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Chromatographic Separation of Mononucleotides derived from Transfer Ribonucleic Acids

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1. A method is described for fractionating alkaline and enzymic hydrolysates of ribonucleic acids and oligonucleotides on DEAE-cellulose columns by using a constant-composition eluent. 2. The relative partition coefficients of the four major and several minor nucleotides present in nucleic acid digests are given. 3. The influence of pH and of molarity of the buffer on the separation is described. 4. Relations for the quantitative determination of the major nucleotides from their absorption zone areas on the chromatographic record are derived. 5. Hydrolysis of oligonucleotides with T2 ribonuclease yields solely the nucleoside 3′-monophosphates, thereby simplifying the subsequent chromatographic separation. 6. Examples of the qualitative and quantitative analysis of oligonucleotides from enzymic hydrolysates of RNA are given.

Many procedures for separating the components of nucleotide mixtures obtained by hydrolysis of ribonucleic acids or of derived oligonucleotides make use of electrophoresis or chromatography on paper or on thin layers of cellulose or modified cellulose as supporting media. Under such conditions a precise measurement of the separated nucleotides by any spectrophotometric method will require prior elution of the nucleotides. Parallel treatment of areas of the support free of nucleotide is essential to act as controls. Recoveries may nevertheless not be quantitative.

Column-chromatographic methods yield the nucleotides in solution ready for assay. Thus Cohn & Volkin (1951) by the use of a series of formic acid–ammonium formate buffers were able to separate the 2′-, 3′- and 5′-forms of Ap, Cp, Gp and Up on a column of Dowex 1 (formate form). Katz & Comb (1963) described a rapid method for measuring the four ribonucleotides after some fractionation on a Dowex 50 column.

Automatic recording of the solute concentrations in column effluents, which facilitates these studies, has been applied to the fractionation of complex mixtures of nucleoside phosphates on polyethyleneimine-cellulose (Miller & Kirkpatrick, 1969; Christianson, Paulis & Wall, 1967) and to the separation on Dowex 1 of nucleotide mixtures resulting from hydrolysis of RNA (Lukášová, Boháček & Soška, 1965).

Uziel, Koh & Cohn (1968) described a method for the separation of nucleosides on a Dowex 50 column by using a single eluent and a spectrophotometric recording technique. A similar method has been adopted by Horvath & Lipsky (1969) for the analysis of pmol quantities of the nucleosides of RNA. A narrow-bore column packed with pellicular cation exchanger was operated at high pressure and elevated temperature, a constant-composition phosphate buffer being used as eluent.

The use of a constant-composition eluent for separating nucleotides was suggested almost simultaneously by Blattner & Erickson (1967) and by Thompson (1967). The latter work on the efficiency of DEAE-cellulose chromatography, which was partly carried out in this laboratory, stressed the necessity of using a microgranular cellulose exchanger of uniform particle size from which fines and heavy particles had been removed. Two methods of packing the exchanger into the column were described, the essential requirement being avoidance of size classification during the settling process.

A method has now been developed, using DEAE-cellulose and an eluting buffer of constant composition, which gives complete resolution of the 2′- and 3′-monophosphates of adenosine, cytidine, guanosine and uridine as well as of the monophosphates of a number of nucleosides present as minor constituents in hydrolysates of transfer ribonucleic acids.

The course of the separation was found to be critically dependent on the pH and the ionic strength of the eluent, and the effects of changes in these variables on the relative partition coefficients of the

nucleotides were investigated to arrive at optimum separation conditions. Measurements of the relative zone positions and their areas enabled the compositions of most oligonucleotides obtained during degradation studies of ribonucleic acids to be determined.

Enzymic hydrolysis of oligonucleotides with T2 ribonuclease has now been adopted to replace alkaline hydrolysis. This procedure, which is complete within 2h, yields solely the nucleoside 3′-monophosphates, thereby simplifying the subsequent chromatographic separation.

MATERIALS AND METHODS

Materials. DEAE-cellulose (Whatman DE32) was submitted to acid–alkali cycling and was freed from fines and large particles as described by Thompson (1967). Fines could be removed more efficiently by using a particle-size grading apparatus similar to that devised by Hamilton (1958) and DEAE-cellulose graded in this manner was used for packing the columns used in fractionating T2-ribonuclease digests.

