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SEPARATION OF NUCLEIC ACID CONSTITUENTS BY COLUMN LIQUID CHROMATOGRAPHY

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Introduction

Among the main factors related to the rapid growth of nucleic acid biochemistry, the development of useful separation methods for nucleic acid constituents should be mentioned in the first place. Without these methods which have found wide application not only in estimating nucleic acid compositions

but also in nucleic acid metabolism as well as in evaluating the effect of drugs on the existant nucleotide pools it would have been impossible to examine and determine the biological function of nucleic acids.

To provide valuable information on the role and structure of nucleic acids, investigators have used a variety of techniques including thin layer, paper, gas-liquid, gas-solid, liquid column chromatography, and electrophoretic methods. Since Cohn[1] first separated nucleic acid constituents in 1949, liquid column chromatography has been the most widely used method in the separation and quantitation of nucleic acids and their constituents. Liquid chromatography on column of ion exchange resins is the most frequently employed method for the separation of nucleic acid bases, nucleosides, and nucleotides, which was originally developed chiefly by Cohn and his colleagues.[2-10] Several earlier reviews on the ion exchange chromatography of nucleic acid constituents may be found.[11-14]

In 1967, there was a considerable breakthrough in this field. Horvath et al.[15] developed stable, efficient ion exchange resins, pellicular packing materials[16], and applied high pressure which permitted the rapid and very sensitive analysis of nucleotides. As the speed of the separation depends to a large extent on the structure and size of the packing material in the column, by this innovation complete elution time could be significantly decreased.

Realizing this situation, it is obviously clear that an investigator aiming at the separation of nucleic acid constituents should make a choice be-

tween classical and high speed chromatography. The present writers, reviewing the latest and remarkable developments in both fields, attempt to give some direction to this choice to achieve better separation and analysis of such compounds.

Properties of the constituents

Nucleic acid constituents may be discussed on four levels of structure: N-bases, nucleosides /sugar derivatives of the N-bases/, nucleotides /phosphate esters of the nucleosides/, and oligonucleotides /polymers of nucleotides/. The fifth level of structure is nucleic acid itself. In the above order, the individual bases which may be purines and pyrimidines are the simplest in structure. Nucleosides and nucleotides may be classified according to their sugar content /pentose or deoxypentose/. Nucleotides may be also differentiated according to their phosphate content /mono-, di-, and triphosphates/ and which C atom is phosphorylated /C-2', C-3', etc./. A great variety of base and sugar modified nucleotides and oligonucleotides have also biological importance. Other biologically important compounds have also nucleotide structures including coenzymes such as the nicotinamide nucleotides, coenzyme A, uridine nucleotide coenzymes, etc.

Since this review deals primarily with the separation of nucleic acid constituents, for further insight into the structure of these compounds and higher molecular weight nucleic acids, the reader is referred to the many remarkable monographs, reviews, and books covering this subject.

It should be also mentioned that in this review the terminology recommended by the IUPAC-IUB Commission on Biochemical Nomenclature and approved by IUPAC and IUB is applied.[18,17]

Due to the similarities and differences in the ionic nature of nucleic acid constituents, their acid-base and dissociation behavior may be extremely important in the chromatographic separation. Because amphoteric behavior is an inherent part of these molecules, it is clear that separations by either cation or anion exchange chromatography were, a priori, equally applicable. Therefore, as Cohn[13] has shown, four different systems could be envisioned:

1. cation exchange
   a. with high competing ion concentration on the acid side of the cationic pK's of the compounds
   b. with lower competing ion concentration on the reverse, alkaline, side of these pK's
2. anion exchange
   a. with high anion concentration on the alkaline side of the anionic pK's
   b. with low anion concentration on the reverse side of these pK's

From bases containing amino groups, cations may be formed most readily and there is some correlation between their pK values in the acid region and their elution order from a strongly cationic exchanger. This correlation may be, however, modified by exchanger acidities or internal pH-s in the salt form. Alkylation or arylation of the bases increases their retention times, increasing their affinity for the resin.

Separations of nucleosides by charge reduction on both cation and anion exchangers have been carried out.

Nucleotides having phosphate groups bounded to nucleosides may be favorably separated by anion exchangers. Cation exchange materials may be also employed in the case of using their salt form and buffered solutions to avoid the breakage of the glycosyl linkages of the purine and cyclic nucleotides. Because nucleotides to be separated by anion exchange chromatography are widely divergent in ionic and chemical nature, gradient elution technique should be used.

The behavior of sixty-four nucleic acid compounds was studied by Garel et al.[19] in a "saline solvent system" containing 1.5 M potassium phosphate pH 7.0, 2-methoxyethanol, and 2-butoxyethanol. From the values of partition isotherms and coefficients, it can be concluded that phosphorylation of nucleosides accounts for negative, but additive effect on partition coefficients. Nucleoside monophosphates have higher partition coefficients than diphosphate derivatives. N-alkylation and ribose - 3'-deoxyribose transformation increase partition coefficients. Partition isotherms of 3'-exoguanylic and 3'-exopyrimidilyc di- and triribonucleotides are also given.[20] Contribution of nucleotide composition and chain length has been evaluated. The logarithm of the partition coefficients of 3'-exopyrimidilyc oligoribonucleotides varies with the relative levels of Ap and Gp, expressed as the Ap/ /Ap+Gp/ ratio whereas the logarithmic values of partition coefficients are a linear function of chain length, although sequential isomers are not distinguishable.

Although analysis of nucleic acid constituents is carried out overwhelmingly by ion exchange chromatography, other techniques may be successful, too. Wasternack[21] has applied Sephadex G-10 packing material to separate pyrimidines. A relation could be demonstrated between a log function of capacity factor analogue values /V_e^o/ and the pattern of substitution of the pyrimidine nucleus. Methylation raised these values in the increasing order of 1-Me, 3-Me, 6-Me, and 5-Me /Table I./. A similar behavior could be observed for halogen derivatives /F, Cl, Br, I/ of uracil.

Nucleic acid bases

The separation of nucleic acid bases /and some nucleosides/ on Sephadex G-10 packing material is affected by a number of conditions. Their influence on the adsorption behavior of the compounds has been studied by Wasternack and Reinbothe.[22] For purine and pyrimidine compounds different mechanisms were suggested as a result of different ΔH^o enthalpy values. Because for most of the compounds examined the basic heterocyclic nitrogen atoms are the primary sites of adsorption, hydrogen bonding between the unshared pairs of electron of these N atoms and the gel hydroxy groups is very important in separations. Therefore, an increase in ionic strength increased retention time, while dioxane, n-propanol, and urea had a reverse effect. Elution order of compounds corresponded to their degree of dissociation. The degree of adsorption decreased with increasing temperature /Figure 1/.

TABLE I

Relation between structure and chromatographic behavior of pyrimidine compounds on Sephadex G-10 /Contribution by Ref. 19/

Compound	V_e^o	log V_e^o
UMP	0.11	- 0.963
CMP	0.13	- 0.903
TMP	0.30	- 0.519
Urd	0.98	- 0.011
2-thiouracil	1.04	0.018
1,3-dimethyluracil	1.08	0.033
1,3,6-trimethyluracil	1.09	0.038
5-fluorouracil	1.10	0.041
Cyd	1.20	0.078
1,6-dimethyluracil	1.25	0.097
5-diazauracil	1.50	0.176
Ura	1.55	0.190
6-methyluracil	1.73	0.238
Cyt	1.77	0.248
5,6-dimethyluracil	2.06	0.313

Sephadex G-10 may be also successfully used to separate a mixture obtained in the acid hydrolysis of methylated DNA.[23] The compounds m^3Gua, m^7Gua, m^1Ade, m^7Ade, and O^6-mGua were separated on a 85x 1.5 cm column, using 0.05 M ammonium formate at pH 6.8.

