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Modern Methods for the Chromatographic Analysis of Nucleic Acids and Their Components

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The literature data for the chromatographic separation of nucleic acids and their components are considered in the review. Particular attention has been devoted to the commonest types of column chromatography, thin layer chromatography using different adsorbents, and electrophoresis. The applicability of each type of chromatography in the study of the primary nucleic acid structure and the formation of complexes by nucleic acids with natural products of other classes is demonstrated. The bibliography includes 597 references.

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I. INTRODUCTION

The development of modern biology, which has led to many important discoveries, would have been impossible without the development of microanalytical methods. Paper chromatography and electrophoresis and ion exchange column chromatography have played an outstanding role in the establishment of this science, permitting the elucidation of the most general characteristics of the structures of nucleic acids and proteins. However, further improvement in the extent of our knowledge and the acquisition of more detailed information in this field required the solution of specific problems in the isolation and determination of the structures of specific individual nucleic acid and protein molecules, only a few of which are sometimes present in the cell. The previous micromethods were relatively unsuitable for the solution of these new problems and, although the old procedures were still used in the determination of the structures of the first individual nucleic acids, the necessity for the development and utilisation of new, much more sensitive analytical methods became quite evident.

Nowadays the employment of thin-layer chromatography in combination with labelled compounds permits the analysis of amounts of compounds not less than 10^{-12} g. So far column chromatography has a lower sensitivity (10^{-7}—10^{-9} g) but it often permits the necessary spectroscopic analysis of the substance. The contribution of ultramicromethods to modern research is continuously increasing.

This review gives a general account of the advances in the field of analytical methods for nucleic acids in the last 10 years. Readers interested in work carried out prior to this can become acquainted with it in reviews published previously[1-5].

In the present review attention has been concentrated on ultramicromethods. Since the principles and chromatographic systems employed in ultramicroanalysis are general, the review includes also less sensitive methods which can be employed in ultramicroanalytical procedures.

The analytical methods for nucleic acids are general and can be used also in other fields of research. The authors hope that the review will be useful not only to chemists, physicists, and biologists working in the field of molecular biology but also to organic chemists employing micromethods in their work.

To facilitate the practical employment of the review, the data are divided into sections combining identical types of compounds to be analysed. The abbreviations and symbols for nucleic acids and their components employed in the review are those recommended by the IUPAC Commission and IUB.[6]

Large numbers of adsorbents of different types are used to analyse nucleic acids and their components. The detailed characteristics of these adsorbents and the names of the firms manufacturing them are given in Lur'e's handbook[7].

II. NUCLEIC ACIDS

The number of different types of nucleic acids in the cell is fairly large and it is therefore extremely difficult to isolate the individual components. Moreover, their limited solubility in aqueous solutions and their capacity for aggregation and non-specific interaction with sorbents greatly reduce the number of methods available for the fractionation of nucleic acids. However, fairly numerous systems are described in the literature for the chromatographic resolution of nucleic acids into individual components. Experience gained in their application shows that, for a successful isolation of a homogeneous substance, it is insufficient to employ a single chromatographic procedure. Stepwise fractionation of nucleic acids is most useful. The nucleic acid mixtures are initially divided into fractions with identical specific features—transfer RNA (tRNA), ribosomal RNA (rRNA), messenger RNA (mRNA), DNA, etc. Selective extraction, a combination of different precipitation methods, centrifugation[8], and chromatographic methods are frequently employed for this purpose. The nucleic acid fractions are then subjected to a more detailed resolution. Among chromatographic methods separation on ion-exchange resins, partition and affinity chromatography, and other procedures are most widely used.

1. ION-EXCHANGE CHROMATOGRAPHY

A. Adsorbents Containing Proteins

One of the types of such chromatography—fractionation on columns with methylated albumin sorbed on Kieselguhr (MAK-chromatography)—has become very common in the separation of all types of nucleic acids. Their mobility on MAK-columns is determined largely by three factors: (1) the length of the polynucleotide chain (the overall molecular charge), (2) the conformation of the nucleic acid in solution, and (3) its nucleotide composition. The first factor determines the strength of ionic binding of the substance chromatographed to the adsorbent. The second and third factors determine the degree of hydrophobic interaction and the interaction via hydrogen bonding. The chromatographic behaviour of nucleic acids on MAK columns shows that the second and third factors play a more significant role in the separation of nucleic acids.

During elution in an NaCl concentration gradient from 0.2 to 1.2 M, nucleic acids are separated into fractions corresponding to their functions in the cell: tRNA, DNA, etc.[9,10]. The separation is carried out at 15—25°C and sometimes at 35°C. An increase of temperature is undesirable, since part of the nucleotide material is then retained by the adsorbent. A decrease of temperature is likewise undesirable in view of the less clear-cut separation. One should recall that the NaCl concentration at which the given type of nucleic acid is eluted can vary for different batches of Kieselguhr and methylated albumin, which can be accounted for by the different degrees of methylation of the albumin. However, the elution sequence [tRNA—DNA—(rRNA, mRNA)] does not as a rule change[9-12].

The elution profile depends on the source of nucleic acid. Thus, in the separation of bacterial nucleic acids on MAK columns, tRNA and both types of rRNA (16S and 23S) are satisfactorily separated from one another and the rapidly labelled RNA are separated into several fractions. In nucleic acids from animal tissues the rRNAs are separated somewhat less satisfactorily and mRNAs are eluted as a mixed fraction[10-11]. If the nucleic acid is denatured, it is eluted from the column at higher salt concentrations than the native form. This is true of both DNA [13-15] and RNA.[16-18] The yield of substance from the column is usually 90—100% for native preparations, but is much lower for denatured nucleic acids (50—70%),[13,14] and when the column is overloaded the deoxynucleotides for example, may not be eluted at all.[19]

The optimal separation conditions on an MAK-column are 0.5—2 mg of the nucleic acid mixture per 10 ml of the MAK suspension with an overall volume of the gradient solution of 800 ml. The methods of preparation of the adsorbents and the chromatographic techniques have been described[9,10,20-25]. In the preparation of the columns one can employ both bovine albumin[10,20,23-25] and human albumin[26,27]. Kieselguhr can be replaced by silica gel[17,28-31] (MASA-chromatography); the adsorbent capacity with respect to the amount of nucleotide material is then significantly increased[30,32].

Chromatography on MAK and MASA columns is also used to separate the nucleic acid fractions into individual components. In the separation of DNA the position of the DNA being eluted depends on the length of the molecule, its conformation, and nucleotide composition. DNA with long molecules and containing more guanine—cytosine pairs (GC pairs) are eluted at higher salt concentrations[24,32]. Certain types of DNA, for example those of *B. subtilis* and calf and mouse thymus are eluted at the same salt concentrations[33]. The *E. coli* and T2 and T4 phage DNA are satisfactorily separated from one another. The T5 phage DNA can be resolved into two components[8,34]. Glycosylation of the DNA bases leads to firmer binding to the sorbent[35].

Chromatography on MAK and MASA columns has been widely used for the separation of the native and denatured forms of DNA.[13,15,36-43]. With the aid of this method, it is possible to isolate DNA-RNA hybrids, the replicated forms of phage DNA and complementary DNA strands[44-47]. MAK chromatography has been proposed by Japanese investigators for the separation of tRNAs [48] and has been successfully used for the fractionation of tRNAs [49-58] from a wide variety of sources[16,22,25,26,49-58] and of its chemically modified derivatives[30,31,59].

Basic proteins and polypeptides adsorbed on Kieselguhr are also used as supports for the chromatography of nucleic acids[60-67]. The chromatographic profile and the characteristics noted previously (the effect of the molecular weight, nucleotide composition, etc.) in fractionation on these adsorbents are the same as in chromatography on MAK and MASA columns[68-71]. An interesting modification of the method is the separation on Celite of a ribonucleoprotein complex (RNP complex)[72]. The RNP proteins are then adsorbed on Celite and are not eluted from the column, while the RNA is fractionated as in the separation on MAK. The chromatography of DNA on protein powders from wool has been described[73]. The behaviour of DNA on such columns resembles its chromatography on MAK.

It is noteworthy that the principle of the chromatography of nucleic acids on proteins sorbed on carriers of the type of Kieselguhr or silica gel is extremely promising, since in this case separation is determined primarily by specific adsorption and hydrogen bonding forces. We believe that the employment for these purposes of other proteins, for example, immunoglobulins, etc. might significantly extend the scope of the fractionation of nucleic acids.

B. Adsorbents Based on Cellulose and Sephadex

In the separation of nucleic acids on ion-exchange polysaccharides the binding of nucleic acids to the resin via ionic interactions is stronger than for adsorbents containing methylated and basic proteins. For this reason, the effect of other factors (conformation and composition of the nucleic acid and non-ionic interactions with sorbent) on the separation is significant only for nucleic acids with a low molecular weight. High-polymer nucleic acids are adsorbed by anion exchangers so strongly that their elution requires high salt concentrations. For this reason, the separation of DNA and high-polymer RNA on ion-exchange celluloses and different types of Sephadex is comparatively rare[8,74,75]. These adsorbents have found extensive applications only in the fractionation of tRNA.[76-78] The chromatography of tRNA on diethylaminoethylcellulose (DEAE-cellulose)[76-85], triethylaminoethylcellulose (TEAE-cellulose)[86], and DEAE-Sephadex[76-78,87-99] has been used in numerous modifications (Table 1).

The behaviour of tRNA from *E. coli* and yeast on ion exchangers has been most thoroughly investigated. tRNA from *E. coli* is eluted from the column at higher salt concentrations, but the elution sequence of the tRNAs in terms

of their specificity with respect to a particular aminoacid is retained. Chromatography on polysaccharide anion exchangers is nowadays used in the first stage of the purification of tRNA. The method permits the separation in a single step of up to 100 g of the total tRNA.[100]

Table 1. Conditions in the separation of tRNA on ion-exchange resins.

Adsorbent	Separation conditions	Concn. of eluent for tRNA		Refs.
		from *E.coli*	from yeast	
DEAE-cellulose	pH 5.0; salt gradient	0.4-0.7 M NaCl	0.4-0.65 M NaCl	76
	pH 7.5; urea, salt gradient	0.33-0.40 M NaCl	0.28-0.35 M NaCl	76
	pH 7.5; 0.36 M NaCl, urea gradient	2-5 M urea	2-6 M urea	76
	pH 4.2; 7 M urea	0.35-0.45 M NaCl	0.30-0.40 M NaCl	76
	pH 4.2; 0.40 M NaCl urea gradient	1-6 M urea	1-6M urea	76
	pH 4.2; 4 M urea, salt gradient	0.42-0.53 M NaCl	0.38-0.50 M NaCl	76
	pH 6.0; 65°, salt gradient	0.8-1.6 M NaCl	0.8-1.3 M NaCl	77
TEAE-cellulose	pH 3.6; 38-40°, 7 M urea, salt gradient	–	0.35-0.55 M NaCl	86
DEAE-Sephadex	pH 6.0; 0.9 M NaCl, temperature gradient	65-20°	65-20°	77
	pH 7.5; 7 M urea, salt gradient	0.45-0.65 M NaCl	0.40-0.60 M NaCl	76
	pH 4.2; 7 M urea, salt gradient	0.55-0.75 M NaCl	0.49-0.65 M NaCl	76
	pH 4.5; salt gradient	0.5-0.80 M NaCl	0.5-0.85 M NaCl	76
	pH 5.3; 1% dimethylformamide, salt gradient	0.75-1.6 M $(NH_4)_2SO_4$*	–	94
	pH 6.2; 5% dimethyl formamide, 1 M phosphate 33°, salt gradient	0.45-0.4 M KCl*	–	94
	pH 7.5; salt gradient		0.37-0.52 M NaCl	95
	pH 7.2; salt gradient	0.375-0.525 M NaCl**	0.008-0.016 M $HgCl_2$	97

* tRNA from *I. utilis*.
** tRNA from rat liver.

The most effective separation of tRNA takes place in chromatography of benzoylated DEAE-cellulose (BD-cellulose). The use of this hydrophobic adsorbent increases the contribution of adsorption forces to the chromatographic separation process. BD-cellulose was first proposed for the separation of tRNA by Tener's group[101,102]. Effective fractionation of tRNA is observed (Fig. 1) for elution by salt solutions at low temperatures. The method has been widely used to isolate the individual tRNAs.[95,97,103-109]. The chromatographic profiles for tRNAs from different sources mainly give rise to similar patterns. Phe-, Cys-, Tyr-, Ser-, and Leu-specific tRNAs are retained on BD-cellulose more strongly than other tRNAs. Phenylalanine tRNA from yeast and rat liver is eluted at higher salt concentrations than the analogous tRNAs from *E. coli*[102,107] and halophilic bacteria[109]. The lower degree of adsorption can probably be accounted for by the absence from these tRNAs of the hydrophobic base Y, which has been found in the $tRNA^{Phe}$ from yeast and rat liver.