The nucleotides Ap, Cp, Gp and Up were each the mixed 2′- and 3′-isomers as supplied by Calbiochem, Los Angeles, Calif., U.S.A. 5-Methylcytosine and pseudouridine were also obtained from Calbiochem.

Pancreatic ribonuclease (EC 2.7.7.16) (five-times recrystallized) was obtained from British Drug Houses Ltd., Poole, Dorset, U.K., T1 ribonuclease (EC 2.7.7.26) and T2 ribonuclease (EC 2.7.7.17) from Sankyo Co. Ltd., Tokyo, Japan, and bovine spleen phosphodiesterase (EC 3.1.4.1) from Sigma Chemical Co., St Louis, Mo., U.S.A.

Transfer RNA was prepared in this laboratory from baker's yeast by the method of Holley (1967).

Other chemicals were of A.R. grade.

Paper and thin-layer chromatography. Sheets of Whatman no. 1 paper (44cm×22cm) were used for paper chromatography. Glass plates (20cm×10cm) for t.l.c. were coated with cellulose powder (MN300; Macherey Nagel und Co., Düren, Germany) just before use.

The solvent systems used were as follows: for 5-methylcytosine, pseudouridine and ribothymidine, *n*-butanol–water (43:7, v/v), and for the substituted guanines, propan-2-ol–11.6M-HCl–water (660:167:173, by vol.).

Preparation of minor nucleotides from an alkaline hydrolysate of tRNA. RNA (2g) in water (20ml) was hydrolysed to mononucleotides by incubation at 37°C for 18h with 1M-KOH (10ml). The pH was then adjusted to 4.1 by addition of 1M-$HClO_4$ and the precipitated $KClO_4$ removed by centrifugation. The supernatant liquid was applied to a DEAE-cellulose column (70cm× 1.7cm) and the mononucleotides resolved by using the optimum conditions for separation of the components developed below. The principal nucleotides were identified on the basis of their elution positions and absorption spectra. Minor nucleotides were further purified by chromatography on a narrow DEAE-cellulose column (96cm×0.6cm). Tentative identification of these nucleotides was made by comparison of their spectral absorption curves with data in the literature and confirmed by paper chromatography and t.l.c. of the nucleotides and free bases with reference standards.

Preparation of nucleoside 3′-monophosphates from tRNA. A solution of tRNA (0.5mg) in 0.1M-ammonium acetate, pH9.0 (0.2ml), was incubated for 1h at 37°C with pancreatic ribonuclease (0.05mg). The solution was then adjusted to pH5 by the addition of 2M-acetic acid and incubated for a further 1h at 37°C with bovine spleen phosphodiesterase (25μg). The solution was diluted with the standard sodium acetate buffer (250μl) and then applied to the 130cm column of DEAE-cellulose for chromatography under the standard conditions.

Preparation of column. DEAE-cellulose was equilibrated with 0.09M-sodium acetate–acetic acid buffer, pH4.40, and packed into a column (130cm×0.6cm) by addition in small portions of the DEAE-cellulose slurry, allowing free drainage, as described by Thompson (1967). Sufficient slurry was added to form a packed length of about 5cm and this was allowed to settle completely before the next addition of slurry. In those experiments where DEAE-cellulose was prepared by the hydraulic grading process an extension tube was connected to the top of the column previously half filled with buffer. The rest of the column and the extension tube were then filled with sufficient DEAE-cellulose slurry and this was allowed to settle under free-flow conditions. When it was required to change the concentration or pH of the buffer, washings for 24h with the new buffer allowed sufficient time for equilibration of the DEAE-cellulose to the new conditions.

Investigations of conditions for chromatography. A series of experiments were performed with the 130cm column of DEAE-cellulose and buffers within the concentration range 0.05–0.12M-sodium acetate and within the pH range 3.95–4.4.