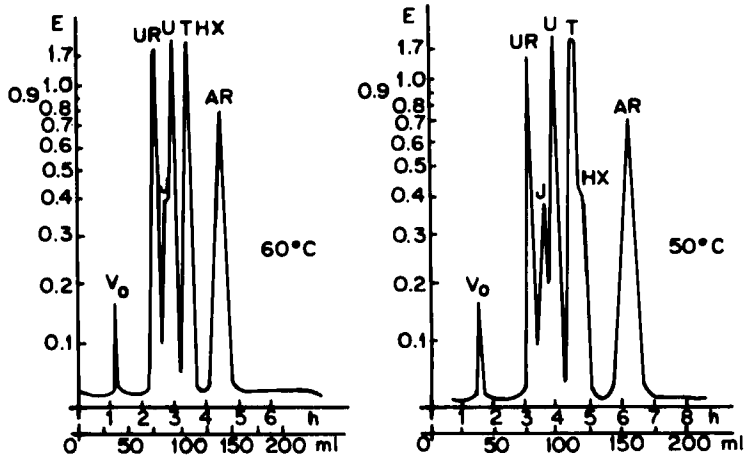

FIGURE 1
Separation of nucleic acid constituents on Sephadex G-10
Components: UR /uridine/, I /inosine/, U /uracil/, T /thymine/, HX /hypoxanthine/, AR /adenosine/; Column: 90 x 1.3 cm; Eluent: 0.01 M $/NH_4/_2CO_3$; pH 9.0; Flow rate: 15 ml/hr /Contribution by Ref.22/

Samples of acid hydrolysates of alkylated rRNA could be also analyzed by ion exchange chromatography.[24,25] Dowex-50 H^+ columns were equilibrated with 0.75 M HCl and developed at a flow rate of 15 ml/hr by using a convex gradient of 0.75 - 2.0 M HCl /Figure 2/. Chromatography on columns of Dowex-50 H^+ failed to separate m^7Gua and m^3Ade as well as Ade and m^7Ade.

Dowex-50 columns for the separation of alkaline, $HClO_4$, and enzymic hydrolysates of alkylated rRNA should be, however, equilibrated with 0.3 M ammonium formate and developed by using a gradient of 0.3 - 1.0 M ammonium formate at pH 8.9./Figure 3/. This column was employed to estimate the molar proportions of m^3Cyt, m^1Ade, and O^6-mGua at the nucleoside level.

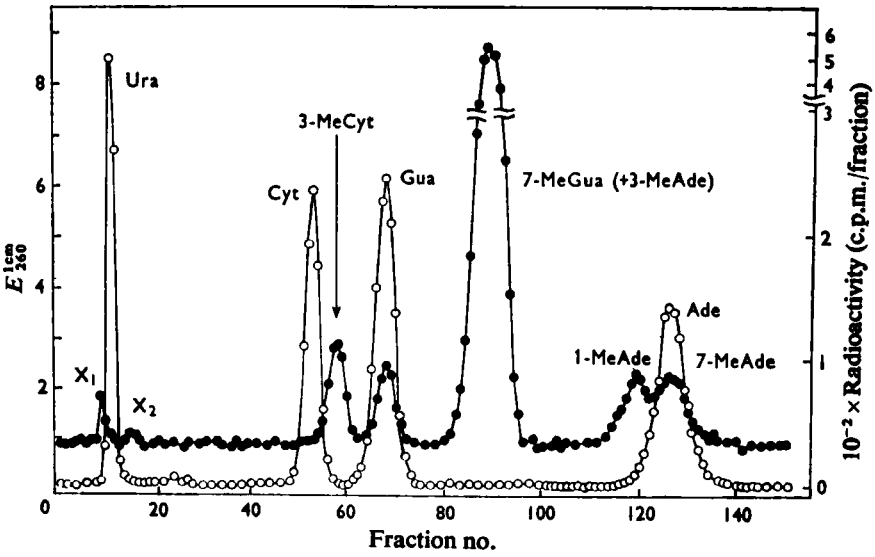

FIGURE 2
Ion-exchange chromatography of an acid hydrolysate of an alkylated rRNA
Column: Dowex-50 H^+, 200-400 mesh, 28 x 1 cm; Eluent: 0.75-2.0 M HCl; UV and radioactivity detection /Contribution by Ref. 25/

Hypothesis of action of 1,2-dimethylhydrazine as a methylating agent could be proved by using ion exchange chromatographic separation on Dowex-50 H^+ column with a 1.0 - 3.0 N exponential HCl gradient.[26]

Characterisation of RNA alkylated by sulphur mustard after hydrolysation has been also carried out on Bio Rad AG 50 W-X_4 resin pre-equilibrated with 1.0 M HCl and eluted with a linear gradient of 1.0-12.0 M HCl.[27]

The shortcomings of these separations are their slow flow rates. On the other side, however, they have relatively high capacity.

In searching faster techniques for the separation of nucleic acid bases, Kelemen and Degens[28] have used a pressure-gradient elution technique by which the separation of bases could be achieved within 6o min at room temperature. The procedure used ECTEOLA cellulose ion exchanger, sodium chloride in NH_4OH and NH_4Cl at pH 10.6 and distilled water adjusted with HCl to a pH of 2.75. Because the positions of Ura and Thy are almost identical, to separate these bases, a 6 hour equilibration of the column with NH_4OH should be employed.
Column efficiency and speed of analysis have been significantly increased by using very fine /particle diameters of less than 2o microns/ ion exchange resins and higher column inlet pressures.[29-31]

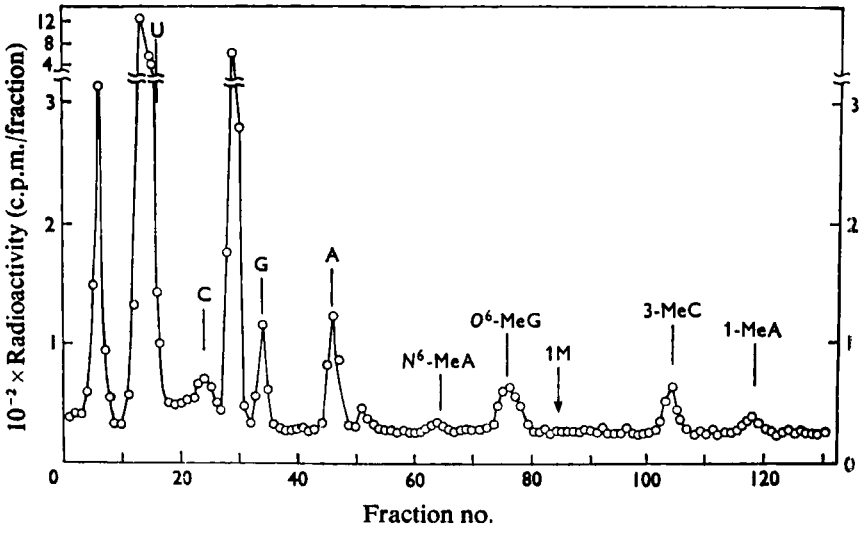

FIGURE 3
Ion-exchange chromatography of an enzymic hydrolysate of an alkylated rRNA, Column: Dowex-50 NH_4^+ 28 x 1 cm; Eluent: 0.3-1.0 M ammonium formate; UV and radioactivity detection /Contribution by Ref.25/

Despite these improvements, the analysis of nucleic acid bases /and nucleosides/ at the subnanomole level was the first time carried out by Horvath and Lipsky.[32] They have used pellicular ion exchange materials developed at that time, which consist of thin shells of ion exchange resin bounded to the surface of glass microspheres. This separation was performed by elution with acidic salt solutions of KH_2PO_4 and $NH_4H_2PO_4$.
The effect of the most important parameters which determine the equilibrium and transport phenomena, such as the chemical composition of the salt in the eluent, the concentration of the salt solution, the pH of the eluent, the column temperature, the applied pressure and flow rate, and the length and diameter of the column, on the separation has been investigated by Horvath and Lipsky using a 300-cm long pellicular cation exchange resin column. The ion exchange capacity of pellicular ion exchangers is generally smaller /2-5 μequiv/g/ than that of comparable conventional resins /2-5 mequiv/g/. When nucleic acid bases are separated by salt elution at fixed pH and temperature, distribution coefficient increases as the salt concentration in the eluent decreases which means less concentrated eluents from pellicular columns than from conventional columns /this may be balanced by using narrower columns/. The pH of the mobile phase significantly influences both the retention time and the band spreading of the sample components /Figure 4/.