The employment of additional procedures—gradient elution by alcohol[110,111] and ether[112,113] solutions—in many instances improves the separation and increases the elution from the column. BD-cellulose proved to be extremely effective in the chromatography of DNA and other types of RNA.[8,114-117] A sorbent analogous to BD-cellulose was proposed recently by Osterman for the fractionation of tRNA.[118]

C. Hydroxyapatite

Hydroxyapatite (HA) is a weak anion exchanger, which permits its employment for the chromatography of anions. The range of applications of HA chromatography is fairly considerable: the behaviour of low- and high-molecular-weight and RNA and DNA from different sources on HA columns has been thoroughly investigated[119-144]. In the elution of nucleic acids from HA columns one usually employs a phosphate buffer at pH 6.8 (Table 2). However, more acid solutions have also been used for elution.

Table 2. The concentration of phosphate buffer and the recovery of nucleotide material from the column in chromatography on HA.

Substance	Concn. of phosphate buffer at pH 6.8, M	Recovery from column, %
Mononucleotides	0.001	100
Oligonucleotides	0.01—0.02	100
Polynucleotides		
polypyrimidine	0.1—0.12	100
polypurine	0.22—0.25	60—80
Polynucleotides in 1% formaldehyde	0.05—0.17	100
RNA	0.11—0.18	100
Denatured DNA	0.11—0.18	50—100
Native DNA		
from higher organisms	0.11—0.18	50—100
from bacteria	0.20—0.22	90
from phages	0.25—0.27	90

A significant factor in chromatography on HA is that the adsorption of nucleic acids is almost independent of the concentration of anions other than phosphate. For this reason, this method is very convenient in rechromatography after other procedures involving the separation and concentration of nucleic acids in salt solutions. One must make certain that the solutions do not contain substances forming complexes with Ca^{2+}. If a phosphate buffer containing other anions (for example Cl^-) is used in the elution of the test substance from HA columns, the concentration of phosphate at which nucleic acids are eluted from the column increases.[138,144] The separation is usually carried out at +4°C or at about 20°C.

In most cases the separation is independent of temperature. However, for certain polynucleotides, for example polyuridylic and polycytidylic, which have an ordered structure at low temperatures, the eluting concentration is higher at low temperatures than at 20°C.[138] An impression has been created that a polynucleotide with an ordered structure is eluted after the same polynucleotide whose secondary and tertiary structures have been disrupted[128,138,139,144]. The method permits the separation of denatured and native DNA,[128,135,139,144-154] of different isoacceptor tRNA,[123,129,130,131,133,140,143,155,156] and different

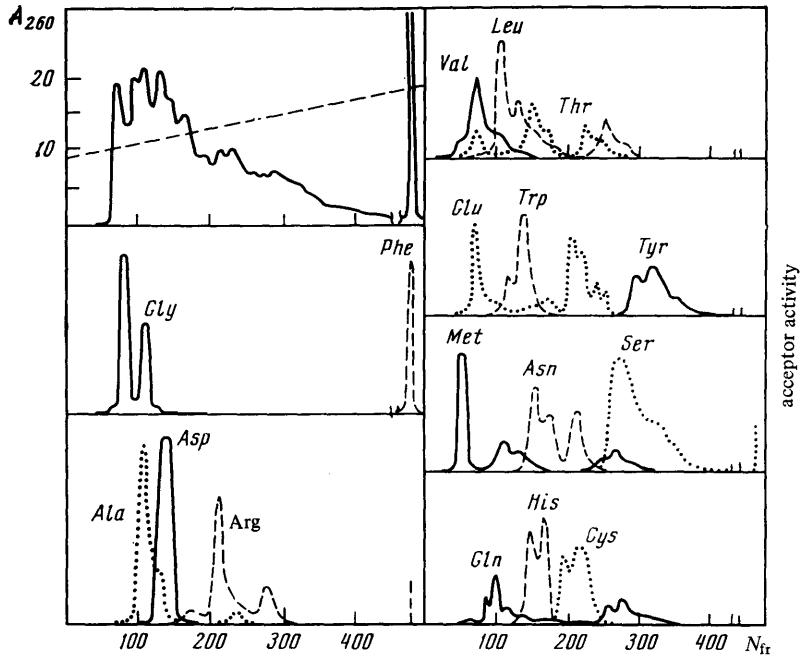


Figure 1. Fractionation of tRNA from *E. coli* on BD-cellulose[102].

forms of cyclic DNA.[157,158] Glycosylated DNA from T-even phages are retained more firmly on HA than the DNA from *E. coli* or from T-odd phages[138,159].

The behaviour of DNA on HA columns depends also on the nucleotide composition. The greater the number of GC pairs in the molecule the more firmly is it bound to HA.[128,139,144]

Both gradient and stepwise elution from HA has been used for the fractionation of nucleotide material. In connection with the stepwise fractionation of nucleic acids on HA, one should bear in mind the presence of "false fractions", i.e. fractions of the substance which on rechromatography are eluted at a lower molarity of the phosphate buffer[128,144]. To avoid this, it is useful to employ gradient elution.

A satisfactory separation requires 10 ml of HA per milligram of the nucleotide material for an overall volume of the gradient eluent of 200—300 ml. The optimal rate of elution is 0.1—0.2 ml min^{-1} cm^{-2}. The preparation of the sorbent and the chromatographic technique have been described[138,144,160-162].

D. Cation Exchangers with Charges Balanced by Polyvalent Metal Cations

Ion exchangers with charges balanced by the cations of polyvalent metals should have properties resembling those of weak anion exchangers of the type of HA. It has been noted that DNA is adsorbed from solutions by Amberlite IRC-50 electrically neutralised by Mg^{2+}. On elution, it was possible to isolate several DNA fractions differing in composition and sedimentation coefficient[163,164]. Chromatography of DNA on Amberlite IR-120 columns electrically neutralised by Al^{3+} leads to the isolation of seven DNA fractions differing sharply in their chromatographic properties[165-167]. The separation profile is independent of the protein impurities[168], RNA,[169] and the pH,[170] but does vary markedly as a function of the method of isolation of DNA,[171] which is probably due to the degradation of DNA during its isolation.

Chromatography on cation exchangers electrically neutralised by Al^{3+} appears to be very promising, since it permits the separation under mild conditions and does not entail the degradation of DNA. The elution of the nucleotide material from the resin is complete.

2. COUNTERCURRENT DISTRIBUTION AND REVERSED-PHASE CHROMATOGRAPHY

The separation of nucleic acids by countercurrent distribution has been proposed at the beginning of the 1960's and immediately proved useful as a qualitative method for the fractionation of DNA and different types of RNA.[172-179] This chromatographic technique has been most widely used for tRNA.[180-182] However, the complexity of the equipment, the small amounts of substances separated, and the fairly low resolving power greatly limited the scope of the method. The principle of countercurrent distribution has been used in chromatography in aqueous organic systems on different adsorbents. The adsorbent is saturated by the organic phase and is then transferred to a column and equilibrated with the aqueous phase. Systems for this type of chromatography were initially taken from studies on the countercurrent distribution of nucleic acids[183-190]. Partition chromatography on columns (reversed-phase chromatography) eliminated the above disadvantages of countercurrent distribution.

The introduction into the organic phase of ammonium salts containing aliphatic hydrocarbon residues significantly improved the resolving power of the method[191-194]. Chromatography on Chromosorb impregnated with tricaprylmethylammonium bromide in tetrafluoropropane (Freon) has been most widely used[192,195-198]. The application of a special type of PTFE impregnated with trioctylpropylammonium bromide[199,200], which was proposed recently, permits a satisfactory separation of tRNA (Fig. 2). The separation curves clearly show that the resolution of tRNA in the "reversed phase" is very promising.

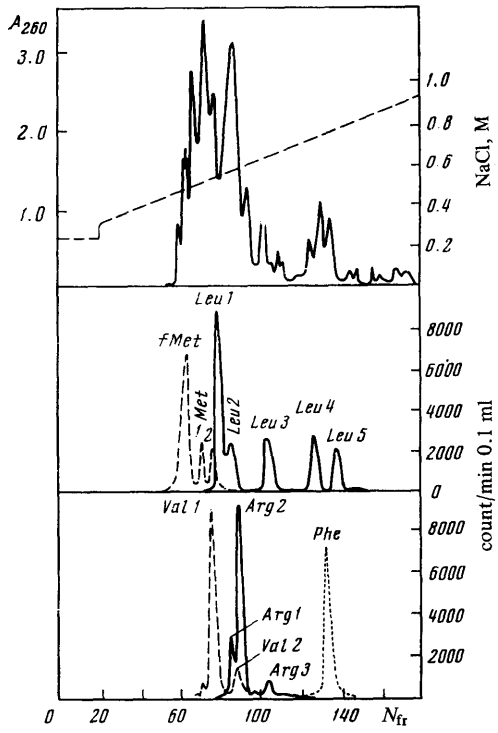


Figure 2. Chromatography of tRNA from *E. coli* on PTFE.[199]

It is noteworthy that reversed-phase chromatography can probably be used also for the fractionation of other types of nucleic acids, since it is well known that aliphatic amine salts of different types of nucleic acids are soluble in aqueous solutions at different NaCl concentrations[201].

3. AFFINITY CHROMATOGRAPHY

The name of this method comes from the English word "affinity", since this type of chromatography employs the interaction between two substances exhibiting affinity with respect to one another. One of these is in solution and the other is on an insoluble matrix (adsorbent). The test mixture is passed through a column containing the adsorbent under optimal conditions for the interaction of the fixed substance and one of the mixture components. In this interaction the adsorbent retains only the component which exhibits affinity with respect to the substance on the adsorbent. The remaining mixture components are not retained. Thus the substance exhibiting affinity with respect to that fixed on the adsorbent is separated from the remaining components of the mixture.

The application of affinity chromatography for the separation of polynucleotides and nucleic acids dates back to 1962 when two types of adsorbents were proposed at the same time. The first of these comprised gels containing nucleic acids (mechanical adsorption)[202] and the second consisted of cellulose with mono- and poly-nucleotides bound by covalent bonds (chemical binding)[203,204]. Mechanical adsorption in agar–agar[202,205-208], acetylated phosphocellulose[202], and polyacrylamide[209] gels permits the preparation of sorbents with a high content of nucleotide material. Nucleic acids and polynucleotides are within the grains.

The gels exhibit a high selectivity with respect to mRNA and adsorb from solutions only their "own" mRNA, while "foreign" mRNA is not retained[202-208]. The retained mRNA can be removed from the gels by a temperature gradient elution or by reducing the ionic strength. The mRNA fractions with a high GC content are then eluted from the column at a higher temperature[207]. Polyuridylic acid in polyacrylamide gel forms complexes with oligo- and poly-adenylic acids[209].

The simplicity of the preparation of the gels containing polynucleotides and the fairly mild conditions in their preparation, which tend to preserve the native form of nucleic acids, constitute undoubted advantages of the mechanical method of preparation of affinity adsorbents. It is noteworthy that in this case adsorption from solutions can be observed only for substances with a lower molecular weight than that of the nucleotide in the gel. This disadvantage can be eliminated by employing gels whose grains are coated on the outside by polynucleotide molecules and are then "cross-linked" to one another under the influence of ultraviolet irradiation[210-212]. It is noteworthy that the gels frequently have a low mechanical strength. These disadvantages must be borne in mind in experiments with these materials.

Nitrocellulose is also used as an adsorbent for the affinity chromatography of nucleic acids. Under certain conditions, nitrocellulose is known to bind irreversibly denatured DNA. The nature of the binding is unknown, but neither native DNA nor RNA are retained by nitrocellulose. Low-molecular-weight fragments of denatured DNA are not sorbed by nitrocellulose[213]. Denatured DNA immobilised by nitrocellulose binds selectivity polynucleotides with affinity for DNA. This method can be used to investigate the formation of complexes with DNA.[214-223]

Proteins immobilised by nitrocellulose, particularly the aminoacids of tRNA ligase, permit the investigation of the complexes of these enzymes with tRNA and the isolation of enriched tRNA preparations[224,225].

