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Preparative Isolation of Purine Oligonucleotides from Partial Hydrolyzates of Depyrimidinated DNA

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Abstract

DNA is chemically degraded to a mixture of pure oligonucleotides. From the resulting partial hydrolyzate, defined oligonucleotides with two to six monomer units and defined mixtures of sequence-isomeric purine oligonucleotides are isolated in preparative amounts. The partial hydrolyzate is fractionated by a chromatographic separation route which uses anion-exchange chromatography on DEAE- and QAE-Sephadex at different pH values, paper chromatography, and reversed-phase HPLC. The sequence of the isolated oligonucleotides and the composition of the mixtures of sequence-isomers were determined from chromatographic data, absorption characteristics, enzymatic degradation, and "fingerprints."

INTRODUCTION

DNA fragments can be synthesized up to milligram amounts automatically. The synthesis of larger quantities is wasteful. An alternative to preparative chemical synthesis is to submit DNA to partial hydrolysis and subsequently isolate defined DNA fragments chromatographically from the DNA partial hydrolyzate obtained. The isolation of purine oligonucleotides, described in the following, demonstrates that defined oligonucleotides are obtainable with a minimum of equipment in preparative amounts without chemical synthesis.

EXPERIMENTAL

Reagents

Tris(hydroxymethyl)aminomethane (Tris), sodium carbonate, sodium hydrogen carbonate, ammonium acetate, and concentrated HCl are used at "p.a." grade, and the remaining chemicals at the "chemically pure" grade: Methanol "for chromatographic use" (Merck, Darmstadt, FRG); QAE-Sephadex A-25 and DEAE-cellulose (Pharmacia, Uppsala, Sweden); DNA (PWA Waldhof, Mannheim, FRG); alkaline phosphatase from calf intestine (E.C. 3.1.3.1), phosphodiesterase from snake venom (E.C. 3.1.4.1) (Boehringer, Mannheim, FRG); membranes for ultrafiltration (Amicon, Lexington, Massachusetts); Nucleosil C 18 7.0 μm (Macherey, Nagel & Co., Düren, FRG).

Paper Chromatography

Paper chromatography is performed on Paper MN 260 (Macherey, Nagel & Co., Düren, FRG), sheet dimensions 58 × 60 cm, in System A, ethanol–1 *M* ammonium acetate (pH 7.5) (7:3), or in System B, 1-propanol–concentrated aqueous ammonia solution–water (55:10:35).

Buffer Solutions

1 *M* Tris–HCl (pH 7.5): 121 g (1 mol). Tris(hydroxymethyl)aminomethane (Tris) is dissolved in 900 mL water. This solution is adjusted to pH 7.5 by the addition of about 50 mL concentrated HCl and subsequent dilution to a final total volume of 1 L. The 1 *M* Tris–HCl buffer solutions (pH 9.4 or 8.1) are prepared analogously. About 5 mL concentrated HCl is necessary to obtain a pH value of 9.4.

1 *M* TEAB (triethylammonium hydrogen carbonate buffer) (pH 8.5): 0.14 L triethylamine is mixed with 0.75 L water and CO_2 is bubbled through this solution until pH 8.5 is reached. Subsequently the solution is diluted with water to a final total volume of 1 L.

1 *M* $NaHCO_3$–Na_2CO_3 (pH 9.6): 65.3 g (0.78 mol) $NaHCO_3$ and 23.5 g (0.22 mol) anhydrous Na_2CO_3 are dissolved in 1 L water.

1 *M* ammonium acetate (pH 7.5): 77 g ammonium acetate is dissolved in 1 L water. The pH is adjusted to pH 7.5.

Methods

I. Depyrimidination and Hydrolytic Cleavage of Herring Sperm DNA

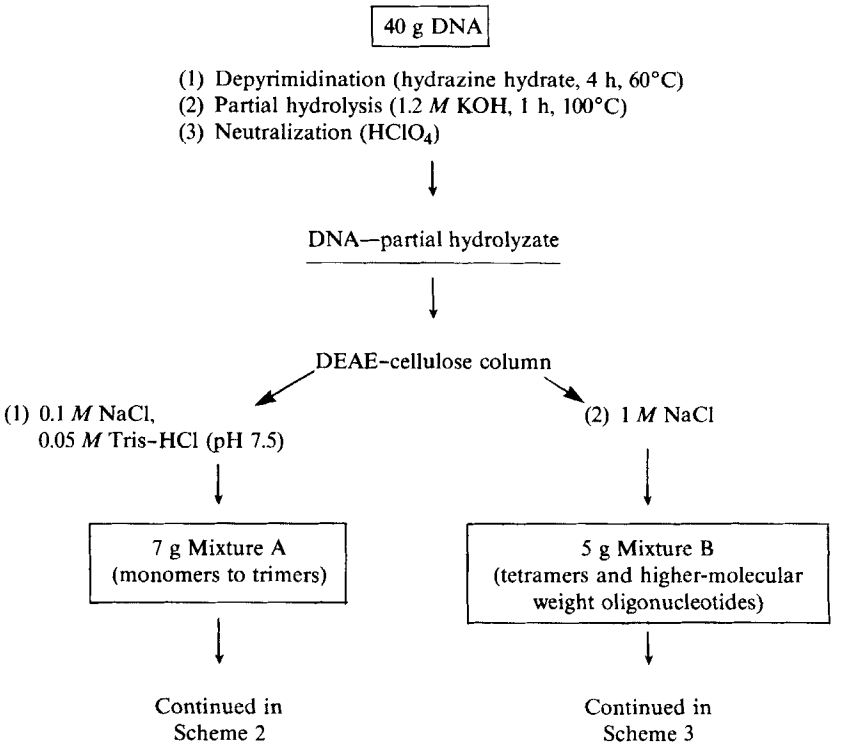
Commercially available herring sperm DNA (40 g) is dissolved in 160 mL hydrazine hydrate and kept at 60°C for 4 h. The resulting brown reaction solution is brought to dryness by means of a rotary evaporator, dissolved in water, and again rotated to dryness. The residue is mixed with 1.5 L of 1.2 *M* KOH and warmed for 1 h on a boiling water bath. The solution is subsequently chilled to about 5°C on an ice bath, neutralized with about 25% perchloric acid, and kept in a refrigerator for 12 h. The precipitate is filtered off on a funnel and repeatedly washed with cold water. The filtrate and washing solutions are combined, raised to 5 L with water, and fractionated by column chromatography.