The double peaks of Gua appeared in the pH range of 4.7 - 5.2 suggest two equilibria giving two different distribution constants or kinetic effects. Increasing

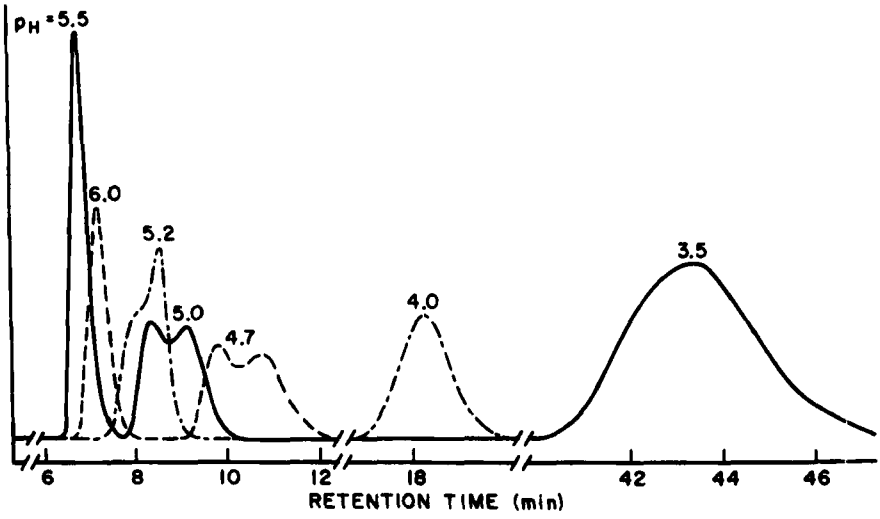

FIGURE 4
Effect of pH on elution pattern of guanine
Column: 300 cm x 1 mm pellicular cation exchanger; Eluent: 0.02 M KH_2PO_4; Flow rate: 14.8 ml/hr; Temperature: 60°C; /Contribution by Ref. 32/

temperature gives sharper and more symmetrical peaks; increasing diffusion rate, on the other hand, however, may cause increasing relative retention times /Figure 5/. For the adjusted retention volumes of bases vs. inverse temperature, Arrhenius type plots could be drawn. For the separation of bases, high column temperature has been found more favorable than the lower one. High pH appears however, to be more effective than high temperature alone. The dependence of plate height on flow velocity has been found to be nearly linear, indicating that band spreading is controlled only by stationary phase mass transfer at higher flow rates. All in all, optimization of the operating conditions with pellicular ion exchange materials closely follows guide lines having been applied to conventional ion exchange resins.

UV detection systems applied to columns packed with pellicular materials requires low noise level and a flow cell of small volume-to-light-path ratio.

Purine and pyrimidine bases could be separated within 16 minutes on a pellicular cation exchange resin column of 300 cm x 1 mm at 70°C, using 0.05 M $NH_4H_2PO_4$ at pH 2.5 or within 5 minutes using 0.025 M $NH_4H_2PO_4$ at pH 4.4.[33]

The modification of the phosphate mobile phase to 0.008 N HNO_3 makes the use of shorter column possible and gives similar resolution for bases.[34]

Using 0.01 M HNO_3 and a Zipax controlled surface porosity cation exchanger column of 1 mm x 2.1 mm, a good separation of bases could be achieved.[35]

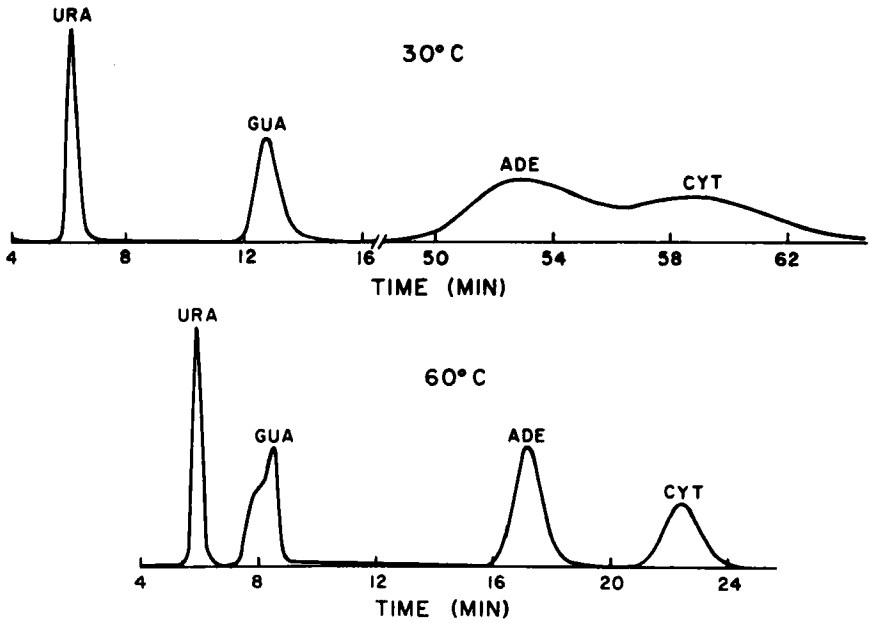

FIGURE 5
Effect of temperature on the separation of bases
Same conditions as in Figure 4, /Contribution by Ref. 32/

A similar separation on a Vydac cation exchanger has been reported, using 0.1 M ammonium acetate at pH 4.9.[36]

Scott and his coworkers[37] have applied vacancy chromatography to the separation of bases on a pellicular ion exchanger column. Vacancy chromatography made possible to evaluate differences in composition between a circulating mobile phase and an injected sample and to allow a single elution peak of some selected component to be observed even though it would be eluted partially unresolved under nonvacancy conditions. In the experiments, 600-µl slugs of the bases in buffer were injected into a column in which the total void volume was only 800 µl. The advantage of this technique is to mask out unwanted peaks which could normally overlap and interfere with the component of special interest.

Unusual bases such as Hyp and m^6Ade were also separated from "regular" bases on a Zipax SCX column.[35]

In addition to ion exchangers, other packing materials may be employed in HSLC to analyse unusual bases. Pfadenhauer[38] have used a silicic acid column of 300 cm to measure human plasma levels of the endogenous oxypurines, Xan and Hyp. The solvent system was ethyl ether - n-propanol - 5% acetic acid in water /35:14:4/ flowing at 45 ml/min.

Nucleosides

Uziel et al.[31] have demonstrated the advantages of enzymatic hydrolysis of nucleic acids to their nucleosides. They were able to separate and quantitate nanomole quantities of nucleosides in less

than 1 hr on a cation exchange resin and to avoid the isomeric peaks that arise from alkaline hydrolysis.