Mention should also be made of DNA immobilised by cellulose as a mechanical affinity sorbent[225,226]. However, this sorbent is so far used widely only for the purification of proteins forming specific complexes with nucleic acids[226-229].

The affinity chromatography of nucleic acids and polynucleotides has been widely used on sorbents containing covalently bound polynucleotides. Such sorbents are synthesised by the condensation of cellulose or phosphocellulose with mono-[203,230-232], oligo-, and poly-nucleotides[204,230,232-235], RNA,[204,230,232] and DNA [236-238] in the presence of dicyclohexylcarbodiimide or Sepharose treated

with cyanogen bromide[239]. RNA—DNA complexes[236-238], the purification of tRNA,[235] and the separation of poly- and oligo-nucleotides[231,233-235] can be investigated by chromatography on such adsorbents.

The employment of enzymes covalently bound to Sephadex[240], and of mercury-containing adsorbents which are capable of specific binding of DNP complexes containing SH groups[241,242], for the chromatography of nucleic acids has also been described. To isolate polynucleotides with a free *cis*-glycol group at the 3′-end of the molecule, one can use borate-containing cellulose[243,244].

Thus it is evident that affinity chromatography can be used successfully to fractionate various nucleic acids and polynucleotides. In contrast to the methods described previously, this type of chromatography makes it possible to employ more refined mechanisms for separation—the affinity of the substance to be isolated for a specially prepared adsorbent. In the application of this affinity, one can use a specific reactive group (the affinity of the SH groups of DNA for the terminal ribose residue), the complementary interaction forces (RNA—DNA complexes, polynucleotide interactions), and the formation of complexes with proteins.

4. GEL FILTRATION AND OTHER SEPARATION METHODS

The separation of nucleic acids on Sephadex and polyacrylamide gels is used only for the partial elimination of other types of nucleic acids and low-molecular-weight compounds. Gel chromatography has been most widely used for the purification of tRNA [245-255] and even partial fractionation of tRNA is then observed[256,257]. The method is likewise suitable for the isolation of large tRNA fragments and for the investigation of their interaction with one another[258,259]. Gel filtration is of limited applicability to nucleic acids with large molecular weights, since their aqueous solutions are very viscous, which greatly hinders chromatographic separation by this procedure.

Nucleic acid fractionation methods, such as paper and thin-layer chromatography, have found only limited applications[260-262].

III. OLIGONUCLEOTIDES

Recently the development of methods for the fractionation of oligonucleotides has been determined by trends associated with the elucidation of the structures of nucleic acids and their fragments responsible for important biochemical functions and by studies on the synthesis of oligonucleotides and nucleic acids. The advances in this field (the determination of the structures of more than 50 tRNAs, of several 5S RNAs, and of large fragments of ribosomal and viral RNA, the synthesis of the DNA copy of alanine tRNA, etc.) can be accounted for in many respects by the achievements in the development of micromethods for the fractionation of oligonucleotides, Column, paper, and thin-layer ion-exchange chromatography, partition paper and thin-layer chromatography, and combinations of these methods with electrophoresis have found extensive applications in the separation of nucleotides.

1. ION-EXCHANGE CHROMATOGRAPHY

The method has a high resolving power and can be used on both preparative and analytical scales. The main factor determining separation is the overall charge of the nucleotide. This quantity can be readily calculated[263,264] at different pH using the pK of the ionic groups of the nucleotides. Since the force of binding to the adsorbent is determined primarily by the charge of the oligonucleotide (the higher the charge the stronger the binding), it follows that, having determined this quantity, it is possible to predice the mobility of the oligonucleotides relative to one another under the conditions of the separation. The sequence of the elution of the oligonucleotides from the column based on the calculation of the charges will undoubtedly be approximate, since, apart from the ionic interaction with the adsorbent, hydrophobic and hydrogen bonding interactions are also observed. However, calculation of the charges of the nucleotides is in many cases undoubtedly useful, since it permits the selection of the optimal pH for the separation.

The literature data for oligonucleotide chromatographic fractionation systems lead to the conclusion that single-stage ion-exchange column chromatography yields satisfactory results only for simple mixtures of oligonucleotides and is insufficiently effective for complex mixtures. In such cases repeated fractionation is necessary.

The principles of the selection of the binary systems (two-dimensional column chromatography), put forward in 1963,[263] proved to be justified in the separation of complex oligonucleotide mixtures[265-270].

In the selection of the binary systems it is desirable to employ different types of adsorbents and to carry out the separation at significantly different pH values. Satisfactory results have been obtained in the successive separation at initially neutral pH values in aqueous solutions of salts in 7 M urea (chromatography by Tener's method)[271-273] on DEAE-cellulose or TEAE-Sephadex and then either in an acid or in an alkaline medium for adsorbents of any type. In 7 M urea solution in the pH range 5.4—8.0 mixtures of oligonucleotides are resolved into fractions, each of which contains an identical number of nucleotide residues (mono-, di-, and tri-nucleotides, etc.). This system can be used to separate both ribo- and dioxyribo-oligonucleotides[271-276] and gives a satisfactory resolution of the fractions down to decanucleotides. Other conditions being equal, best results are achieved on DEAE-Sephadex[266,270]. The use of urea is not obligatory for homo-oligonucleotides[277,278].

The separation in an acid medium is usually carried out at pH 2—5. Among the adsorbents which give rise to the best results mention may be made of Dowex-1,[263,268,279] DEAE-cellulose[275,280,281], DEAE-Sephadex[263,264,270], and TEAE-cellulose[282]. DEAE-Sephadex and DEAE-cellulose are as a rule employed in fractionation in an alkaline medium. A satisfactory separation is observed at pH 8.5—9.0.[263,283,284] As an example of the two-dimensional fractionation of oligonucleotides, one may quote the separation of the oligonucleotides in the pyrimidyl-ribonuclease (pyridyl-ribonuclease) hydrolysate of tRNA^{Val} from yeast[285] (Fig. 3).

It is noteworthy that in both acid and alkaline media the individual isomeric oligonucleotides are separated[263,286].

A few words should be said about chromatography on hydroxyapatite. This sorbent was proposed recently for oligonucleotides[144,287,288]. The efficiency of the separation of oligonucleotides on HA in 7 M urea in a phosphate buffer concentration gradient (0.001—0.2 M) at pH 6.8 is higher than in chromatography by Tener's method on DEAE-Sephadex.

In order to achieve a satisfactory resolution in the fractionation of oligonucleotides on ion-exchange resins,

the separation is usually carried out on columns with a diameter : height ratio in the range from 1 : 10 to 1 : 50, an amount of the gradient eluent equivalent to 100—200 column volumes, and a rate of elution of 0.2—0.6 ml min^{-1} cm^{-2}. It is then desirable to employ an adsorbent whose particles have the same dimensions. The accounts of methods of fractionation of ion exchangers with respect to dimensions published by Hamilton[289] and Koprda and Fojtik[290] may be recommended. The homogeneity of the dimensions of the adsorbent particles (a scatter of 5—10 μm) is particularly necessary in experiments on columns and on the ultramicroscale.

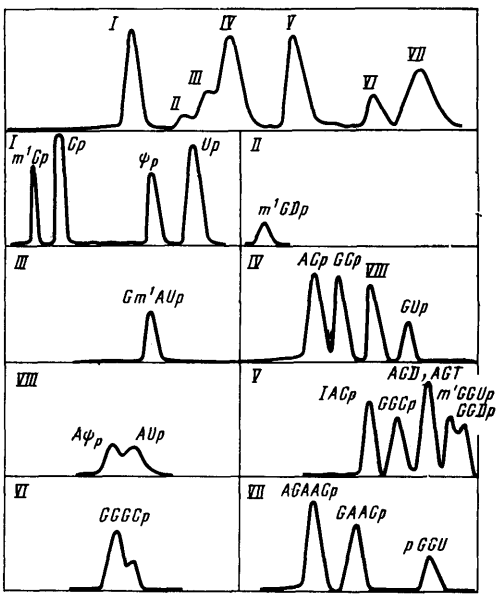


Figure 3. Separation of the oligonucleotides in the pyrimidyl-ribonuclease hydrolysate of $tRNA_1^{Val}$ from yeast on an ultramicroscale[285].

The sensitivity of the chromatographic column methods may be increased in two ways—by increasing the sensitivity of the detector[291] and by reducing the scale of the chromatographic process. The decrease of the chromatographic scale permits the reduction of the amount of the substances to be separated by 2—3 powers of 10 for the same detector sensitivity[285,291]. Thus, in experiments on 0.6 × 100 mm columns and with 300 μl of the gradient eluent, it is possible to separate 10^{-7}—10^{-9} mole of nucleic acids and oligonucleotides[285,292-294]. A significant factor in experiments on the ultramicroscale is that published chromatographic systems, tested on large amounts of substances[285,292-294], can be used.

The fractionation of oligonucleotides on ion-exchange papers[295-298] is less common than column chromatography, since it does not allow the application of gradient elution and has a lower resolving power. Moreoever, the low mechanical strength of ion-exchange paper makes this method relatively unpromising. As a result, chromatography on ion-exchange papers is hardly used nowadays as an independent method.

The disadvantages of fractionation on ion-exchange papers were eliminated by the fractionation of oligonucleotides in thin layers of ion-exchange cellulose. The application of this method to the components of nucleic acids is due to Randerath[299-302]. The ease of preparation of the sorbents and the simplicity of the preparation of the plates make this method extremely attractive. The thin-layer chromatography of oligonucleotides is usually carried out on polyethyleneimine cellulose (PEI-cellulose) in two-dimensions. The sensitivity of the method with the detection of fractions based on ultraviolet absorption is extremely high, amounting to 0.5—1 μg of the substance in the spot and can be significantly improved by using radioactive preparations *in vivo* or by the artificial introduction of a radioactive label, as suggested for oligonucleotides[303,304]. Thin-layer chromatography on PEI-cellulose is distinguished by a high resolving power[305-307] (Fig. 4).and does not require either complicated equipment or long separation times. Moreover, mixtures of PEI-cellulose with other adsorbents, for example with cellulose[308], can be used to prepare the plates, which gives rise to great possibilities for varying the separation conditions.

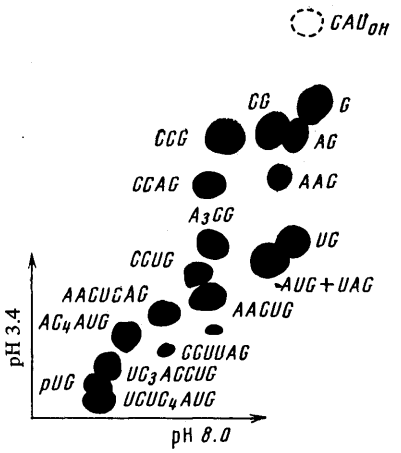


Figure 4. Separation of the pyrimidyl-ribonuclease hydrolysate of 5S RNA from *E. coli* on PEI-cellulose plates[307].

Various salt systems are used to develop the chromatograms[305,306,309,310]. To facilitate the experiment, one frequently employs volatile buffer solutions based on pyridine, or based on lithium salts, since these are effectively extracted by methanol. This makes it easy to desalinate the oligonucleotides directly on the plates. Unfortunately, thin-layer chromatography on PEI-cellulose is still not used on a sufficiently large scale for the fractionation of oligonucleotides. However, advances in the separation of various mixtures of oligonucleotides[305-310], studies on the formation of complexes by polynucleotides with oligonucleotides[311], and also the excellent results obtained in the separation of complex mixtures of long oligonucleotides in combination with electrophoresis (see below) make it possible to state confidently that the fractionation of oligonucleotides in

thin layers of PEI-cellulose is one of the most promising separation procedures. The preparation of adsorbents for thin-layer chromatography and the technique of the separation have been described[305,312-314].

2. PARTITION CHROMATOGRAPHY

The chromatography of oligonucleotides on various adsorbents in aqueous organic media is not a new method. It was discovered at the beginning of the 1950's but it still has not lost its importance in the study of nucleic acids and their components. The classical version of this chromatography—two dimensional paper chromatography—has played a significant role in the study of the structure of tRNA [315-319] and in the analysis of the cleavage products of DNA.[320,321] The commonest solvent system is isobutyric acid—0.5 M NH_3 at pH 3.7 for chromatography in one direction and either a 1:1 butanol—formic acid mixture at pH 3.8 [315,317-319,321] or a mixture of butanol with volatile buffers[316,320,322] for chromatography in the other direction. Apart from the above systems, solvents containing acetonitrile[323] and ethanol[324] can be recommended for the separation of simple mixtures of oligonucleotides. The knowledge or the R_f values in different systems for synthetic oligonucleotides and their derivatives may be of great assistance to chemists engaged in synthesis[325-336].