II. Column Chromatography on Anion Exchangers

The preparative column chromatographic separations are performed at room temperature. The flow rate is achieved by means of a pump. Fractions of about 20 mL are collected. The UV absorbance of every tenth (or, if necessary, of every fifth) fraction is measured at 250, 260, and 280 nm. The values measured at 260 nm were plotted versus the elution volume, resulting in the elution profiles shown. After the gradient the columns are eluted with 1 *M* NaCl. Peak fractions within the vertically dotted lines in the figures are combined and desalted by ultrafiltration.

III. Prechromatography of the Depyrimidinated DNA Hydrolyzate on a DEAE-Cellulose Column at pH 7.5 (see Scheme 1)

The DNA hydrolyzate is dissolved in 5 L water and pumped through a DEAE-cellulose column (50 × 7 cm) with a flow rate of about 1 L/h. After the application, the column is eluted with 5 L water until the absorbance at 260 mm, drops below 1 A unit. The eluate is discarded. In the second step the column is eluted with about 12–15 L of 0.1 *M* NaCl buffered at pH 7.5 with 0.05 *M* Tris-HCl until the absorbance of the eluate decreases to less than 4 A units at 260 nm. (Note: Identified as A_{260} units throughout the remainder of this paper.) In the third step the purine nucleotides that are still adsorbed are eluted with 1 *M* NaCl. The solution of purine nucleotides which have been eluted with 0.1 *M* NaCl and 0.05

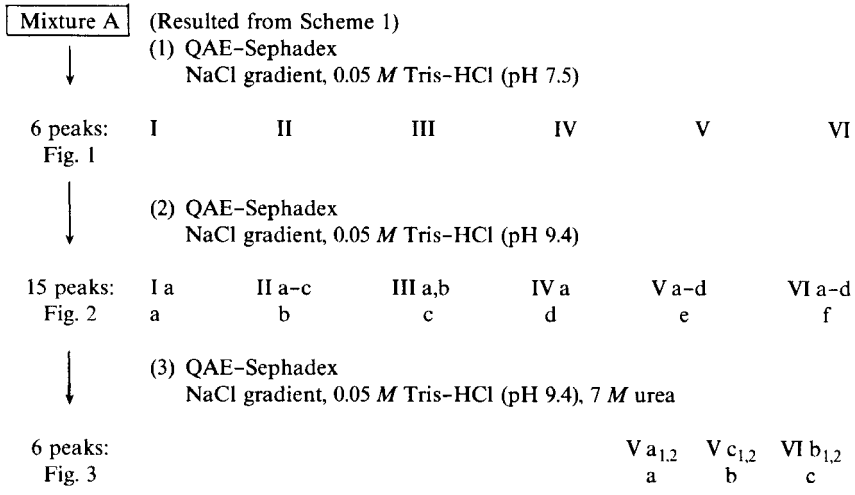


SCHEME 1.

M Tris-HCl is concentrated by rotary evaporator until the salt precipitates. The concentrated solution is freed from the salt and is ultrafiltrated (UM 2 membrane) until the filtrate does not show any reaction with $AgNO_3$. The desalted solution yields about 7 g of low-molecular-weight purine nucleotides as a yellowish powder after lyophilization (Mixture A). The higher-molecular-weight purine nucleotides, which had been eluted with 1 *M* NaCl, are obtained as a light brown powder in about 5 g yield (Mixture B).