A portion (0.1ml) of the alkaline hydrolysate of RNA diluted with the eluting buffer to contain about 10mg of nucleotide/ml was applied to the column. Elution was continued with the same buffer pumped at 2.4ml/h. The effluent was monitored at 254nm by means of an LKB Uvicord spectrophotometer with a flow cell with optical path length 5mm, and collected in 1.2ml fractions. The nucleotide content (μmol) of each fraction was determined by measuring the extinction on a Unicam SP.500 series 2 spectrophotometer. Areas on the extinction record were determined by the product of peak height and peak width at half height. Peak height was read directly from the chart, which was calibrated logarithmically, and peak width was measured directly on the chart in mm.

Identification of the eluted nucleotides was carried out by comparison of the absorption curves between 230nm and 300nm with those of standard nucleotides, with a Unicam SP.800A recording spectrophotometer. The positions of zones were identical with those obtained from a mixture containing 250μg of each of the nucleotides Ap, Cp, Gp and Up chromatographed under the same conditions.

Composition of oligonucleotides. Small oligonucleotides used as examples for the hydrolytic and chromatographic procedures were obtained in this laboratory by pancreatic or T1 ribonuclease hydrolysis of a partially purified cysteine tRNA (Holness & Atfield, 1971). The oligonucleotides were hydrolysed by one of the following

methods and then applied to the column as described above.

(*a*) Alkaline hydrolysis. The oligonucleotide (2–10 E_{260} units) in water (200 μl) was incubated for 18h at 37°C with 10M-KOH (20 μl). The pH of the medium was then adjusted to 4.1 by addition of 1M-$HClO_4$. The precipitated $KClO_4$ was removed by centrifugation and the supernatant liquid applied to the column.

(*b*) Hydrolysis with T2 ribonuclease. The oligonucleotide (1–5 E_{260} units) in the form of a dry residue in a small round-bottomed tube (1cm × 5cm) was dissolved in 0.05M-sodium acetate buffer, pH5.0 (20 μl), and after incubation with T2 ribonuclease (20 units) for 2h at 37°C the contents of the tube were diluted with the standard acetate buffer (250 μl) and applied to the column.

In this context an E_{260} unit is defined as the quantity of nucleotide or oligonucleotide which, dissolved in 1ml of water or buffer, has an extinction of 1.0 at 260nm when contained in a cell of 1cm light-path-length.

RESULTS AND DISCUSSION

It is convenient to express the chromatographic mobilities of the individual components relative to that of 3′-Gp, the slowest moving nucleotide. We can write

$$V_n = V_0 + \alpha_n V_s$$

V_n and α_n are respectively the elution volume and partition coefficient for nucleotide n and V_0 and V_s are the void and stationary-phase volumes.

For the reference nucleotide, 3′-Gp, the elution volume and partition coefficient are V_g and α_g respectively,

thus $$V_g - V_0 = \alpha_g V_s$$

whence $$(V_n - V_0)/(V_g - V_0) = \alpha_n/\alpha_g.$$

V_0 was determined by introducing 1ml of water into the column in the place of nucleotide hydrolysate and recording the conductivity of the effluent liquid under otherwise normal conditions. For the 130cm column a significant dip in the conductivity record occurred after the passage of about 30ml of buffer.

Fig. 1 shows the relative partition coefficients of the principal nucleotides and of pseudouridylic acid as a function of the pH of the eluting buffer. The influence of ionic strength at pH4.10 and pH4.40 is shown in Fig. 2. When 0.09M-sodium acetate buffer at pH4.40 was used as the eluent as described by Thompson (1967), 3′-Ap and 2′-Gp could not be resolved. Changes in the ionic strength of the buffer at pH4.40 led to no improvement (Fig. 2). Decreased pH gave better resolution, but below pH4.10 2′-Ap, 2′-Up and 3′-Up had very similar elution volumes. A 0.07M-sodium acetate buffer, adjusted to pH4.10 or 4.15 with acetic acid, was finally adopted as the optimum.

5-Methyl-Cp, ψp and dimethyl-Gp could also be separated from the 2′- and 3′-isomers of Ap, Cp, Gp and Up, as shown in Fig. 3. As the amount of 3′-Up was insufficient in this experiment its presence is indicated solely by an inflexion on the side of the prominent 2′-Up peak. The resolution of 2′- and 3′-Up obtained in another experiment is shown as a broken curve plotted on the same horizontal scale.