The solvent systems used in paper chromatography have proved extremely effective also in the separation of oligonucleotides in thin layers of cellulose[337-339] and silica gel[336,340]. Nevertheless partition chromatography is nowadays rarely used, since, in view of the availability of other methods of separation, the difficulties associated with the desalination of the oligonucleotides before and after separation make the experiments on the separation of oligonucleotides by this procedure extremely laborious.

3. ELECTROPHORESIS AND VARIOUS COMBINATIONS OF CHROMATOGRAPHIC METHODS

The presence of a negative charge on oligonucleotides permits their separation by electrophoresis. At a potential gradient E the oligonucleotide molecule migrates to the anode. The rate of migration V is described fairly satisfactorily by the equation $V = EQ/K$, where E is the potential gradient, Q the overall charge of the oligonucleotides, and K a constant which depends on the dimensions and shape of the molecule and on the medium in which the separation takes place (adsorbent, composition of the ions, etc.). K increases with the ionic strength of the buffer solution in which the separation takes place and decreases with increase of temperature[341,342]. If the electrophoresis is carried out on a neutral carrier, for example on paper or in a thin layer of cellulose, the rate of migration is higher the higher the overall charge of the substance. A different picture arises when the electrophoresis is carried out on ion-exchange columns. In this case the rate of migration of the substance is higher the smaller its charge. The decrease of the rate of migration of substances with a large charge is due to the fact that they are more firmly retained by the ionic groups of the carrier. Both types of carriers are used to fractionate oligonucleotides.

Best results are obtained when the electrophoresis is carried out in an acid medium in two perpendicular directions on acetylated cellulose and DEAE-paper. In this version the method was proposed for the first time by Sanger et al.[343] for [^{32}P]-oligonucleotides. The separation on acetylated cellulose is carried out in a pyridine—acetate buffer at pH 3.5 or in the same buffer in 7 M urea[343-348]. The technique of the electrophoresis has been described[342-354].

DEAE-paper[349] at pH 9.7 is also used instead of cellulose acetate. The replacement of the acetylated cellulose by Whatman 3 MM paper leads to somewhat worse separation[350].

The solvents employed for separation in the second direction are a mixture of 2.5% formic acid and 8.7% acetic acid (by volume) at pH 1.9, 7% formic acid[344-347], or a buffer solvent at pH 3.5 and 6.5.[346,349] The electrophoresis is carried out on DEAE-cellulose[343-349], aminoethylcellulose (AE-cellulose) and ECTEOLA-cellulose[349].

Sanger's method gives a satisfactory separation with a resolution of up to 200 fractions of oligonucleotides (Fig. 5) and has been widely used to analyse the structures of nucleic acids.

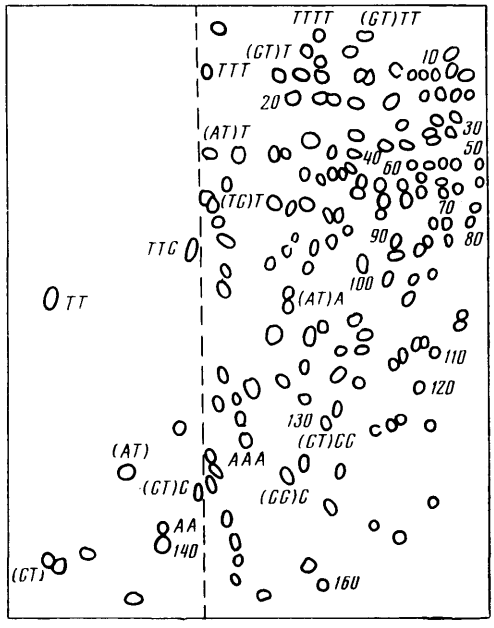


Figure 5. Separation of [^{32}P]-deoxyribo-oligonucleotides by two-dimensional high-voltage electrophoresis[349].

Interesting modifications of this method involve the employment of the electrophoresis in combination with ion-exchange chromatography on thin layers of PEI-cellulose[306,308] and the "homochromatographic" method[351-356]. The essential feature of "homochromatography" is as follows. When a plate with a thin layer of ion-exchange cellulose is placed with one end dipping in a solution of a mixture of oligonucleotides and the solution is allowed to rise a certain distance, the oligonucleotides are separated in the following way along the plate. At the top of the plate are the mononucleotides with the dinucleotides below, etc. i.e. the oligonucleotides are distributed in bands of equal chain length.

Hence the name of the method—"homochromatography". It is noteworthy that this distribution in bands of substances of equal chain length is possible only if the amount of oligonucleotide is greater than the capacity of the ion exchanger bands, since only then would longer oligonucleotides displace shorter oligonucleotides in consequence of their less strong binding to the ion exchanger.

In the chromatography of [^{32}P]-oligonucleotides in "homomixtures" of non-radioactive oligonucleotides an effective separation of long oligonucleotides (down to 40–50 nucleotide residues and less) is achieved. The fractionation is carried out at elevated temperatures (50–60°C), which gives rise to better equilibration and rules out the possibility of the formation of complexes by complementary oligonucleotides. The compositions of the "homomixtures" have been published[294,345,351,352].

The two-dimensional method for the fractionation of oligonucleotides permitted the determination of the structures of a number of tRNA,[354,357-367] low-molecular-weight RNA,[317,351,368-370] large fragments of 16S and 23S ribosomal RNA,[346,371,372,379] and phage RNA.[353,373-378] The possibility of the employment of this method for the analysis of the DNA structure has also been demonstrated[349,355,356]. One disadvantage, associated with the impossibility of identifying the substance by ultraviolet spectroscopy, which is particularly important for minor components, is not of fundamental importance. For nucleic acids which are difficult to obtain in a radioactive form, a radioactive label may be introduced into the terminal nucleotide[303,304,379], which greatly extends the scope of the method. The method is undoubtedly one of the best for the fractionation of oligonucleotides.

IV. NUCLEOTIDES

1. THIN-LAYER CHROMATOGRAPHY

A. Cellulose

This method is apparently most widely used at present for the analysis of mixtures of nucleotides, nucleosides, and nucleic acid bases. The preparation of cellulose layers has been described in fair detail in reviews[305,380-383] and other publications[384-387].

Randerath carried out a comparative separation of nucleotides in a thin layer of cellulose and on paper in four different solvents under identical conditions[384,388]. He established that the solvent systems developed for paper can be used for separation on a thin layer.

A multiplicity of systems have been used for the fractionation of nucleotides in a thin cellulose layer[380,382,385-390]. The corresponding R_f values for certain solvents are listed in Table 3. In most cases, the nucleotides are separated both with respect to the bases and with respect to the carbohydrate residue. To achieve a satisfactory reproducibility of the results, the specimen to be chromatographed must be neutralised and freed from salts and proteins[385]. In the separation of nucleotides with similar R_f values one can re-run the solvent in the same direction.

Nucleotides are satisfactorily separated by two-dimensional chromatography[385,390-395] or by a combination of thin-layer chromatography and thin-layer electrophoresis[337,396-398]. For example, Bergquist[337] separated the enzymatic tRNA hydrolysate by electrophoresis in one direction (phosphate buffer, pH 2.45), then by chromatography in another direction using the 50 : 50 : 2 t-butyl alcohol–0.08 M formic acid–isopentyl alcohol solvent system, and finally by chromatography again in the same direction using the 86 : 14 n-butyl alcohol–water solvent system. All four ribonucleotides and the isomeric 5'- and 3'-phosphates of adenylic and guanylic acids were separated by thin-layer electrophoresis and subsequent chromatography on cellulose using the 80 : 18 : 2 saturated ammonium sulphate–1 M sodium acetate–isopropyl alcohol solvent system[396]. This method was also used for determining the nucleotides composition of RNA. The R_f values for each nucleotide have been published[396].

Table 3. The R_f values in the chromatography of ribonucleotides and deoxyribonucleotides in a thin layer of cellulose[385,395].

Nucleotide	Solvent I	Solvent II	Solvent III	Solvent IV	Nucleotide	Solvent I	Solvent II	Solvent III	Solvent IV
AMP	0.34	0.34	—	0.38	dAMP	0.25	0.27	0.07	0.54
CMP	0.39	0.73	—	0.34	dCMP	0.53	0.63	0.09	0.49
GMP	0.26	0.53	—	—	dGMP	0.15	0.47	0.05	0.33
UMP	0.62	0.71	—	0.37	dTMP	0.89	0.54	0.18	0.58

Solvent composition: I) Isopropyl alcohol–HCl–water (65 : 16.7 : 18.3); II) n-butyl alcohol–water (86 : 14); III) n-butyl alcohol–water–90% formic acid (60 : 30 : 10); IV) n-butyl alcohol–acetone–acetic acid–5% aqueous ammonia–water (3.5 : 2.5 : 1.5 : 1.5 : 1.0 for deoxyribonucleotides and 4.5 : 1.5 : 1 : 1 : 2 for ribonucleotides).

Two-dimensional chromatography in various solvent systems permits a successful separation of the alkaline hydrolysates of RNA [396-400] or enzymatic hydrolysates of DNA.[401]

B. Silica Gel

Among inorganic sorbents, silica gel is frequently used in the chromatographic analysis of nucleic acid components, usually without a binder[402-420], although in some cases binders are also employed[421-428]. The separation on silica gel is as a rule poorer than on cellulose[300,381,384,426,427] and therefore it is rarely employed for analytical purposes and for the separation of unprotected derivatives. However, since the capacity of silica gel layers greatly exceeds that of cellulose, separation on silica gel is frequently used for preparative purposes, particularly in the case of synthetic derivatives of nucleic acids[402,415,418,419,426]. Various silica gels are employed for the separation[426-432]. The R_f values for nucleotides have been published[426]. Nucleoside 2'(3')-monophosphates can be separated from the 5'-phosphates but neither can be separated with respect to the bases. When the solvent is rerun in the same direction, the separation is improved. Silica gel treated with 5% tetraborate has been suggested for the fractionation of a mixture of adenosine, 3'- and 5'-adenylic acid, and cyclic AMP.[411]

The separation of the nucleotides in an alkaline hydrolysate of RNA was achieved by employing a 9 : 1 mixture of cellulose and silica gel[433]. Under these conditions, the presence of salts in the hydrolysate had hardly any influence on the separation of the nucleotides.

C. Ion-Exchange Resins

Methods for the chromatography of nucleotides and their derivatives in a thin layer of ion-exchange cellulose have been rapidly developed and adopted in the last 10 years. Ion-exchange celluloses can be prepared by treating unmodified or modified cellulose with high-molecular-weight basic or acid derivatives[391,434].

A comparative study of the fractionation of nucleotides and oligonucleotides on various kinds of ion-exchange cellulose[434-437] showed that cellulose treated with PEI is superior to other ion-exchange celluloses as regards the clear-cut separation of compounds. Evidently the results of the separation depend on the PEI-content, which determines the capacity of the layer[438]. A satisfactory separation has been achieved on layers containing 0.5—1% PEI,[380,388,435,438]. Cellulose plates prepared as described by Randerath are usually employed[309,311,380,388,391,435,436,438].

In contrast to layers of unmodified cellulose, a large volume of solution may be deposited on a PEI-cellulose plate without partial drying, since nucleotides are fairly firmly bound to the ion exchanger and are therefore concentrated in the centre of the spot. It was noted that, for optimal separation of nucleotides, the pH of the specimen deposited should be approximately equal to that of the first solvent[439]. The solutions analysed must be very dilute. For analytical purposes, it is best to employ concentrations ranging from 0.0005 to 0.005 M and in micropreparative studies (with several milligrams of the test substance) from 0.005 to 0.02 M.[305]

Nucleotides are separated on PEI-cellulose plates with development by aqueous electrolyte solutions[305,438,440-449]. In certain cases organic solvents are added to electrolyte solutions[305]. Both stepwise and continuous gradient elution is usually carried out in the fractionation of nucleotides. The mobility of each substance is largely determined by the negative charge, which depends on the pH. Best and most clear-cut results in the separation of nucleotides are obtained at acid pH (1—3 M formic acid with added LiCl).[305,438,439,443]. Table 4 lists the R_f values for various mononucleotides.

