and flow-rate,but not on the pore size or chemical nature of the packing.

6.2.4. Further evidence of absence of interaction between DNA fragments and column packing

There is evidence that the size-dependent separation of DNA is not due to electrostatic or hydrophobic interaction. The retention times of DNA fragments were not significantly affected by the addition of NaCl up to 0.5 *M* or 20% (v/v) acetonitrile. Moreover, separation by slalom chromatography was achieved even with cation-exchange columns, which should repel polynucleotides. Two cation-exchange columns bearing sulphopropyl groups, TSK-SP-5PW and TSK-SP-NPR (a non-porous polymer with a particle size as small as 2.5 μm (54]), were examined [52]. SP-5PW gave similar chromatograms to that obtained with Asahipak of corresponding particle size: a high-resolution zone in range 15–30 kbp. SP-NPR seems to resemble GS-310 packed with 5-μm particles to some degree. These results confirm that there is no interaction between the chromatographic media and DNA fragments.

6.2.5. Effect of temperature

Temperature has a singificant effect on the slalom mode separation. The RRTs of DNA fragments increase significantly when the temperature is lowered [52]. This suggests that the viscosity of the eluent has an important effect, probably because it determines the steepness of velocity gradient of laminar flow.

6.3. The present and future of slalom chromatography

6.3.1. Advantages and limitations of slalom chromatography

Slalom chromatography is a very useful tool in the field of DNA research. An example of the efficient separation of restriction fragments is shown in Fig. 9. At present, slalom chromatogra-

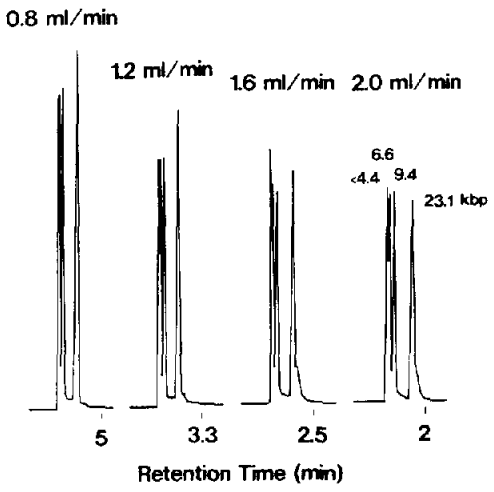

Fig. 9. Chromatography of *Hin*dIII digest of λ phage DNA on a Capcell Pak C_1 column (5-μm silica-based particles, 250 mm × 4.6 mm I.D., Shiseido) at different flow-rates (unpublished result).

phy is the only chromatographic method applicable to the size-dependent separation of large DNA molecules. It is unique because it is based on the hydrodynamic principle that the mobility of fibrous molecules in a column is determined by their length, and truly size-dependent separation occurs. Its advantages are summarized below.

(1) Both preparative and analytical use are possible. As an analytical procedure, it provides information on the length of polynucleotides.

(2) The experimental procedure is very simple and rapid. Ordinary HPLC apparatus and a gel permeation column are sufficient as equipment.

(3) Results can be easily predicted.

(4) Only isocratic elution programme is necessary, and there is no need for column washing or reequilibration.

(5) The separation and recovery of DNA fragments are very rapid in comparison with gel electrophoresis. Recovered DNA fragments are free of undesirable contamination originating from the Agarose gel.

(6) DNA can be detected without the use of a harmful reagent, such as ethidium bromide.

(7) This procedure provides a new effective tool for physicochemical and hydrodynamic studies of DNA.

On the other hand, there are also some limitations.

(1) At present, the range of separable sizes for DNA molecules is not wide: fragments of 5–50 kbp are separable using commercially available packing particles. However, further extensive studies will undoubtedly improve the situation.

(2) The resolution efficiency is still inferior to that of gel electrophoresis.

(3) The flow-rate must be relatively high. This may cause physical degradation of extremely large DNA fragments. However, DNA fragments of less than 50 kbp proved to be generally very stable under the conditions of most experiments (*e.g.* flow-rates less than 1.2 ml/min).

6.3.2. Comparison with other size separation methods for DNA

Pulsed-field gel electrophoresis is widely used for the size separation procedure of extremely large DNAs [55,56]. This technique is based on the difference in ability of DNA molecules to change direction of migration responding to a frequently changing electric field. Because larger DNA molecules take longer to reorient, size-dependent separation is achieved. This method has features in common with slalom chromatography: both are based on the ability of DNA molecules to adapt to a frequently changing environment (direction of electric field or flow) that depends on size. Although the size range of DNA separable by pulsed-field electrophoresis is much wider than that of slalom chromatography, the latter has a variety of merits (already pointed out), and thus the two methods are complementary. Capillary electrophoresis also seems to be very promising as a procedure for the size-dependent separation of nucleic acids. If the principles of slalom chromatography and capillary electrophoresis can be combined, it may become a much more effective procedure.

6.3.3. How to make the best use of slalom chromatography

Slalom chromatography is very efficient and

interesting from the viewpoints of both investigation and application. This principle will undoubtedly provide us with a valuable tool for nucleic acid research, as the invention of gel-permeation chromatography has contributed to the biological sciences, especially in the field of protein research. Although only applications to DNA have been reported so far, it sould be also useful for RNA research. Some possible applications are listed below.

(1) Size-dependent separation of DNA.

(2) Estimation of size of DNA.

(3) Monitoring and analysis of size change of DNA.

(4) Separation of DNA and RNA based on conformation, or topology.

(5) Analysis of interaction of DNA with other molecules, such as DNA-binding proteins.

(6) Distinction of types of circular DNA, *e.g.* super coil, relaxed and single strand.

(7) Studies of the physicochemical properties of nucleic acids, *e.g.* rigidity, elasticity, bendability, etc.

(8) Hydrodynamic studies of nucleic acids.

The principles of separation of all chromatographic modes currently applied to biomolecules are based on equilibrium phenomena occurring between the mobile and stationary phases, which are caused by such phenomena as electrostatic interaction, hydrophobic interaction, differential solubility, etc. A packing material that serves as a support for one of these phenomena is always needed. Slalom chromatography, however, is based on a completely different principle, *i.e.* the hydrodynamic phenomenon that is caused by a current of liquid passing through narrow spaces with a particular shape. The column packing serves only for the construction of these spaces. Therefore, this phenomenon is important from the viewpoint of the physicochemistry of macromolecules, and it should be investigated on the basis of hydrodynamic theory rather than equilibrium kinetics.

It was only the combination of hard, spherical packings and the application of high flow-rates that led to the observation of this phenomenon. It is very unlikely that slalom chromatography is the only technique that could be based on hydrodynamic principles. Extensive studies of a variety of fields will contribute greatly to the development of slalom chromatography and accelerate the discovery of a number of unknown separation mechanisms, and result in the expansion of the horizons of chromatography.

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