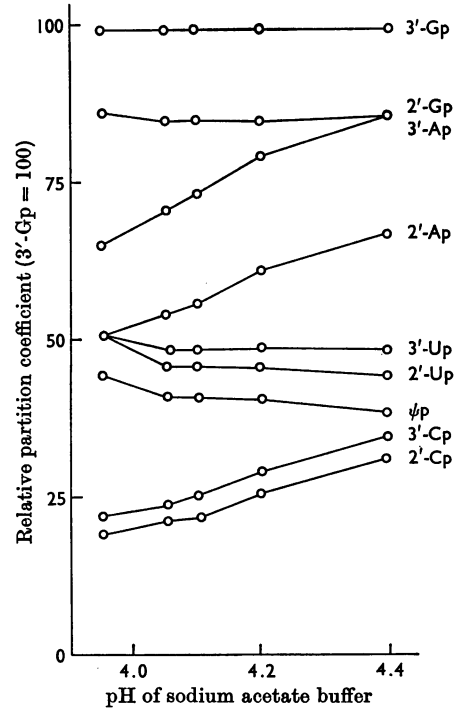

Fig. 1. Dependence of relative partition coefficients of ψp and the 2′- and 3′-isomers of Ap, Cp, Gp and Up on the pH of the eluting buffer. All experiments were performed on a DEAE-cellulose column (130cm × 0.6cm) with 0.07M-sodium acetate buffer adjusted to the required pH with 2M-acetic acid.

Confirmation of the assignments of relative positions of 2′- and 3′-isomers was obtained by chromatography of the nucleotide mixture resulting from hydrolysis of tRNA with pancreatic ribonuclease and spleen phosphodiesterase. The latter enzyme degrades oligonucleotides to give nucleoside 3′-monophosphates only. Four u.v.-absorbing peaks were found. The partition coefficients relative to 3′-Gp were 3′-Cp, 0.244, 3′-Up, 0.480 and 3′-Ap, 0.735, confirming the assignments in Fig. 3.

The nucleosides of the four common bases were eluted near the void volume but were just resolved with relative partition coefficients C, 0.013, U, 0.051, A, 0.080 and G, 0.114. This order is in agreement with that found by Staehelin (1961) for the nucleotides on DEAE-cellulose at pH8.6 and