Mixtures of nucleotides can be successfully separated by the stepwise elution with a solvent at an increasing electrolyte concentration[438]. The first solvent is allowed to rise to a certain height after which the plate is transferred, without drying, to a chamber with another solvent, etc. Alkaline RNA hydrolysates and mixtures of nucleoside 5′-monophosphates are separated with the aid of one-dimensional chromatography on PEI-cellulose layers using stepwise elution with acetic acid and LiCl solutions[309,444]. The rate of migration of the components of the alkaline RNA hydrolysate decreases in the following sequence: nucleosides > 2′- + 3′-CMP, 2′-AMP > 3′-AMP > 2′- + 3′-UMP > 2′- + 3′-GMP. The same elution sequence holds for the 5′-isomers and the corresponding derivatives containing deoxyribose[380,444]. Sometimes elution using a salt concentration gradient is employed[440-445].

In the stepwise chromatography of a mixture of nucleoside mono-, di-, and tri-phosphates in 0.6, 0.8, and 1.25 M NaCl without intervening drying of the plates, a clear-cut separation of these derivatives is achieved[310,436,446,447].

The components of alkaline RNA hydrolysates are usually satisfactorily separated by continuous ascending chromatography[380,440]. The technique employing a continuous linear salt gradient has been described in fair detail[448].

Deoxyribonucleotides can be separated from ribonucleotides in solvents containing borate[437,438,450-452]. The borate complexes of ribonucleotides have an additional negative charge and move more slowly than deoxyribonucleotides[438]. The following solvent systems are usually employed in the separation of ribonucleotides and deoxyribonucleotides: 2 : 1 2% H_3BO_3—2 M LiCl for nucleoside monophosphates and 4 : 3 4% H_3BO_3—4 M LiCl for nucleoside triphosphates[438,453]. Since satisfactory fractionation of deoxyribonucleotides is achieved in the reverse system, it is sometimes used to separate enzymatic DNA hydrolysates[442,453,454].

Table 4. The R_f values (×100) for nucleotides and their derivatives in thin-layer chromatography on PEI-cellulose[380,438].

Substance	Solvent*												
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
AMP	11	52	65	68	80	80	80	80	80	80	61	69	74
IMP	13	59	74	40	60	73	80	19	53	78	04	52	72
GMP	06	40	51	28	45	57	65	41	50	72	07	45	62
CMP	15	64	75	70	80	80	80	80	80	80	76	79	80
UMP	20	74	80	51	72	80	80	20	64	80	06	62	77
ADP	00	26	54	03	10	32	75	03	29	70	00	19	52
IDP	00	30	63	00	04	14	49	00	03	55	00	05	30
GDP	00	17	45	00	02	09	34	00	13	61	00	05	27
CDP	00	33	64	03	20	45	80	04	35	73	00	26	56
UDP	00	41	71	02	07	24	60	00	11	60	00	08	40
ATP	00	00	34	00	00	04	24	00	04	33	00	01	12
ITP	00	09	39	00	00	02	11	00	02	17	00	00	05
GTP	00	05	25	00	00	00	07	00	02	24	00	00	05
CTP	00	11	41	00	02	05	29	00	04	37	00	02	16
UTP	00	14	49	00	00	02	17	00	02	20	00	01	07

*Solvent composition: I) 0.25 M LiCl; II) 1.0 M LiCl; III) 1.6 M LiCl; IV) 0.5 M sodium formate—formic acid buffer, pH 3.4; V) 1.0 M sodium formate—formic acid buffer, pH 3.4; VI) 2.0 M sodium formate—formic acid buffer, pH 3.4; VII) 4.0 M sodium formate—formic acid buffer, pH 3.4; VIII) 1.0 *N* HCOOH; IX) 2.0 *N* HCOOH—0.5 M LiCl; X) 2.0 *N* HCOOH—2.0 M LiCl (1 : 1); XI) 1.0 *N* CH_3COOH; XII) 1.0 *N* CH_3COOH—3 M LiCl (9 : 1); XIII) 1.0 *N* CH_3COOH—4 M LiCl (8 : 2).

Mixtures of nucleotides or RNA hydrolysates can also be fractionated by two-dimensional chromatography on PEI-cellulose layers[439,449]. Before passing the second solvent, it is important to remove the first from the plate. Randerath[380,438] proposed previously that, after drying, the plate be washed with methanol for this purpose. However, when a volatile solvent is used in the first direction, for example formic acid, it is sufficient to simply dry the plate before the second chromatographic step[439]. A mixture of ribonucleotides containing various nucleoside mono-, di-, and tri-phosphates was fractionated by ascending two-dimensional chromatography and PEI-cellulose[455] (Fig. 6). Stepwise elution by LiCl solutions was used in the first direction and stepwise elution by a sodium formate buffer (pH 3.4) in the second direction. The entire separation of the nucleotide mixture lasts about 3 h. The fractionation of a complex mixture of ribonucleosides and deoxyribonucleoside triphosphates by two-dimensional chromatography has been described[450]. In later studies[452,456-458] analogous chromatography was used to separate biological materials. For best separation,

extracts from the biological material are sometimes subjected to preliminary purification before chromatography, but in most cases chromatography is carried out without such purification. The chromatography of specimens containing large amounts of salts has been described by Foster et al.[441]

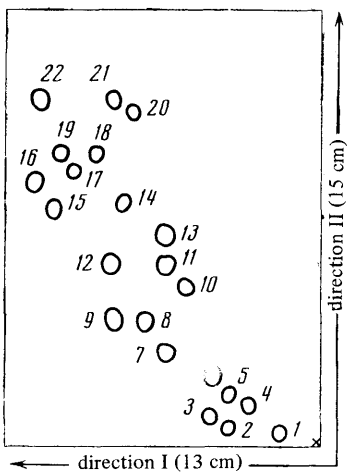


Figure 6. Two-dimensional chromatography of nucleotides on a PEI-cellulose layer. First direction: 0.2 M LiCl for 2 min, 1 M LiCl for 6 min, and 1.6 M LiCl up to 13 cm. Second direction: 0.5 M buffer for 30 s, 2 M buffer for 2 min, and 4 M buffer up to 15 cm (sodium formate buffer, pH 3.4)[455]: 1) GTP; 2) ITP; 3) UTP; 4) ATP; 5) CTP; 6) GDP; 7) IDP; 8) UDP; 9) UDPGA; 10) ADP; 11) CDP; 12) GDPM; 13) GMP; 14) IMP + TPN; 15) UDPG; 16) UDPAG; 17) ADPG; 18) UMP; 19) CDPG; 20) AMP; 21) CMP; 22) DPN.

Since PEI-cellulose has a high capacity, it can be used also in micropreparative separations. Layers 1–1.5 mm thick, which permit the fractionation of milligram amounts, are used for this purepose[305,438]. In thin-layer chromatography the fractions are desalinated by washing the entire plate with methanol and subsequent elution (usually with volatile buffers or with dilute HCl solutions) of the individual spots[380,438,439]. For the standard method of preparation of the plates, the average deviations of the R_f values do not exceed those for the usual thin-layer chromatography, amounting to ±0.005–0.025 on the basis of 10 determinations[459]. However, the reproducibility of the R_f depends on the amount of cellulose. When the plates are stored for about 100 days (in the dark at room temperature), the R_f values hardly change[459].

Quantitative extraction of nucleotides from a PEI-cellulose layer has been described in fair detail[380,382,442,443,458,460]. The optimal amounts of substances for a quantitative determination are 5–50 nanomoles[380,442]. The sensitivity of the method can be greatly improved and substances in amounts down to 1 nanomole and less can be determined by reducing the volume of the eluent and by measuring the absorption in microcuvettes or by employing a radioactive label[443,458].

Together with cellulose, DEAE- and ECTEOLA-cellulose layers have been used for the analysis of ribonucleotides and deoxyribonucleotides phosphorylated to different extents[385,392,440,461-481]. The principal advantage of these celluloses is the rapidity of separation. For example, a mixture of 10^{-2} mole of ADP and ATP is separated in 4–5 min on ECTEOLA-cellulose[461,464,467]. The preparation of DEAE-cellulose[302,385,440,463] and ECTEOLA-cellulose[462,475] plates has been described. Dilute solutions of hydrochloric or formic acids are usually employed as eluents[302,385,392,461,463,464,467]. The use of dilute sodium chloride solutions gives a less clear-cut separation[461]. Randerath[461,464] claims that DEAE- and ECTEOLA-cellulose layers give approximately identical results, the fractionation on an ECTEOLA-cellulose layer being the faster.

The rate of migration of different nucleotides decreases in the sequence C > A > G > U. Adenine and cytidine nucleotides with the same content of phosphate groups are poorly separated in a dilute acid, but can be separated in 0.1 M NaCl.[461,464] The R_f values for mono-, di-, and tri-nucleotides on DEAE-cellulose in dilute formic or hydrochloric acid solutions are listed in Table 5.

Table 5. The R_f values for nucleoside mono-, di-, and tri-phosphates in thin-layer chromatography on DEAE-cellulose[461,463,467].

Substance	In hydrochloric acid				In formic acid		
	0.01 *N*	0.02 *N*	0.03 *N*	0.04 *N*	1%	4%	10%
AMP	0.45	0.65	—	—	0.87	0.96	0.97
ADP	0.24	0.48	0.68	—	0.19	0.49	0.68
ATP	0.06	0.11	0.20	0.56	0.01	0.03	0.11
GMP	0.36	0.60	0.75	—	0.55	0.73	0.87
GDP	0.09	0.27	0.51	—	0.04	0.18	0.56
GTP	0.05	0.07	0.14	0.41	0.01	0.01	0.06
CMP	0.46	0.65	—	—	0.90	0.96	0.97
CDP	0.31	0.53	—	—	0.35	0.55	0.71
CTP	0.09	0.13	0.31	0.64	0.10	0.05	0.16
UMP	0.31	0.49	—	—	0.33	0.51	0.68
UDP	0.07	0.15	0.25	—	0.02	0.06	0.14
UTP	0.04	0.04	0.08	0.18	0.01	0.01	0.02
IMP	—	—	—	—	0.33	0.54	0.76
IDP	—	—	—	—	0.03	0.07	0.23
ITP	—	—	—	—	0.01	0.01	0.04
Inorganic phosphate	—	—	—	—	0.44	0.67	0.81

Satisfactory results are obtained in the analysis of a mixture of ribonucleotide and deoxyribonucleotide mono-, di-, and tri-phosphates with identical heterocyclic bases by two-dimensional chromatography on DEAE-cellulose. A clear-cut separation with respect to the number of phosphate groups takes place in the first direction and with respect to the sugar residue in the second[385,465].

The employment of two-dimensional gradient elution (1.0 M NH_4HCO_3–0.15 M NH_4HCO_3 for the first direction and 2.0 M NH_4HCO_3 at pH 4.2–0.2 M NH_4HCO_3 at pH 2.8 for the second) permits the separation of a mixture of the mono-, di-, and tri-phosphates of the four principal nucleotides with the exception of CTP and UTP.[440] This method can be used to analyse biological materials[462,463,466,476]. The authors suggest gel filtration in a thin layer on G-25 Sephadex before chromatography in order to separate them from polysaccharides and

fluorescent materials which are always present[440]. The nucleotide material is eluted from Sephadex by water and lyophilised.

A DEAE-cellulose layer can be used also to separate ribonucleoside and deoxyribonucleoside 2′- amd 3′-phosphates in alkaline or enzymatic RNA and DNA hydrolysates[385,477,478]. Nucleotide 2′- and 3′-phosphates are chromatographed in 0.05 *N* HCl for both ribonucleoside and deoxyribonucleoside derivatives in the first direction and in 0.035 *N* HCl for ribonucleoside derivatives and 0.02 *N* HCl for deoxyribonucleotide derivatives in the second[385,464,477]. 2′ and 3′-Adenylic acids are fractionated under these conditions[385,464]. The separation of the 2′- and 3′-isomers of all nucleotides is achieved on DEAE-cellulose containing 10% of $CaSO_4$.[478]

2. PAPER ELECTROPHORESIS AND ELECTROPHORESIS IN A THIN LAYER OF CELLULOSE

Nucleotides and their derivatives contain different ionisable groups so that electrophoresis on paper and in a thin layer of cellulose is a valuable method for their analytical and preparative separation. The relative mobilities of nucleotides can be calculated at any pH from their charges using the pK_a of the ionisable groups, which are known[265].