Busch[37] has employed cation exchanger column and ammonium formate buffers in routine works and studied the dependence of retention values on pH /Figure 6/. In the pH range of 3.0 - 4.0, most of the bases and nucleosides strongly vary their relative retention time. Relative retention times can be also shifted by varying temperature /Table II/. The separation of NAD^+, AMP, Xan, Ino, Hyp, Ado, Gua, and Ade could be successfully carried out on a

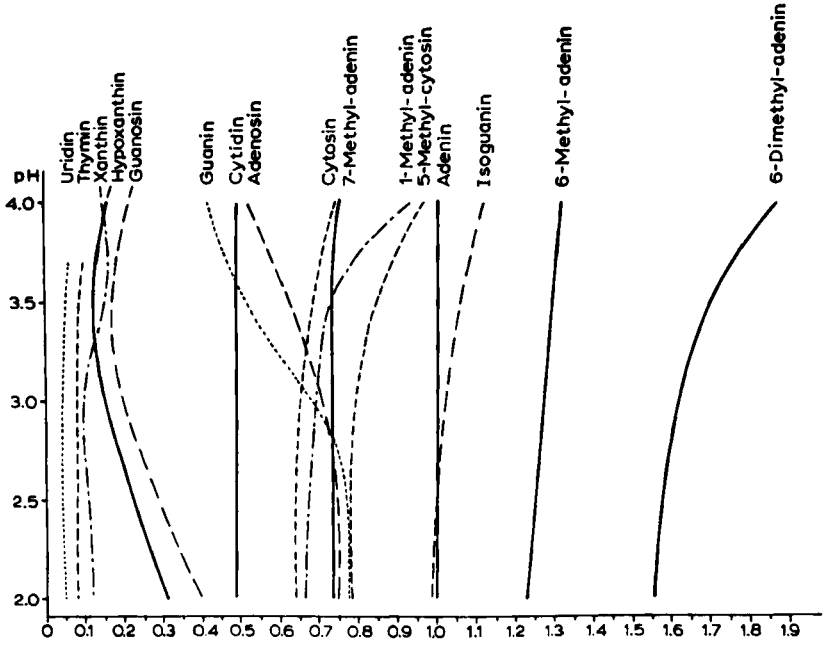

FIGURE 6
Dependence of relative retention on pH
Column: Dowex-50 Na^+ 200-400 mesh, 20 x 1 cm; Eluent: ammonium formate buffer with 0.35 M NaCl,/Contribution by Ref. 39/

Table II

Relative retention of bases and nucleosides at different temperatures /Contribution by Ref. 39/ /Same conditions as in Figure 6/

Compound	21-23°C	37.5°C
Hyp	0.12	0.18
Guo	0.17	0.20
Cyd	0.48	0.49
Gua	0.52	0.49
Ado	0.64	0.56
Cyt	0.69	0.77
m^1Ade	0.72	0.83
m^5Cyt	0.82	0.93
Ade	1.00	1.00
m^6Ade	1.29	1.35
m_2^6Ade	1.68	1.71

Dowex-50 H^+ ion exchanger column /25 cm x 1 cm/, using stepwise elution of water to 2 N HCl /first step 0.005 N HCl, second step 0.5 N HCl/. 3'- or 5'-deoxynucleosides and deoxynucleotides have been separated on Dowex-50 cation exchanger using 0.3 M ammonium formate at pH 9.2.[40] Separation of the similar ribo-compounds could be also carried out on a longer column using the same buffer but adjusted to pH 8.9. For the separation of all four nucleosides and 5'-nucleotides, a two step gradient elution method of 0.015 M /pH 3.2/ - 0.3 M /pH 9.2/ ammonium formate has been applied. This

separation is extremely pH-dependent. A variation of 0.1 in pH affects the elution position of Guo so that it interferes with either Cyd or Ado. The separation of dThd from ribonucleosides, which was carried out in the chromatographic system above, provides an easy method for the identification of DNA contamination in RNA preparation. Chromatography of nucleosides, Ado, Guo, Ino, and Urd was successful on a Dowex anion exchange column, using 0.4 M ammonium formate mobile phase.[41] These nucleotides were also separated from AMP, GMP, and ATP on this column, using gradient elution of 0.4 - 3.0 M ammonium formate buffer. Separation and identification of nucleosides obtained from methylated RNA can be carried out on a Dowex-50 NH_4^+ column with ammonium formate mobile phase.[24] The positions of elution of various nucleosides are shown in TableIIIas percentages of those of Ado; the elution volume for Ado was about 300 ml at pH 8.9 and about 120 ml at pH 6.0. During the digestion and subsequent chromatography, the principal product, m^7Guo, may split into several components. In addition to this, other known products of methylation of RNA that could not be isolated at the nucleoside level, including m^3- and m^7Ado, could be detected as bases.

In their epoch-making work, Horvath and Lipsky[32] studied the influence of chromatographic conditions on nucleosides, too. The variation of the salt concentration in mobile phase had the same effect on the nucleosides as on the bases. From the variation of the adjusted retention volumes of nucleosides it can be seen that the equilibrium

TABLE III

Elution of nucleosides obtained from methylated RNA /Contribution by Ref. 24/

Column material: Dowex-50 NH_4^+; Eluent: ammonium formate; I., 0.3 - 1.0 M pH 8.9; II., 1.0 - 2.0 M pH 6.0

Compound	I.	II.
m^1Ado	327	272
m^6Ado	135	12o
Cyd	49	-
m^3Cyd	286	256
Guo	88	83
m^1Guo	92	-
m^2Guo	126	-
O^6-mGuo	160	122
m^7Guo	265	323
Ino	51	55
Urd	33	28
m^3Urd	53	33

constants decrease with increasing pH. For fast separations of nucleosides /and bases/, the pH range from 5.2 to 5.5 was found most adequate to achieve full separation even at high flow velocities. There is a pH, however, where the effect of the temperature on the retention is the most pronounced. Optimum pH and temperature for the separation of the four nucleosides are about 5.5 and 40°C, respectively /Figure 7/.

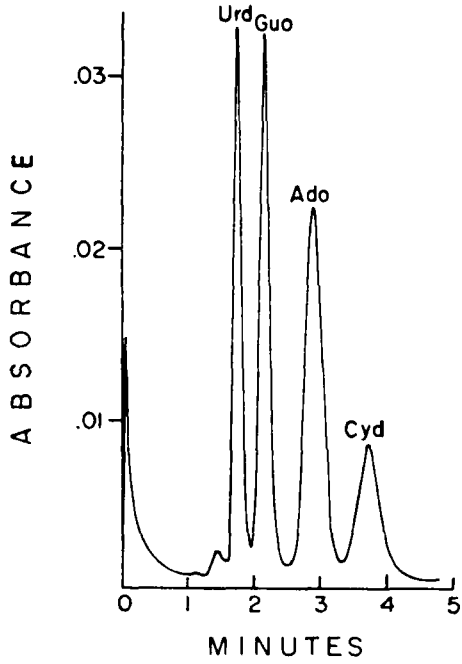

FIGURE 7
Rapid separation of ribonucleosides
Column: pellicular cation exchanger, 151.7 cm x 1 mm;
Eluent: 0.020 M $NH_4H_2PO_4$ pH 5.6; Temperature: 39°C;
Flow rate: 25.5 ml/hr /Contribution by Ref. 32/

A standard mixture containing 0.1 µg of each ribonucleoside /Urd, Guo, Ado, Cyd/ was separated in less than 2 min by eluting from a conventional cation exchanger with 0.4 M ammonium formate at a flow rate of 80 ml/min /Figure 8/.[42]
On another conventional cation exchanger /Aminex A-6/, these nucleosides could be separated by 0.5 M ammonium formate at pH 4.65.[43]
A mixture of deoxyribonucleosides prepared that the mole ratio of /dAdo+dThd/ to /dGuo+dCyd/ was 30 to 1 were analyzed on a Bio Rad A-7 cation exchanger column at 55°C, using 0.4 M ammonium formate at pH 4.55 /Figure 9/.