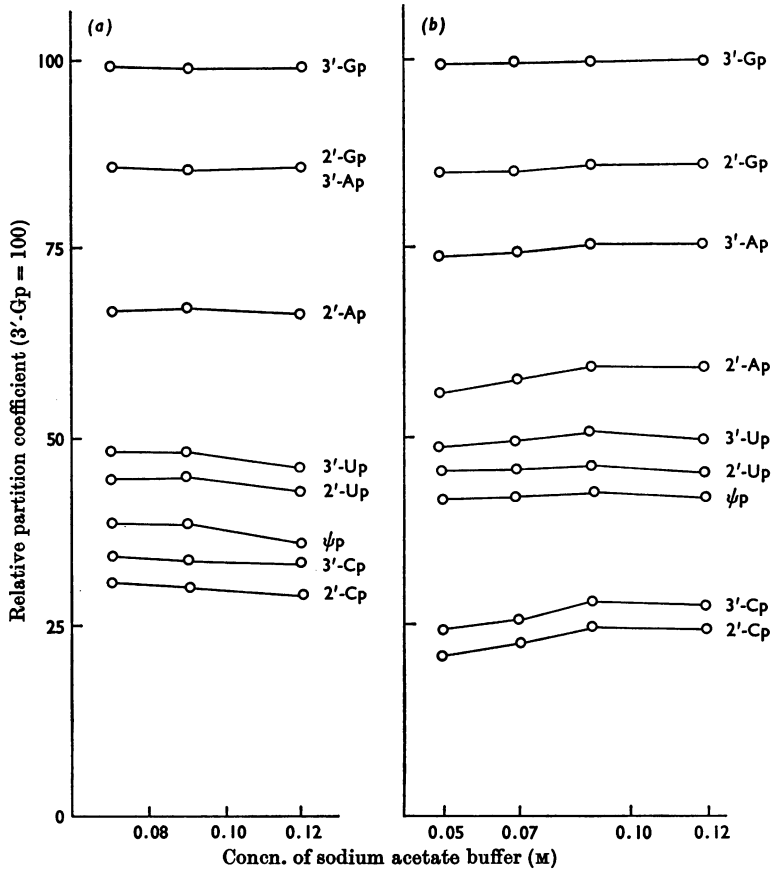

Fig. 2. Dependence of relative partition coefficients of ψp and the 2'- and 3'-isomers of Ap, Cp, Gp and Up on the concentration of the eluting buffer, (*a*) at pH 4.40 and (*b*) at pH 4.10.

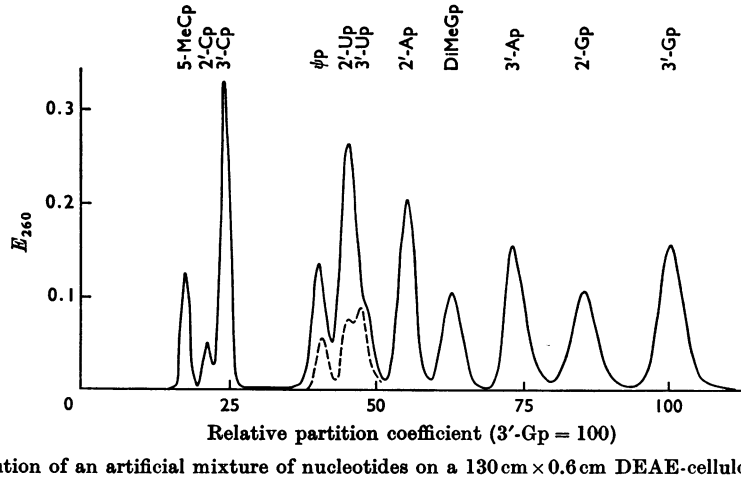

Fig. 3. Resolution of an artificial mixture of nucleotides on a 130 cm × 0.6 cm DEAE-cellulose column with 0.07 M-sodium acetate buffer, pH 4.10, as the eluent. The mixture contained 5-MeCp, 2'-Cp, 3'-Cp, ψp, 2'-Up, 3'-Up, 2'-Ap, diMeGp, 3'-Ap, 2'-Gp and 3'-Gp previously isolated from an alkaline digest of tRNA. The resolution of ψp, 2'-Up and 3'-Up was better shown when equal molar proportions of these nucleotides were chromatographed (----).

Table 1. *Dependence of recorded peak area (y) on the nucleotide content (x): regression coefficients of the equation* $y = a + bx$

Peak area is expressed as the product of recorded extinction at peak maximum and the width of the peak in mm at a point corresponding to half the maximum extinction. The nucleotide content of each zone was derived from the summed extinctions at 260nm of each column fraction collected in that zone by using the following molar extinction coefficients; Ap, 13100; Cp, 6800; Gp, 10300; Up, 9000, at 260nm and pH4.10.

Nucleotide	No. of observations	Intercept (a)	Regression coefficient (b) ± S.D.
Ap	19	0.00	17.35 ± 0.78
Cp	17	0.04	8.07 ± 0.33
Gp	22	−0.07	16.95 ± 1.03
Up	8	−0.13	11.74 ± 0.54

Table 2. *Duplicate determinations of percentage recovery of nucleotides on chromatography under standard conditions with or without prior treatment under conditions used for alkaline hydrolysis of oligonucleotides*

Samples of standard solutions of nucleotides (Ap 3.78, Cp 2.28, Gp 2.61 and Up 3.03 E_{260} units) were chromatographed separately under standard conditions on the 85cm × 0.6cm column of DEAE-cellulose.