The four principal ribonucleotides and deoxyribonucleotides are separated at pH 3.5. At this pH the 2′- and 3′-isomers of guanylic acid have different mobilities[265,343,482,483].

Certain minor nucleotides can also be separated from other nucleotides by paper electrophoresis, but for a complete analysis, for example of a tRNA hydrolysate, additional separation and by thin-layer or column chromatography (see the relevant Sections) is necessary[343,482,483]. The mobilities of ribonucleotides are listed in Table 6.

Table 6. The relative mobilities of ribonucleotides* in paper electrophoresis[265].

Nucleotide	pH 3.5**	pH 2.1***
4,5-Dihydrouridylic acid	1	–
Ribothymidylic acid	0.98	–
Pseudouridylic acid	0.98	–
Inosinic acid	0.90	–
Guanylic acid	0.73	0.38
7-Methylguanylic acid	0.98	–
1-Methylguanylic acid	–	0.30
N^2-Methylguanylic acid	–	0.30
N^2N^2-Dimethylguanylic acid	0.76	0.25
Adenylic acid	0.50	0
N^6-Methyladenylic acid	0.46	–
N^6N^6-Dimethyladenylic acid	0.42	–
2-Methyladenylic acid	0.25	–
Cytidylic acid	0.41	0

* The mobility of uridylic acid was adopted as unity.
** 0.05 M ammonium formate at pH 3.5.[343,348]
*** 0.05 M phosphate buffer at pH 2.1.[482]

Mono-, di-, and tri-phosphates are separated best in a citrate buffer at pH 5.0 or 7.0 depending on the number of dissociable phosphate groups. In the presence of bivalent cations the mobility of nucleoside triphosphates decreases[265].

2′- and 3′-Ribonucleotides can be separated from 5′-ribonucleotides in a borate buffer at pH 9.2.

Electrophoretic separation of nucleotides after hydrolysis can be used to determine the incorporation of various nucleotide analogues in nucleic acids. For example, the p*K* of 5-fluoro-, 5-bromo-, 5-chloro-, and 5-iodouridylic acids, 2-thiouridylic acid, and 8-azaguanylic acid are lower than those of uridylic, guanylic, and thymidylic acids and therefore at pH 9.0 these nucleotides migrate faster than all others[265].

The components of the RNA or DNA hydrolysates can also be separated by electrophoresis on cellulose layers[397,461,483-486]. Best separation is then achieved on carefully degassed cellulose washed with a solution of the sodium salt of EDTA[485].

Fractionation of 2′, 3′-ribonucleotides and deoxyribonucleotides is achieved at pH 3.4–3.5.[397,484,486] Adenine and inosine nucleotides are separated at pH 5.1.[486] Deoxyribonucleoside mono-, di-, and tri-phosphates can be easily separated from one another with respect to each base at pH 5.5.[484]

The employment of thin-layer electrophoresis permits the improvement of the separation of certain nucleotide mixtures which are difficult to fractionate by partition or ion-exchange chromatography. For this purpose, the electrophoresis is usually carried out in one direction in two-dimensional chromatography[337,396-398]. A microversion of electrophoresis was employed by Edström to analyse the nucleotides obtained fron the nucleic acids in a single cell [487,488].

3. ION-EXCHANGE COLUMN CHROMATOGRAPHY

Ion-exchange column chromatography on polystyrene resins, introduced by Cohn[1], has been used successfully for the fractionation of various natural nucleic acids on analytical and preparative scales. The general principles of separation by ion exchange column chromatography were examined by Cohn as early as 1955. Ion-exchange column chromatographic methods have been described in fair detail in three articles in the "Methods in Enzymology" series[489].

A mixture of ribo- or deoxyribo-nucleotides[490,491] can be very conveniently separated on a strong anion exchanger of the type of Dowex-1 in acid[492] or alkaline[493-496] systems. In acid systems the elution sequence of the nucleotides is as follows: cytidylic, adenylic, uridylic, and guanylic acids. In columns with a large height : diameter ratio guanylic acid sometimes overtakes uridylic acid[497]. Isomeric nucleotides can be separated into the 5′-, 2′-, and 3′-phosphates and are eluted from the column in this sequence[497].

In alkaline systems the elution sequence of nucleotides changes as follows: cytidylic, uridylic, adenylic, and guanylic acids[495].

In the simplest cases the relative dimensions of the column are unimportant, but in the separation of complex multicomponent mixtures tall columns (height : diameter = 50 : 1) give a satisfactory separation[497].

In the majority of systems used to separate a known mixture of nucleic acid derivatives on columns, the concentration or the pH of the eluent are increased in steps. However, in selecting the separation conditions or in the separation of a complex mixture, gradient elution is employed in which the eluent composition varies smoothly in accordance with the specified gradient. A linear gradient is usually employed.

Chromatographic separation of nucleotides on ion-exchange columns has been described in greater detail in other reviews[1,5,491,497].

The ability of purine and pyrimidine derivatives to absorb radiation intensely in the region 260—290 nm has been used to develop micromethods for the chromatography of nucleotides permitting the determination of small amounts of the latter and greatly reducing the duration of the fractionation[498-501]. Thus Anderson and coworkers[498,499] and somewhat later Hori et al.[500] described a method for the automatic analysis of nucleic acids whereby it is possible to separate and analyse the nitrogenous bases, nucleosides, and nucleotides in 24 h on a single column with Dowex-1 × 8 (200—400 mesh) resin using a linear acetate buffer concentration gradient at pH 4.4. For qualitative analysis, 0.02 μmole of substance is sufficient. Specimens containing large amounts of salts (for example, RNA and DNA hydrolysates) are subjected to preliminary desalination by gel filtration and then dissolved in a buffer solution. Pyrimidine and purine bases and nucleosides are eluted first and then nucleotides. Under these conditions, 2′-UMP is not completely separated from 3′-UMP; with the exception of 5′-CMP, 5′-nucleotides are not separated from 2′- and 3′-nucleotides and 5′-IMP and 5′-AMP are not separated at all. However, this hardly reduces the practical value of the method. It can be used to separate the 5′-nucleotides obtained from RNA by nuclease hydrolysis and 2′- and 3′-nucleotides formed in the alkaline hydrolysis of RNA. Deoxyribonucleotides are separated under analogous conditions[498].

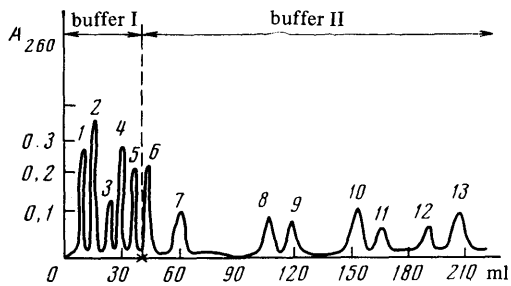


Figure 7. Chromatography of nucleotides, nucleosides and bases on Aminex A-4 cation-exchange resin (0.9 × 50 cm column); citrate (I) and acetate (II) buffers used for separation; rate of elution 60 ml h^{-1};[503] 1) UMP-5′ + IMP-5′ + UMP-2′ and UMP-3′ + ATP; 2) GMP-5′ + ADP + GMP-2′ + GMP-3′; 3) CMP-5′; 4) uridine + AMP-2′; 5) AMP-5′ + CMP-2′ + CMP-3′ + AMP-3′; 6) uracil; 7) inosine + thymine; 8) hypoxanthine; 9) guanosine; 10) adenosine; 11) cytosine; 12) guanine; 13) adenine.

The employment of AG-50W × 4 cation-exchange resin for the fractionation of ribo- and deoxy-ribonucleotides was described in 1967 by Blattner and Erikson[502], who achieved a clear-cut separation of the four principal 5′-deoxyribonucleotides after the enzymatic hydrolysis of DNA and of 2′- and 3′-ribonucleotides after the alkaline hydrolysis of RNA. They employed 0.1 M ammonium formate at pH 3.2 as the eluting solution in the first case and 0.25 M ammonium formate at pH 4.1 in the second. The sequence in which the nucleotides are eluted from the column is the same for deoxyribonucleotides and ribonucleotides: T(U) > G > C > A. The presence of Ca^{2+} and Mg^{2+} ions, snake venom phosphodiesterase, and pancreatic deoxyribonuclease in the specimen does not interfere with the separation. A change in pH or in the molarity of the buffer impairs the separation. The employment of a volatile buffer for elution makes it possible to obtain the nucleotides in a pure form after the evaporation of the solutions.

A clear-cut separation of four 5′-mononucleotides was achieved also on another cation-exchange resin Aminex A-4 (Fig. 7)[503].

Nucleotides phosphorylated to different extents can be readily separated quantitatively with the aid of DEAE-Sephadex A-25 in an ammonium bicarbonate or triethylammonium acetate gradient at pH 7.0.[504,505] Nucleotides are eluted from the column in order of increasing acidities[504].

The efficiency of column chromatography and the speed of analysis were subsequently significantly improved by employing very fine anion- and cation-exchange resins and high pressures[506-509]. New types of resin such as Zipax[507-510], AA-15,[506] and a surface-layer cation-exchange resin[511] were developed for this purpose. The application of highly sensitive detector systems permits nowadays the quantitative analysis of nucleic acid hydrolysates at the level of 10^{-12} mole without the use of a radioactive label[511].

V. NUCLEIC ACID BASES AND NUCLEOSIDES

1. THIN-LAYER CHROMATOGRAPHY

Partition chromatography in a thin layer of cellulose is widely used for the analytical determination and identification of the nucleic acid bases and nucleosides[380,382,385-401]. As in paper chromatography, the usual solvent systems for the separation of the bases and nucleosides are mixtures of water or aqueous solutions of salts, acids, or bases with organic solvents (alcohols, acids, etc.). Table 7 lists the R_f values for various nucleic acid bases, their derivatives, and nucleosides in a series of solvents[385]. These systems are fairly simple and give reliably reproducible results. Comparison of the R_f values in the above systems provides an idea about the influence of the pH of the solution and the salt concentration in the systems on the chromatographic mobilities of the substances being separated. The amphoteric properties of nucleic acid bases, as a result of which the pH of the system can alter the polarity of the bases and nucleosides and hence the partition coefficient, plays an important role in this process. The introduction of substituents alters significantly the polarity and the dissociation constants, so that, knowing the position and nature of the substituent, it is possible to predict, at least by analogy, the R_f value.

A satisfactory separation of the bases by thin-layer chromatography has been achieved with the aid of different versions of Kirby's system[380,401], widely used in the paper chromatography of the bases. Thus the separation of all four bases after the acid hydrolysis of DNA was achieved by one-dimensional chromatography in a 65 : 17 : 18 MeOH—HCl—H_2O mixture[380,401]. The standard system for paper chromatography isopropyl alcohol—HCl—water has also been used successfully[385,512].

The bases and the corresponding nucleosides can be readily separated on cellulose with elution by water[386]. A number of other less common systems have been described for the separation of bases and nucleosides[386,390,392,393,513-523].

Table 7. The R_f values for bases and nucleosides in thin-layer chromatography on cellulose[38].