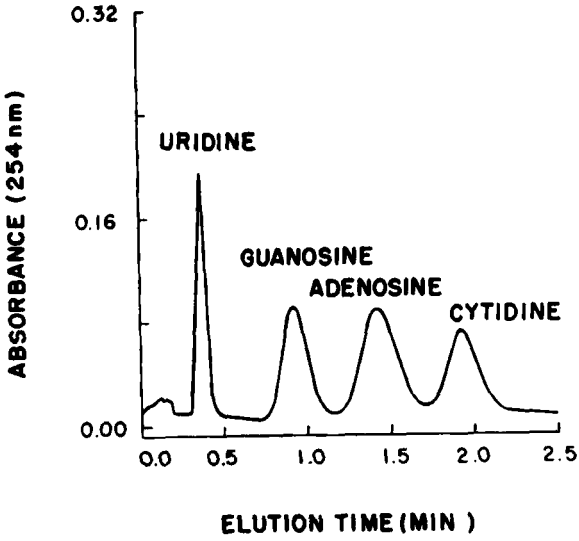

FIGURE 8
Rapid separation of ribonucleosides
Column: VC-10 cation exchanger, 7-14 μm, 25 x 0.24 cm; Eluent: 0.4 M ammonium formate pH 4.5; Flow rate: 80 ml/hr; Temperature: 75°C /Contribution by Ref. 42/

Using similar conditions and shorter column, nucleosides with substituted adenine bases could be separated /Figure 10/. For the separation of Qrd, Urd, Ura, Xao, and m^7Xao on this column, a mobile phase of 0.1 M ammonium formate /pH 4.5/ was adequate at 27°C.

In the purity control of m^1AMP, hydrolyzed to nucleoside level, Ado, m^6Ado, m_2^6Ado impurities were found, using cation exchanger column /30 cm x 2.3 mm/ and 0.4 M ammonium formate /pH 4.65/ mobile phase.[44] The purity control of 1-N-oxide-adenosine, after enzymatic digestion, could be also carried out on this column.

The α- and β-anomers of cycloprpyl derivatives of Ado could be successfully separated on a Zipax SCX

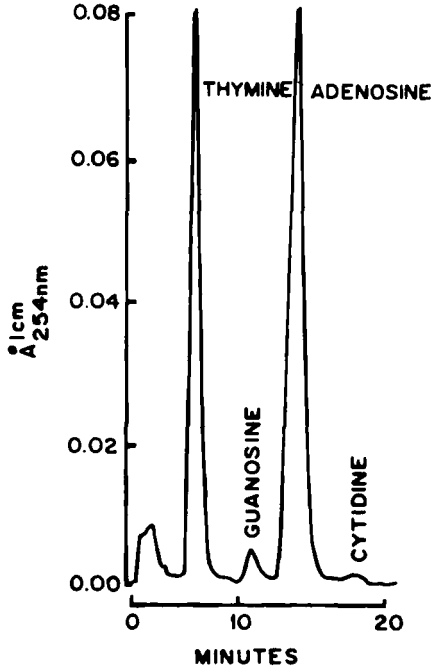

FIGURE 9
Separation of deoxyribonucleosides
Column: Bio Rad A-7 cation exchanger, 7-10 μm, 25 cm x 2.4 mm; Eluent: 0.4 M ammonium formate pH 4.55; Temperature: 55°C, Flow rate: 10 ml/hr /Contribution by Ref. 33/

column /50 cm x 2 mm/ using 0.025 M $NaNO_3$ mobile phase of 1.5 ml/min flow rate.[45]

Singhal and Cohn have applied cation-exclusion chromatography on anion exchangers to the separation of various modified and unmodified nucleosides.[46-48] Figure 11 illustrates the influence of pH on peak positions, resolution, and HETP. Pyrimidines appear before purines, and cations emerge before noncations at pH 4.0. As the pH is lowered, the two cations, Cyt and Ado become more positively charged, therefore are more excluded, i.e. nearer

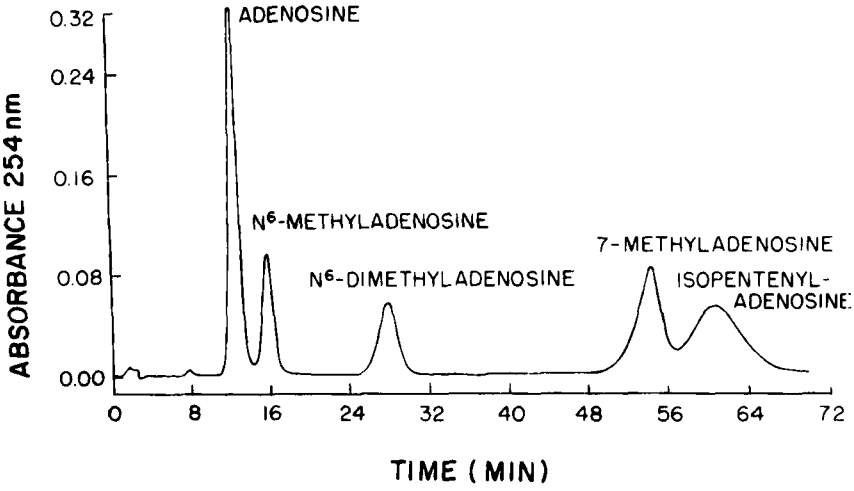

FIGURE 10
Separation of nucleosides with substituted adenine bases
Column: Bio Rad A-7 cation exchanger, 10 μm, 15 cm x 2.4 mm; Eluent: 0.4 M ammonium formate pH 4.50; Temperature: 60°C; Flow rate: 11.4 ml/hr /Contribution by Ref. 33/

the front /Figure 11/. The addition of EtOH to the eluent has a marked effect on nucleosides /Guo, Ado, m^5Urd/ that are more lipophilic in nature; the elution volumes shortened. The result presented in Figure 12 indicate that 21 ribonucleosides show some degree of separation under the conditions applied. Only three nucleosides appear in the same position. The method provides a good tool for isolation and quantitation of thiouridine /^{4}S/ in tRNA, too.

A direct comparison between ion-exclusion and ion-exchange separations /at different pH values and particle sizes/ of the common nucleosides indicates that the former carried out on columns filled with small beads of uniform size is superior or at least equal to the later.