Nucleotide	Recovery of standard nucleotide after chromatography: without alkali treatment (%)	with alkali treatment (%)
Ap	97.1, 98.4	84.2, 80.2
Cp	97.5, 106.4	84.9, 76.9
Gp	83.0, 80.8	84.0, 89.2
Up	102.8, 100.8	108.6, 110.0

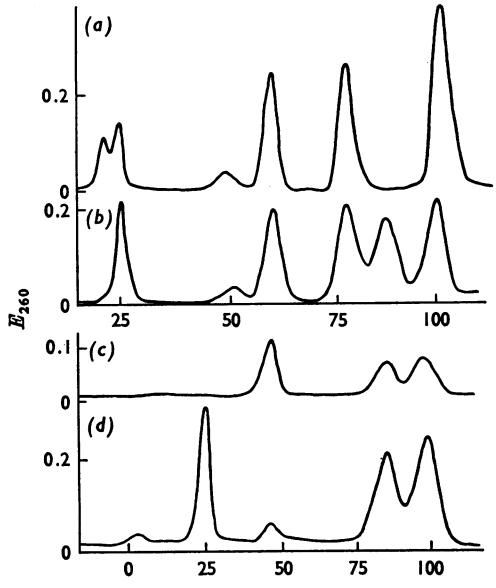

Fig. 4. Uvicord recorder tracings showing the chromatographic resolution of constituent nucleotides liberated by alkaline hydrolysis of di- or tri-nucleotides obtained (*a*) from T1 ribonuclease hydrolysate and (*b*), (*c*), (*d*) from a pancreatic ribonuclease hydrolysate of a partially purified cysteine tRNA. The oligonucleotide (2–10 E_{260} units) was hydrolysed with alkali and chromatographed on DEAE-cellulose columns with 0.07M-sodium acetate buffer, pH4.10; examples (*a*) and (*b*) on an 85cm column, examples (*c*) and (*d*) on a 60cm column. (*a*) The trinucleotide CpApGp gives rise to 2′-Cp, 3′-Cp, 2′-Ap, 3′-Ap and 3′-Gp. (*b*) The trinucleotide ApGpCp gives rise to 3′-Cp, 2′-Ap, 3′-Ap, 2′-Gp and 3′-Gp. (*c*) The dinucleotide GpUp gives rise to 3′-Up, 2′-Gp and 3′-Gp. (*d*) The dinucleotide GpCp gives rise to 3′-Cp, 2′-Gp and 3′-Gp.

with that observed by Cohn (1953, 1950) using Dowex 1 for the separation of the nucleic acid bases at pH10.6 and for the separation of the nucleotides at pH2–3; pyrimidine derivatives are generally eluted before the purine derivatives.

Quantitative determination. Peak areas from the recorder chart were compared with the nucleotide content measured directly at 260nm for a number of peaks of the standard nucleotides. The peak area for each nucleotide was found to be a linear function of the nucleotide content of the form $y = a + bx$. Table 1 shows the values of the constant (a), the regression coefficient (b) and the standard deviation of the regression coefficient for each nucleotide. The values of a in each case are not significantly different from zero.

Recoveries of the standard nucleotides after chromatography under standard conditions are shown in column 2 of Table 2. Column 3 shows the results of a further experiment in which the nucleotides were first heated in potassium hydroxide as described for the hydrolysis of oligonucleotides and then chromatographed. The low recovery of

Gp is due to the presence of G in the standard. This can be measured in the early column eluates and accounts for 13% of the total. The high recovery of Up after alkali treatment may result from deamination of Cp to give Up. A variable loss can occur by co-precipitation of nucleotides with potassium perchlorate.