Substance	Solvent*					
	I	II	III	IV	V	VI
Adenine	0.31	0.38	0.88	0.35	0.40	0.10
2-Methyladenine	0.38	0.43	0.91	0.45	0.49	0.08
6-Dimethyladenine	0.50	0.68	0.88	0.65	0.55	0.08
6-Methyladenine	0.45	0.52	0.91	0.52	0.52	0.08
Cytosine	0.41	0.24	0.59	0.31	0.39	0.59
5-Methylcytosine	0.51	0.27	0.64	0.35	0.44	0.51
5-Hydroxymethylcytosine	0.39	0.10	0.64	0.25	0.33	0.66
Uracil	0.70	0.31	0.57	0.35	0.47	0.54
6-Methyluracil	0.72	0.36	0.63	0.44	0.55	0.44
1,3-Dimethyluracil	0.98	0.70	0.82	0.80	0.73	0.56
Thymine	0.80	0.45	0.65	0.57	0.57	0.44
Guanine	0.26	0.33	0.87	0.39	0.41	0.10
1-Methylguanine	0.16	0.06	0.50	0.13	0.25	0.15
2-Methylguanine	0.53	—	0.74	—	—	0.11
2,2-Dimethylguanine	0.32	—	0.75	0.32	—	0.10
Adenosine	0.23	—	—	—	0.41	—
Deoxyadenosine	0.27		0.82	0.37	0.42	0.09
Cytidine	0.36	—	0.65	0.27	—	0.52
Deoxycytidine	0.50	—	0.65	0.35	—	0.47
Methyldeoxycytidine	0.59	—	0.73	0.40	0.42	0.39
Guanosine	0.22	—	0.50	0.09	0.26	0.27
Deoxyguanosine	0.14	—	0.59	0.17	0.36	0.23
Uridine	0.62	—	—	—	—	—
Deoxyuridine	0.77	—	0.61	0.33	0.47	0.45
Thymidine	0.88	—	0.66	0.52	0.55	0.37
Pseudouridine	0.42	—	0.42	0.08	0.24	0.57

*Solvent composition: I) isopropyl alcohol—HCl—H_2O (65 : 16.7 : 18.3); II) n-Butyl—alcohol—water (86 : 14); III) isobutyric acid—water—25% NH_4OH (400 : 208 : 0.4); IV) isopropyl alcohol—water—conc. (28%) NH_4OH (85 : 15 : 1.3); V) n-butyl alcohol—water—formic acid (77 : 13 : 10); VI) sat. $(NH_4)_2SO_4$—1 M sodium acetate—isopropyl alcohol (80 : 18 : 2).

The R_f values for methylated purines and purine nucleosides have been quoted by Broom and coworkers[517,520]. Data for the separation of deoxyribonucleosides and their R_f values have also been published[395,402,524]. Ribonucleosides can be readily separated from deoxyribonucleosides or ribonucleosides with substituted *cis*-glycol hydroxy-groups in alkaline systems containing borate[382]. The formation of borate complexes with the *cis*-glycol group of the ribose residue has a marked influence on chromatographic mobility.

In this case, when the mixture of bases is too complex for a satisfactory separation by one-dimensional chromatography, two-dimensional chromatography can be employed. An example of a successful application of this method is probided by the separation of a mixture of bases using the 70 : 20 : 10 MeOH—HCl—H_2O system in the first direction and the 60 : 20 : 20 : 2 BuOH—MeOH—H_2O—conc.NH_4OH in the second system[380,525]. A mixture of 6-methyladenine, adenine, cytosine, and 5-methylcytosine can be readily separated into individual compounds by two-dimensional chromatography using the solvent system I in the first direction and the solvent system II in the second (for solvent systems I and II, see Table 7), while the employment of only one system does not lead to a clear-cut separation of the mixture[385].

In the two-dimensional fractionation of the enzymatic hydrolysate of tRNA from *E. coli* and yeast there is a clear-cut separation of 8 nucleosides including minor nucleosides: dehydrouridine, inosine, pseudouridine, and ribothymidine[521]. Electrophoresis is sometimes employed in one direction in two-dimensional thin-layer chromatography[401,484]. For example, a mixture of the five principal and eight methylated bases found in DNA and RNA was separated by this method and it was shown that it yields better results than one-dimensional chromatography[385]. The application of high-voltage electrophoresis and two-dimensional chromatography helps the separation of the majority of bases and deoxyribonucelosides present in DNA hydrolysates[401].

Thus the fractionation of nucleic acid derivatives in a thin layer of cellulose is used nowadays in all cases where paper chromatography was employed previously, but separation in a thin cellulose layer is of shorter duration than paper chromatography, more clear-cut, and the sensitivity is higher by a factor of approximately 10.

The employment of labelled nucleic acid preparation and also of chemical methods for the introduction of radioactive isotopes into nucleic acid components increases the sensitivity of the method to 10^{-12} mole[343,526-528]. In particular, Randerath oxidised the *cis*-glycol group of the ribose residue by periodate with subsequent reduction by sodium borotritide in order to introduce the isotope label into ribonucleosides in the analysis of RNA hydrolysates. The resulting mixture of tritium-labelled compounds was resolved into components by two-dimensional chromatography in thin layers of cellulose or silica gel[304,529-533]. The positions of the individual compounds on the chromatograms were determined by radioautography. The quantitative analysis was carried out by measuring the radioactivity of the eluted substances. The method has a high resolving power and sensitivity[304,529-531]. It was developed and applied to determine the nucleotide composition of RNA in those cases where a minimal amount of RNA is available and cannot be labelled *in vivo*. The authors estimate the sensitivity of the method as several picomoles[304], while the lower limit of spectrophotometric analysis is one nanomole. By studying the factors influencing the sensitivity of the method, Randerath achieved a level of sensitivity which proved to be sufficient for the detection of minor components of tRNA.[530,532,533] However, 2-*O*-methylnucleosides cannot be identified by this method, because they are not oxidised by periodate, and 4-thiouridine is converted into [^{3}H]uridine by reduction with borotritide[530,531,534]. In view of the instability of the borotritide, the reduction is carried out in an alkaline medium, where certain minor bases of tRNA are known to decompose[531,534]. The imidazole ring of 7-methylguanine is partly decomposed (about 35%) and 1-methyladenine is converted into N^6-methyladenine. 3-Methylcytosine also decomposes to the extent of 10—15%.[531,534,535]

Randerath employed his highly sensitive ultramicromethod for systematic studies on the composition of the bases of the tRNA isolated from various normal and neoplastic mammalian brain tissues[536]. The nucleoside derivatives are separated largely on cellulose layers.

Among other adsorbents giving satisfactory results in the fractionation of nucleic acid bases and nucleosides in a thin layer, mention may be made of polyamide[382,392,537]. Table 8 lists the R_f values for ten bases and nucleosides. The use of distilled water as a solvent greatly improves the mobilities of all the compounds, while the addition of a salt reduces the mobility[537]. Best results were achieved in aqueous sodium chloride solutions, although sodium

acetate or borate can also be used[537]. The polyamide layers can be employed repeatedly[537].

Chromatography in a thin layer of silica gel is sometimes used to separate the synthetic analogues and certain derivatives of nucleosides and bases[402,404,414,418,419,426,427,538,539]. Although silica gel layers are, in fact, used for the fractionation of the free bases and nucleosides[300,410,426], they give less satisfactory results than cellulose[300,426,427].

Table 8. The R_f values for bases and nucleosides in chromatography on polyamide layers[537].

Substance	Solvent*				Substance	Solvent*			
	I	II	III	IV		I	II	III	IV
Adenine	0.75	0.85	0.54	0.51	Inosine	0.32	0.30	0.23	0.85
Hypoxanthene	0.48	0.52	0.37	0.74	Guanosine	0.22	0.17	0.17	0.74
Guanine	0.00	0.00	0.00	0.44	Uridine	0.39	0.50	0.37	0.90
Uracil	0.58	0.72	0.54	0.83	Cytidine	0.30	0.37	0.23	0.90
Adenosine	0.65	0.80	0.50	0.65	Thymidine	0.56	0.75	0.51	0.78

*Solvent composition: I) heptane–butyl alcohol–acetic acid (4 : 4 : 1); II) carbon tetrachloride–acetic acid–acetone (4 : 1 : 4); III) toluene–pyridine–ethylene chlorohydrin–0.8 M NH_4OH (5 : 1 : 5 : 3); IV) 0.5 M aqueous NaCl.

2. PAPER ELECTROPHORESIS AND ELECTROPHORESIS IN A THIN CELLULOSE LAYER

The fractionation of nucleic acid bases and nucleosides by electrophoresis on paper or in a thin layer of cellulose has no special advantages over thin-layer or paper partition chromatography of these compounds. However, by combining this method with chromatography in thin cellulose layers, it is sometimes possible to improve the separation of certain mixtures containing, apart from the four main components, also minor components—methylated in the base moiety or in the 2′-hydroxy-group of ribose[382,484]. The most striking example of such application of electrophoresis and chromatography is provided by the separation of a mixture of five main and eight methylated bases with the aid of two-dimensional chromatography[484] (Fig. 8).

Deoxyribonucleosides and 2′-*O*-methylribonucleosides can be separated from ribonucleosides in a borate buffer at pH 9.2.[265,484] Borate complexes of ribonucleosides migrate as anions and the relative mobilities of adenosine, cytidine, guanosine, and uridine are respectively 0.64, 0.74, 0.88, and 1.0.[265] The four main ribonucleosides and deoxyribonucleosides are separated by paper electrophoresis at pH 3.2. The mobilities of uridine (thymidine), guanosine, and adenosine are respectively 0, 0.05, and 0.29 (the mobility of cytidine was adopted as 1.0)[265]. Adenine, guanine, cytosine, and uracil can also be separated by electrophoresis at pH 3.2.[265]

3. ION-EXCHANGE CHROMATOGRAPHY

The principles and methods of the separation of nucleic acid bases and nucleosides on ion-exchange resins have been described in fair detail in Cohn's papers and reviews[11,489,491,497]. Since all the data published hitherto on the fractionation of these components on ion-exchange resins proved to be comparable with one another and with Cohn's data (the same or similar ion-exchangers were employed in all cases), our discussions will be restricted only to those few ion-exchange resins which are used nowadays in micropreparative studies. The problem of the separation of nucleosides and bases by adsorption chromatography on gel filtration materials of the type of Sephadex and biogels, which have been recently used successfully for this purpose, will be discussed in somewhat greater detail in this Section.

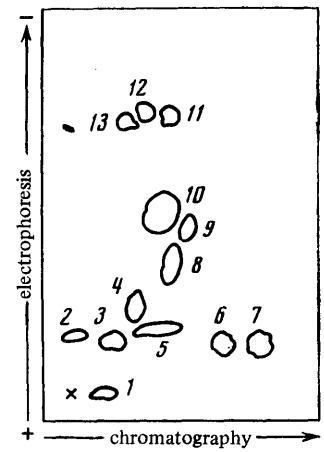


Figure 8. Separation of methylated bases on a thin layer of microcrystalline Pevikon cellulose. First direction: electrophoresis in 0.1 M sodium acetate at pH 3.5, 1000 V, and 20 mA for 3 h. Second direction: chromatography in the 65 : 16.7 : 18.3 isopropyl alcohol–HCl–water system[484]: 1) guanine; 2) 1-methylguanine; 3) 2-dimethylguanine; 4) adenine; 5) 2-methylguanine; 6) uracil; 7) thymine; 8) 6-methyladenine; 9) 6-dimethyladenine; 10) 2-methyladenine; 11) 5-methylcytosine; 12) cytosine; 13) 1-methyladenine.

In 1968 Cohn and coworkers modified Anderson's nucleotide analyser[498,499] and used it to separate nucleosides on cation-exchange resins such as Amberlite GG-120 (type III), Bio-Rad A-6, and Dowex 50 × 4.[540] The four main nucleosides obtained by the hydrolysis of RNA are readily separated on elution with 0.4 M ammonium formate at pH 4.65. The sequence in which nucleosides are eluted from the column is as follows: U > G > A > C. Minor nucleosides such as N^6-dimethyladenosine 7-methylguanosine, and N^2-dimethylguanosine are readily separated from one another and so do not interfere with the separation of the main nucleosides. Pseudouridine ribothymidine, uridine, inosine, and 4-thiouridine are incompletely separated. Although the authors investigated only the separation of nucleosides, a similar nucleoside analyser can probably also be used to analyse nucleic acid bases[502,541–543]. For example, a method was proposed in 1970 for the fractionation of a mixture of nucleotides, nucleosides, and bases on the cation-exchange resin Aminex A-4 [503] (Fig. 7). The separation was carried out using stepwise elution, initially with sodium citrate at pH 3.0 and then with 0.25, 0.50, and 1.00 M

sodium acetate at pH 6.4. All four 5′-mononucleotides are separated when the first buffer is passed through the resin. The second buffer gives rise to individual peaks of the nucleosides and nucleic acid bases. The employment of this cation-exchanger was also described previously for the separation of purine bases from pyrimidine bases and for the separation of hypoxanthine, guanine, and adenine[544].

A convenient method for the isolation and separation of purine bases from biological extracts using AG50W × 4 (H^+) resin has been proposed[545]. The elution is carried out with 0.05 *N* HCl, which results in the separation not only of purine bases but also of the entire nucleotide material present in the extract.