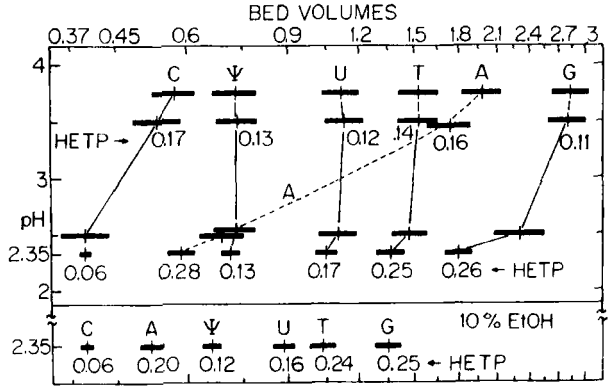

FIGURE 11
Effect of pH on resolution, elution volume, and HETP in cation exclusion-chromatography
Columns: Bio Rad Aminex-25, /1/. 10.8 x 0.635 cm and /2/. 50 x 0.5 cm /only for the analysis at pH 2.35/; Eluents: 0.01 M $HCOONH_4$ at pH 3.75 and 3.50; 0.05 M HCOOH for pH 2.50; and 0.10 M HCOOH for pH 2.35; the bottom analysis mobile phase contained 10% EtOH. Flow rates: 0.57 and 1.0 ml/min for columns /1/ add /2/, resp.; Temperature: 50°C, /Contribution by Ref. 46/

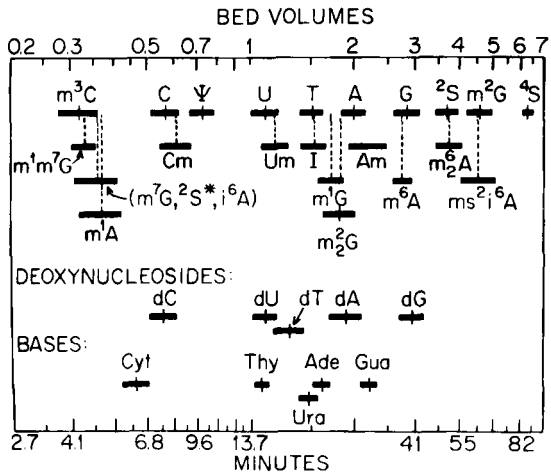

FIGURE 12
Cation-exclusion chromatography of nucleosides and bases
Same conditions as in Fig. 11, /Contribution by Ref. 46/

Nucleotides

The separation of 5'-nucleotides and nucleosides on a DEAE -Sephadex A-25 column of 60 cm x 0.9 cm has been recently reported.[49] The column was equilibrated for 2 hr with 0.0 7 M LiCl mobile phase and then a linear gradient to 0.3 M LiCl was employed. The complete separation took about 18 hours. The elution volumes of the compounds are tabulated in Table IV.

A DEAE-cellulose column / 12 x 1 cm/ pre-equilibrated for 2 hr with 0.0 25 Tris-formate pH 7.8 was applied to alkaline hydrolyzed RNA mixture.[50]

TABLE IV.

Elution volumes of nucleotides and nucleosides on a DEAE-Sephadex A-25 column /Contribution by Ref.49/

Compound	V_e /ml/
Cyd	46
CMP	292
CDP	404
CTP	460
Urd	218
UMP	472
UDP	536
Thd	353
TMP	478
Ado	107
AMP	376
ATP	514

Using 0.085 M Tris-formate and 7.0 M urea, the elution of the mononucleotides could be eluted as a single peak; for the elution of the second peak containing the dinucleotides, 0.17 M Tris-formate and 7.0 M urea was used as mobile phase. Chromatography of alkaline nucleotides obtained from 8-^{14}C-Ade supplemented ethanol/acetate grown Clostridium Kluyveri cells was carried out on a Dowex 1-X8 column using a linear gradient, 0 to 9 N HCOOH + 0.9 N $HCOONH_4$.[51] The nucleotides were localized by UV and radioactivity detection /Figure 13/.

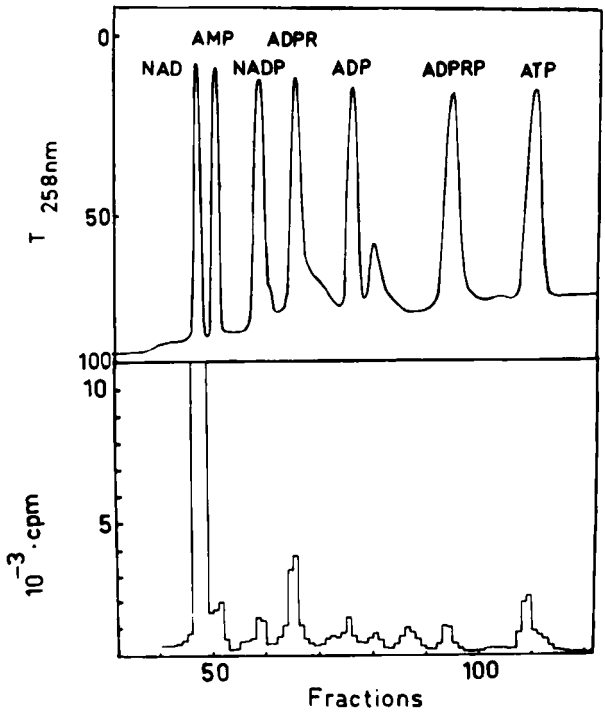

FIGURE 13
Chromatography of adenine nucleotides from C. Kluyveri cells, Column: Dowex 1-X8, 200-400 mesh, 2 m x 1 mm; Eluent: ammonium formate gradient; Temperature: 30°C, /Contribution by Ref. 51/

In the sequence studies of poliovirus RNA, chromatography on DEAE-cellulose was employed.[52] The elution of the hydrolysates was carried out with a gradient of NaCl in 7;0 M urea, 0.02 M Tris-HCl /pH 72./, 2.5 mM EDTA. The elution pattern indicated that the digest did not contain ppNp or pppNp nucleotides; thus poliovirus RNA is not 5'-terminated with a tri- or pyrophosphate.

A procedure has been devised for the separation of acid-soluble tissue nucleotides on a 90 x 1 cm DEAE-Sephadex A-25 column, using consecutive concave gradients of the volatile salt triethylammonium acetate at constant pH 4.7.[53] The complete resolution of these nucleotides in a single chromatographic run has not been achieved.

For the separation and estimation of the four major nucleotides of RNA and those of DNA, Dowex-1 anion exchanger has been used.[54] This analysis was completed within 9o min. For the fractionation of deoxyribonucleotides, 0.15 M formic acid was passed through the column until dCMP was completely eluted, then a complex gradient was used. At the separation of ribonucleotides, 0.2 M formic acid was the first eluent before the complex gradient. Because formic acid used in the separation has a high UV absorption, its contribution to the optical density of each peak should be measured under conditions identical to the normal procedure.

Virkola[59] has elaborated an automated gradient elution method of ion exchange chromatography to the determination of acid-soluble nucleotides at the nanomole level. This system applied Dowex-2 resin /formate form/, a reciprocal pump, and UV detection.

Horvath and Lipsky[55] used a liquid ion exchanger and 0.5 M KH_2PO_4 + 1 N NaOH to separate nucleic acid constituents.

The relationship between the chemical structure and elution position of nucleotides, nucleosides /and other compounds/ in an anion exchange system has been extensively studied by Katz and Burtis.[56] Pyrimidine derivatives eluted early, with the relative position being strongly influenced by the carbonyl group with an α-hydrogen. Therefore, Cyt and Cyd, each having one carbonyl group eluted earlier than Urd, Ura, and Thd, each having two carbonyl groups. The methyl group at position 5 in Thd appears to favor later elution. In purine derivatives, the carbonyl groups with α-hydrogen play the same role as with pyrimidine derivatives. Thus, there are great differences in the elution values of Hyp, Xan, and uric acid, which have one, two, and three of these structural elements. When the α-hydrogen is replaced by α-methyl groups, these compounds elute earlier than the corresponding α-hydrogen analog. An amine /or methylamine/ group in the 2 or 6 position favors later elution as compared with the analogous carbonyl compounds. Horvath and his coworkers[15] achieved rapid separation of nanomole quantities of ribonucleoside mono-, di-, and triphosphates by using a pellicular basic ion exchanger and gradient elution with a phosphate or formate buffer /Figure 14/. There is a linear relationship between salt concentration and the retardation factor. At high temperature, a significant reduction in tailing could be observed. Because the order of elution was the same as that with Dowex-1 columns, the retention