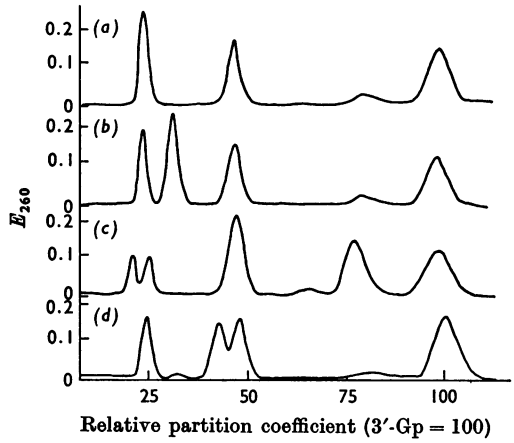

Fig. 5. Uvicord recorder tracings showing the chromatographic resolution of constituent nucleotides released by T2 ribonuclease hydrolysis of oligonucleotides derived from a partially purified cysteine tRNA. The nucleotide composition given does not necessarily indicate the nucleotide order within the oligonucleotide. The oligonucleotide (1–5 E_{260} units) was hydrolysed with T2 ribonuclease and chromatographed on an 85 cm DEAE-cellulose column with 0.07 M-sodium acetate buffer, pH 4.15. Oligonucleotides: (*a*) (C,C,U)G; (*b*) (C,C,?,-U)G; (*c*) (5-MeC,C,U,U,A)G; (*d*) T-ψ-C-G.

Application to the determination of composition of nucleotides. The resolution obtainable with this system depends mainly on the length of the column. For tri- and tetra-nucleotides hydrolysed with alkali an 85 cm column is adequate and for dinucleotides a column 60 cm long is sufficient. Fig. 4 depicts the Uvicord record for the separation of the hydrolysis products of a number of oligonucleotides. Examples (*a*) and (*b*) were obtained by using the 85 cm column and examples (*c*) and (*d*) with a 60 cm column. When a diester linkage is broken by alkaline hydrolysis an equilibrium mixture of 2′- and 3′-isomers results, whereas the terminal nucleotide that is linked to the remainder of the chain only through the 5′-carbon atom is released as the 3′-phosphate only. Thus oligonucleotides (*b*)–(*d*) obtained by enzymic degradation with pancreatic ribonuclease show a single peak due to the terminal pyrimidine residue whereas oligonucleotide (*a*) obtained by T1 ribonuclease hydrolysis shows a single peak due to the terminal Gp residue.

In contrast enzymic hydrolysis with T2 ribonuclease splits almost all internucleotide linkages (Takemura & Miyazaki, 1969), and yields solely the 3′-isomers. This simplification of the hydrolysis products enables adequate resolution to be obtained on an 85 cm column for more complex oligonucleotides. Fig. 5 records the Uvicord traces obtained for

Table 3. *Derivation of the composition of the hydrolysed nucleotides from the Uvicord records shown in Fig.* 5

The relative partition coefficients and zone areas were measured directly on the chart as described in the text. Ribothymidylic acid was distinguished from Up by its spectral properties and by the chromatographic properties of the derived nucleoside. The regression coefficient for Up was used to calculate the relative molar proportions of rTp and ψp.

Oligonucleotide	Relative partition coefficient of component	Identity	Area of zone on chart	Molar ratio (Gp = 1)
(*a*)	0.24	Cp	164	1.9
	0.47	Up	96	1.1
	1.00	Gp	150	1.0
(*b*)	0.24	Cp	105	1.7
	0.31	?	156	?
	0.47	Up	105	1.2
	1.00	Gp	131	1.0
(*c*)	0.20	5-MeCp	50	0.8
	0.25	Cp	43	0.7
	0.47	Up	176	1.9
	0.76	Ap	151	1.1
	1.00	Gp	137	1.0
(*d*)	0.25	Cp	85	0.9
	0.43	ψp	93	0.7
	0.49	rTp	109	0.8
	1.00	Gp	195	1.0

a number of oligonucleotides hydrolysed by this method.

The relative partition coefficients and zone areas measured directly on the Uvicord recorder chart are shown in Table 3. In three cases the composition can be deduced immediately but oligonucleotide (*b*) contains an unidentified component with relative partition coefficient 0.31.

Runs on the 85cm column are complete within 24h and those on the 60cm column, which can be operated at a higher flow rate, are complete within 12h. Samples can be injected without interrupting the buffer flow and provided care is taken to avoid overlapping of zones it is unnecessary to wait until the completion of each run before starting the next.

Columns are usable for many weeks and require repacking then only because compression of the DEAE-cellulose results in a decreased flow rate and particle sedimentation within the column can lead to decreased resolving power. Even under these conditions, however, the values of the relative partition coefficients remain constant and are a reliable guide to identification.

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