4. GEL FILTRATION AND ADSORPTION CHROMATOGRAPHY ON GELS

Gel filtration or fractionation of compounds on molecular sieves (dextran gels) of the type of Sephadex was described as early as 1959 [546] and has been widely used to separate various nucleic acid derivatives in accordance with their molecular weights. Thus nucleotides are readily separated from RNA or oligonucleotides[547,549] and are always eluted before nucleosides and bases, which constitutes a rapid and technically very simple method for the separation of these derivatives into groups of approximately equal molecular weights[549,550]. Sephadex, a relatively inert, insoluble, and hydrophilic material, is manufactured with different porosities (G-10, G-15, G-25, G-50, G-75, G-100, and G-200) and in the form of ion-exchange celluloses (CM- and DEAE-Sephadex), which combine the properties of molecular sieves and ion exchangers[551,552]. The elution of substances from columns is almost quantitative. About 3% is lost as a result of irreversible adsorption[553].

Soon after the introduction of gel filtration, Gelotte showed that nucleic acid derivatives are retained by the gel to different extents and are eluted from the latter by different volumes of the eluent, depending on the structure[553-557]. It was shown that, apart from the molecular weight, the separation of compounds on different types of Sephadex is greatly influenced both by the ionic strength and the pH of the eluting buffer and by the reversible adsorption of the substances[549,550,554-561]. The mechanism of the adsorption on Sephadex has not been established yet, but it is suggested that the adsorption involves the formation of hydrogen bonds between the heterocyclic bases and the ether bridges in the matrix[553,556,557]. Purine derivatives as a rule exhibit a greater affinity for different types of Sephadex than pyrimidine derivatives[549,550,553,556,557]. The separation on G-10, G-15, and G-25 is virtually the same for pyrimidine derivatives, while the use of more cross-linked gels, for example G-10, for purines results in an increased affinity for the matrix[549,550,553,556].

The relative volume of the eluent or the elution constant R_m for different derivatives is independent of the amount of gel and, when the conditions are standardised, can serve as the first criterion for the identification of the compound[556]. The elution constants for 50 purine and pyrimidine derivatives on a G-10 column in 0.01 ammonium carbonate at pH 9 are listed in the same report. In a single step it is possible to separate up to 16 compounds[556]. The elution constants of nucleosides and bases at other pH values have been published[559,561]. All four principal deoxyribonucleosides and ribonucleosides can be successfully separated on Sephadex G-25 or G-10.[141,547,550,562] The amount of each nucleoside in the mixture was $(1 - 10) \times 10^{-9}$ mole. The ratio of the column diameter to its height should be in the range 1:50–1:150 to ensure a clear-cut separation[547,562]. The sequence of the elution of the nucleosides from the column under these conditions is U(T) > C > G > A. The bases are separated in the same sequence, but in the presence of nucleosides the separation of adenosine and guanine is incomplete[553,556].

A mixture of nucleosides and bases can be analysed also in the presence of nucleotides, since the latter are eluted under these conditions as a separate peak ahead of nucleosides[547,553]. The amount of nucleotides should not exceed 50% of the total amount of substance injected. However, if longer columns are employed, then at lower rates of elution it is possible to separate a 12:1 mixture of nucleotides and nucleosides[547].

The fractionation of nucleosides and G-10 can be achieved also at neutral pH. However, an additional separation of uridine and cytidine, which overlap either partially (elution with phosphate buffer at pH 7.0) or completely (elution with water), is then necessary. Adenosine and guanosine are completely separated[549,558].

A similar behaviour has been observed at the same pH values also for nucleic acid bases[557]. Elution with 0.05 M phosphate buffer at pH 7.0 leads to a clear-cut fractionation of purine bases, including the minor bases, and to an incomplete separation of the pyrimidine bases. It is noteworthy that under these conditions not only hypoxanthine, xanthine, adenine, and guanine are separated, but also various methylated purine derivatives. The separation of methylated purines shows that the introduction of the methyl group decreases the degree of reversible adsorption of the compound[557].

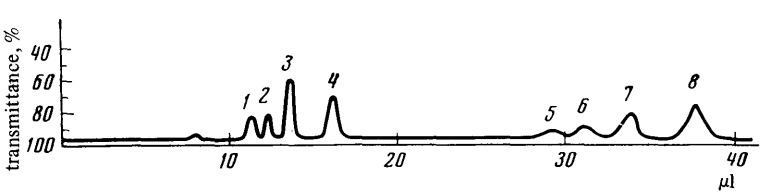


Figure 9. Chromatography of a mixture of deoxyribonucleosides and ribonucleosides on a fractionated Biogel P-2 (0.8 × 0.68 column); rate of elution 2.8 ml h^{-1}; volume of eluate 50 μl; 1) uridine; 2) guanosine; 3) deoxyguanosine; 4) thymidine; 5) cytidine; 6) adenosine; 7) deoxycytidine; 8) deoxyadenosine[566].

Guanylic and adenylic 2′:3′-cyclic phosphates can also be successfully separated on a G-10 Sephadex column[549]. Nucleotides are not separated from one another[141,557,562-565].

Another material most frequently employed recently for the separation of alkaline and acid DNA hydrolysates is polyacrylamide gel (Biogel P-2 and P-6).[136,158,566-574]. The advantage of polyacrylamide gel over different types of Sephadex is that a smaller volume of the gel is needed for separation, which shortens the elution time; the compounds are eluted in much smaller volumes and quantitatively[566]. It is recommended that nucleosides be separated on a fractionated Biogel P-2 at high pH.[158,566,572,573] The fractionation of the Biogel and the preparation of the columns have been described[566,572]. A mixture of ribonucleosides and deoxyribonucleosides was separated in a 0.1 M ammonium bicarbonate buffer with 1×10^{-3} M $Na_2B_4O_7$ at pH 10.1 (Fig. 9)[566,573]. The presence of nucleotides or high salt concentrations in the initial mixtures does not interfere with the separation of nucleosides, since both nucleotides and salts are readily separated from them[566,572,575,576].

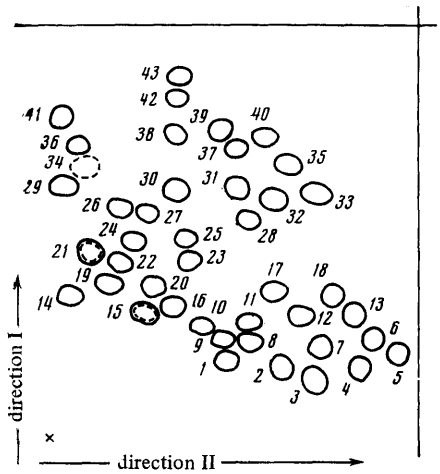


Figure 10. Two-dimensional chromatography of a complex mixture of nucleotides, nucleosides, and nucleic acid bases on a thin layer of cellulose. First direction: 6:3:1 n-propyl alcohol—25% ammonia—water system. Second direction: 2:79:19 isopropyl alcohol—sat.ammonium sulphate—water system[519]: 1) ATP; 2) GDP; 3) GTP; 4) ITP; 5) CTP + UTP; 6) CDP + UDP; 7) IDP + TTP; 8) GMP-5′; 9) TPN; 10) GMP-3′ + ATP; 11) GMP-2; 12) IMP-5′ + TDP; 13) CMP-5′ + CMP-2′ + CDPG + UMP-5′; 14) uric acid; 15) TPNH; 16) AMP-5′ + DPN + ADPG; 17) TMP-5′; 18) CMP-3′ + UMP-2′ + UMP-3′; 19) AMP-3′; 20) AMP-2′; 21) DPNH; 22) guanine; 23) orotic acid; 24) xanthine + GMP-2′:3′; 25) guanosine; 26) 7-methylxanthine; 27) deoxyguanosine + xanthosine; 28) 5-aminothymine; 29) adenine; 30) hypoxanthine; 31) inosine; 32) isocytosine; 33) uridine; 34) 2-aminopurine; 35) uracil + cytidine + cytosine + deoxyuridine; 36) adenosine; 37) 4-methyluracil; 38) 2-thiouracil; 39) thymidine + thymine; 40) 3-methylcytosine + deoxycytidine; 41) deoxyadenosine; 42) nicotinic acid; 43) nicotinamide.

VI. MIXTURES OF NUCLEOTIDES, NUCLEOSIDES, AND NUCLEIC ACID BASES

Partition[518,519] or ion-exchange thin-layer chromatography[438,577], electrophoresis[484,487], and ion-exchange[498-504,543] or adsorption[549,553,556,568,570] column chromatography can be used to fractionate complex mixtures of different nucleic acid components as well as for the separation of compounds of the same type.

Thin-layer chromatography on cellulose and cellulose ion-exchangers has been mainly used recently for the rapid analysis of microamounts of a mixture of nucleotides, nucleosides, and bases. Clear-cut fractionation of a large number of compounds on cellulose can be achieved with the aid of two-dimensional chromatography[518,519].

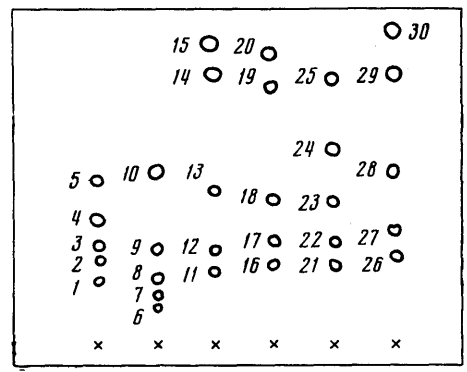


Figure 11. Chromatography of a mixture of nucleotides, and nucleosides, and bases on a layer of PEI-cellulose[577]; gradient: 0.6 ml of 0.1 M LiCl, 0.6 ml of 0.2 M LiCl, 0.6 ml of 0.5 M LiCl, 0.6 ml of 1 M LiCl, and 1.2 ml of 2 M LiCl: 1) ATP; 2) ADP; 3) AMP; 4) adenine; 5) adenosine; 6) GTP; 7) GDP; 8) GMP; 9) guanine; 10) guanosine; 11) UTP; 12) UDP; 13) UMP; 14) uracil; 15) uridine; 16) ITP; 17) IDP; 18) CMP; 19) cytosine; 20) cytidine; 21) ITP; 2) IDP; 23) IMP; 24) hypoxanthine; 25) inosine; 26) TTP; 27) TDP; 28) TMP; 29) thymine; 30) thymidine.

The separation of 22 compounds on MN-300 cellulose using the 57:4:39 isobutyric acid—concentrated ammonia—water solvent system in the first direction and the 7:3 methanol—1 M ammonium acetate system for two runs in the second direction may be quoted as an example[518]. Not only nucleoside mono-, di-, and triphosphates but also the 3′- and 5′-nucleotides are separated from one another fairly distinctly.

Fig. 10 illustrates the chromatographic separation of more than 60 nucleotides derivatives[519]. Special mention should be made of the satisfactory fractionation of the bases and the corresponding ribonucleosides, deoxyribonucleosides, the mono-, di-, and tri-phosphates of various nucleosides, and the 2′-, 3′-, and 5′-phosphates of ribonucleosides. Compounds with the same chromatographic mobility can be refractionated in another solvent.

Separation of the bases, nucleosides, and nucleotides with a high resolution has been achieved on PEI-cellulose using a salt gradient[578] (Fig. 11). Since the R_f values for nucleotides depend on the salt concentration[438], the bases and nucleosides can be readily separated from nucleotides by employing as the initial solvent distilled water or a 1:1 methanol—water mixture[438,442,578].

The separation of a complex mixture of bases, nucleosides, and nucleotides on columns has been described[498-500,503,504,543,549,553,556,557,568,570].

Gas—liquid chromatography (GLC), proposed initially only for the separation of acetyl, methyl, and isopropylidene derivatives of nucleosides[579] and methylated bases[580,581], has also been used recently to separate nucleic acid derivatives. In consequence of the low volatility and instability in the chromatographic process of the substances to be separated, these derivatives can be used only for qualitative analysis. The above difficulties were partly eliminated in the chromatography of trimethylsilyl derivatives of nucleosides[581-585]. Many modifications of the method are employed[581,583-591]. Chromatography of bis(trimethylsilyl)trifluoroacetamide derivatives has been most widely used[589,590,592-596]. However, in this case too certain nucleosides (cytidine, adenosine) give rise to several products after silylation[595,596]. For cytidine, this difficulty was obviated by modifying the cytidine derivatives with hydroxylamine derivatives before silylation[596]. GLC can be used to analyse the nucleotide composition of DNA and RNA,[589,593] but the sensitivity of the method is still insufficient (1—5 μg of nucleic acid per analysis). The GLC method can be usefully employed to analyse the compositions of synthetic oligonucleotides. The separation of rare nucleic acid components has been described[586,590,594,596,597].

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