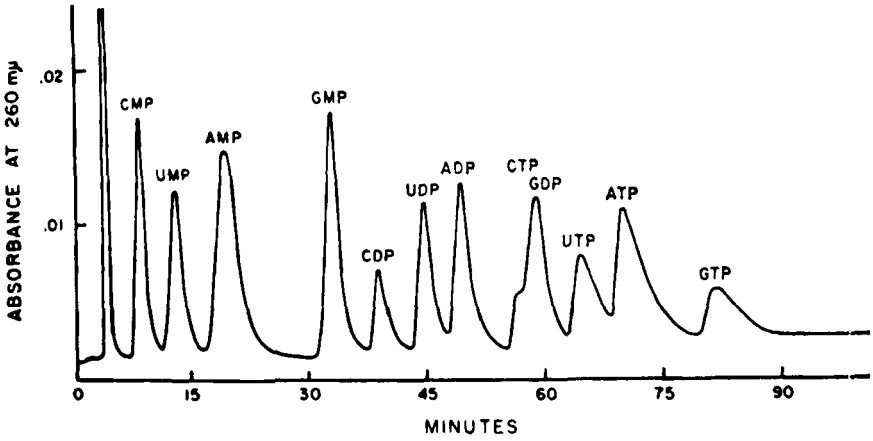

FIGURE 14
Separation of ribonucleoside mono-, di-, and triphosphates
Column: pellicular anion exchanger, 193 cm x 1 mm; Eluent: linear gradient of ammonium formate from 0.04 M to 1.5 M at pH 4.35; Temperature: 71°C; Flow rate: 12 ml/hr, /Contribution by Ref. 15/

mechanism for the classical and pellicular ion exchanger resin was nearly the same.

Using pellicular anion exchanger column, a mixture of mononucleotides can be cleanly separated within 1 hr.[57] To separate 2' and 3' isomers and all the other compounds in this mixture, two different mobile phases should be used.

Ribonucleoside-5'-monophosphoric acids could be also separated on a 1 m x 2.1 mm column of Zipax SAX, using 0.002 M KH_2PO_4 /pH 3.75/ mobile phase at 60°C.[35] On this column, mono-, di-, and triphosphates of nucleosides, nucleotides in beef and rat liver extracts as well as in pig whole blood could be separated and determined.[58] The determination of AMP, ADP, and ADPR impurities in NAD could be also carried out on such a column, using

an exponential gradient of KH_2PO_4 /pH 3.3/ from 0.002 M to 0.5 M at 2%/min.

High speed ion exchange chromatography /HSIEC/ was employed by Brown[60-62] to the rapid separation of nucleotides in cell extracts /Figure 15 and 16/. Enzymic peak shifts were used as a method of verifying peak identities, utilizing the specificity of enzyme reactions with a nucleotide or class of nucleotides. By using this technique to remove completely a large nucleotide peak having similar retention time to another nucleotide of special interest, we are then able to show conclusively the nucleotides that are present.[63]

Other nucleotide extracts have been also analyzed by HSIEC.[64-67]

Brooker has reported the determination of picomole amounts of enzymatically formed Ado-3':5'-P by HSIEC.[68] Pellicular ion exchange resin was converted to the chloride form by 0.1 N HCl and then washed to neutrality with water. The column temperature was 80°C and the flow rate 12 ml/hr of HCl /pH 2.20/.

An Ado-3':5'-P phosphodiesterase assay was combined with HSIEC by Pennington[69] using pellicular anion exchange resin column and 0.013 M HCl as mobile phase. Separation of AMP, cyclic 3'-5' AMP, and IMP was carried out at 75°C.

In the application of pellicular ion exchange chromatography for mixtures of ^{32}Pi AMP, ADP, and ATP at the picomole level reported by Shmukler[70], the separation was accomplished in 15 minutes. Shmukler using this column also analyzed the acid-soluble nucleotides of rat brains.[71] In this procedure, a linear concentration gradient was applied, formed

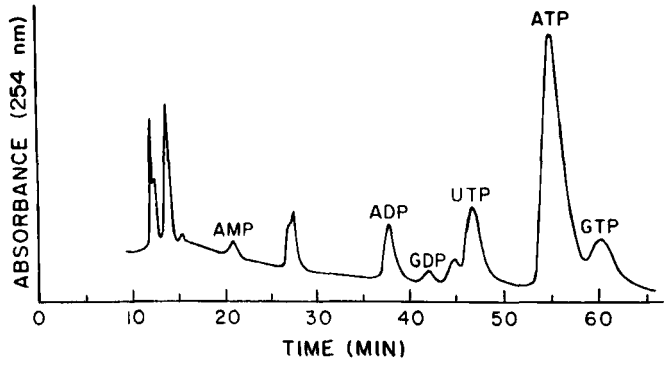

FIGURE 15
Nucleotide extracts from urine leukemia cells
Column: pellicular anion exchanger, 3 m x 1 mm; Eluent: 0.015 M KH_2PO_4; 0.25 M KH_2PO_4 in 2.2 M KCl pH 4.5; Temperature: 75°C; Flow rate: 12 ml/hr; 6 ml/hr /Contribution by Ref. 6o/

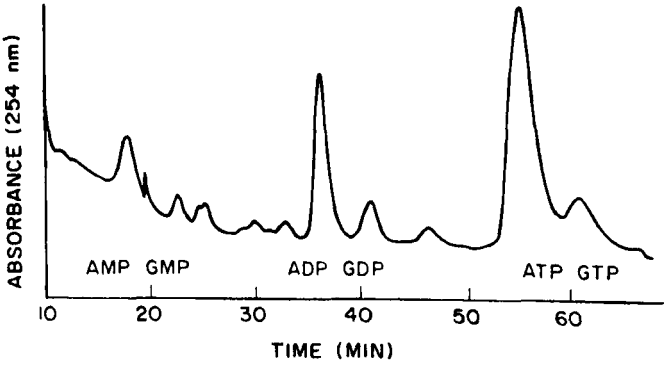

FIGURE 16
Nucleotide exctracts from Sarcoma 180 cells
Same conditions as in Fig. 15, /Contribution by Ref. 60/

by pumping 0.25 M KH_2PO_4 + 2.2 M KCl at a rate of 12 ml/hr into a gradient mixer containing 45 ml of 0.01 M KH_2PO_4 + 0.001 M H_3PO_4. Fourteen nucleotides were eluted within 5o minutes. A number of pairs of

nucleotides were, however, not separable from each other: CDP-UDPG, UDP-TNP, ADP-IDP, ATP-ITP, and GMP-cylic 3':5'AMP

Another linear gradient between 0.01 M KH_2PO_4 /pH 2.6/ to 0.15 M KH_2PO_4 was employed to separate nucleotide monophosphates and Ado-3':5'-P as well as flavin nucleotides and AMP /Figure 17/.[72]

Quantitative determination of beef heart nucleotides were presented by Drobishev et al.[73] They have used Dowex-1X8 column, Cl^- form, a concentration gradient of HCl-NaCl. The procedure was rather pressure-forced than a high-speed one.

Stahl and his coworkers[44] have used a conventional cation exchange resin to separate 5'-AMP and Ado-3': 5'-P by HSIEC. This separation was not full.

Another conventional anion exchanger was employed to separate a standard mixture of nucleotides and estimate flavour nucleotides in beer by a pressure-forced system.[74]

In the monograph written by Burtis and Gere[33], a wide variety of applications of pellicular resins to nucleotide separation is presented.

Oligonucleotides

In the total synthesis of the gene for an alanine transfer RNA from yeast, Khorana and his coworkers have employed Agarose or Sephadex gels to separate the product oligonucleotides of several synthesis steps. Elution was carried out with 0.1 M triethylammonium carbonate at 4°C. In their advanced work on polynucleotide synthesis, they frequently applied a new column adsorbent, trityl cellulose, and DEAE-cellulose for the separation of products.[76]

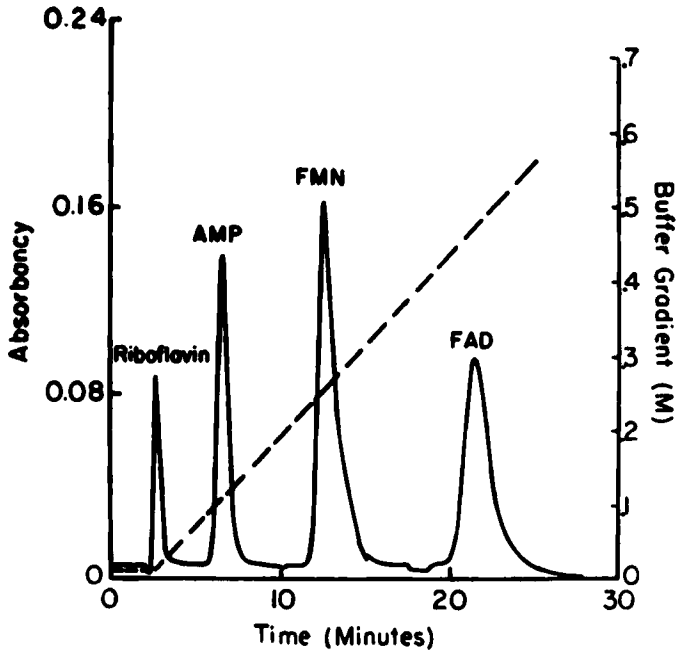

FIGURE 17
Separation of flavin nucleotides and AMP
Column: pellicular anion exchange resin, 2.5 m x 1 mm; Eluent: 0.01 M KH_2PO_4 + 0.01 M H_3PO_4 pH 3.45 to 0.25 M KH_2PO_4 + 2.2 M KCl; Temperature: 70°C; Flow rate: 24 ml/hr, /Contribution by Ref. 72/

DEAE-cellulose column chromatography of a micrococcal nuclease digest of partially labeled Coliphage 186 DNA is shown in Figure 18.[77]
The separation of 2'-5' dinucleoside monophosphates from the corresponding 3'-5' isomers could be carried out on a DEAE-Sephadex A-25 column.[78] The components were eluted with a linear gradient of ammonium bicarbonate from 0.02 M to 0.15 M. The separation of oligonucleotides on such columns depends not only on their net charge but also on secondary binding forces. Because these forces are in accordance with the base composition of oligo-

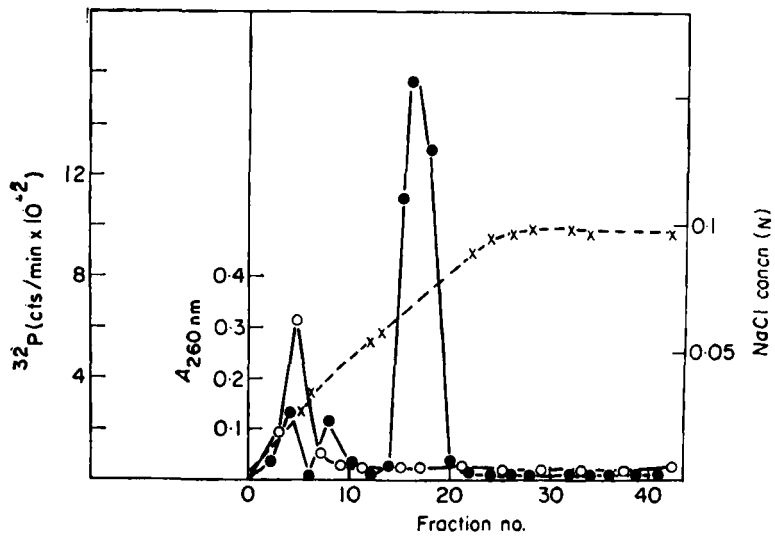

FIGURE 18
DEAE-cellulose chromatography of dephosphorylated oligonucleotides from partially labeled 186 DNA Column: DEAE-cellulose, 22 x 0.3 cm; Eluent: linear gradient formed by 100 ml of 7 M urea - 0.01 M ammonium acetate - formic acid /pH 5.0/ and 100 ml of the same solution containing 0.06 M NaCl /Contribution by Ref. 77/

nucleotides, those containing pyrimidines elute earlier than those containing purines. It is also very likely that the different elution patterns of the 2'-3' and 3'-5' dinucleotides are due to conformational differences.

In the syntheses of deoxyoligoribonucleotides, Heimer and his coworkers[79,80] frequently employed DEAE-cellulose columns to purify the products /Figure 19/.

Properties of a new dihydroboryl-substituted methacryl acid polymer /"borate gel"/ and its application to the column chromatographic separation of oligonucleotides are reported by Schott et al.[81]

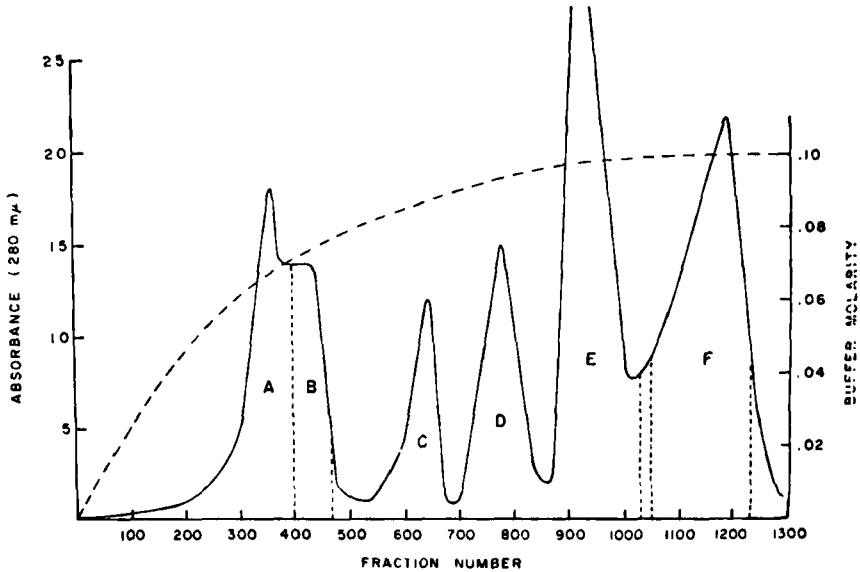

FIGURE 19
Chromatography of d-EtSpA^{Bz}pA^{Bz} oligonucleotide
Column: DEAE-cellulose, 92 x 7 cm; Eluent: convex gradient of 0.1 M triethylammonium bicarbonate and distilled water, /Contribution by Ref. 79/

Treatment of the polymer with acetone is essential in order to optimize the gel. Mixtures of mono- and oligonucleotides were applied on the top of the column pre-equilibrated with 1.0 M $Et_3NH_2CO_3$. Elution with this solvent was carried out until the first nucleotide peak had passed. Subsequently, oligonucleotides were released from the gel by distilled water elution. The capacities of the borate gel seem to depend strongly on the chain length of the material to be chromatographed. Four dinucleotides, ApCp, GpCp, ApUp, and GpUp prepared by digestion of E. coli sRNS with pancreatic RNase were chromatographed on a pellicular

anion exchange resin column.[57] The R values of these compounds were 18.2, 32.3, 33.7, and 51.6. Following the techniques and correlations outlined by Cohn[11] for the separation of dinucleotides, Burtis[33] separated CpC, UpC, ApC, and GpC by HSIEC. Pellicular anion exchanger and gradient elution of KH_2PO_4 from 0.01 to 1.0 M was applied. The method may be extend to tri- and tetranucleotides, too.

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