IV. Fractionation of Mixture A on a QAE-Sephadex Column at pH 7.5 (1st step, Scheme 2; see Fig. 1)

About 20 g (320,000–330,000 A_{260} units) of Mixture A is dissolved in 200 mL water and subsequently pumped through a QAE-Sephadex A-25



SCHEME 2.

column (50 × 5 cm), previously equilibrated with 0.05 *M* NaCl and 0.05 *M* Tris-HCl (pH 7.5). The flow rate is adjusted to about 800 mL/h. The separation is performed using a four-step NaCl gradient buffered with 0.05 *M* Tris-HCl at pH 7.5 as follows: 1) 8 L of 0.05 *M* NaCl, 2) 17 L of 0.1 *M* NaCl, 3) 10 L of 0.15 *M* NaCl, 4) 1.5 L of 1.00 *M* NaCl. The fractions of Peak III_1 and III_2 (see Fig. 1) are worked up together and are referred to in the following as Peak III. Combined fractions are desalted by ultrafiltration (UM 2 membrane).

1. Rechromatography of the Lyophilizates (Peaks I-VI, Fig. 1) on a QAE-Sephadex Column at pH 9.4 (2nd step, Scheme 2; see Fig. 2). The lyophilizates are dissolved in water and applied to the columns previously equilibrated with the respective starting buffers. Elution is performed according to the conditions listed in Table 1 in an increasing NaCl gradient, buffered at pH 9.4 with 0.05 *M* Tris-HCl. For separating the lyophilizates of Peaks I, II, and IV-VI, columns of 70 × 3 cm are used, which are eluted at a flow rate of about 600 mL/h. The lyophilizate of Peak III is rechromatographed on a column of 50 × 5 cm with a flow rate of about 800 mL/h. The peak fractions are desalted by ultrafiltration (UM 2 membrane).

2. Rechromatography of the Lyophilizates (Peaks Va, Vc, and VIb, Fig. 2) on a QAE-Sephadex Column A-25 at pH 9.4 with Addition of 7

M *Urea (3rd Step, Scheme 2; see Fig. 3).* The lyophilizates are dissolved in 7 *M* urea and pumped on to the columns which have been equilibrated with 0.13 *M* NaCl, 0.05 *M* Tris-HCl (pH 9.4), and 7 *M* urea. The elution is achieved according to the data of Table 2 in an increasing NaCl gradient, which is buffered at pH 9.4 with 0.05 *M* Tris-HCl and to which 7 *M* urea has been added. On separating the lyophilizates of Peaks Va and VIb, columns of 70 × 3 cm are used, the flow rate being about 600 mL/h. The lyophilizate of Peak Vc is chromatographed on a 45 × 2 cm column with a flow rate of 400 mL/h. The peak fractions are desalted by ultrafiltration (UM 2 membrane).

3. Fractionation of the Sequence Isomers d(A, G) of the Lyophilizate (Peak IIIb, Fig. 2c) on a QAE-Sephadex Column A-25 at pH 8.4

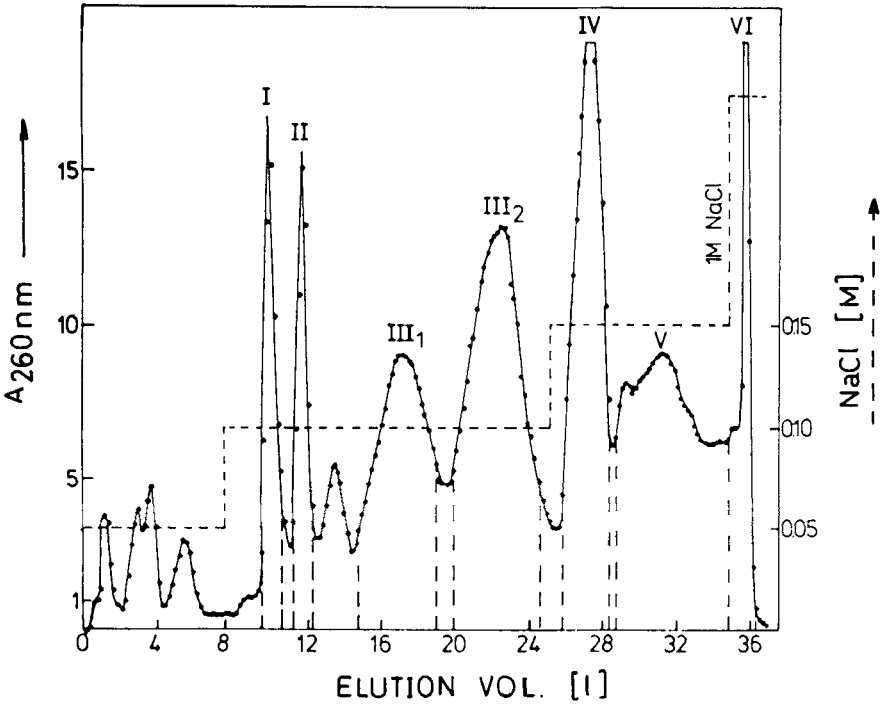


FIG. 1. The separation (1st step, Scheme 2) of 20 g Mixture A from Scheme 1 on QAE-Sephadex A-25 column (50 × 5 cm). The elution is performed using a four-step NaCl gradient, buffered at pH 7.5 by 0.05 *M* Tris-HCl, at a flow rate of 800 mL/h. Peak fractions within the vertically dashed lines are pooled, desalted, lyophilized, and rechromatographed (see Fig. 2).

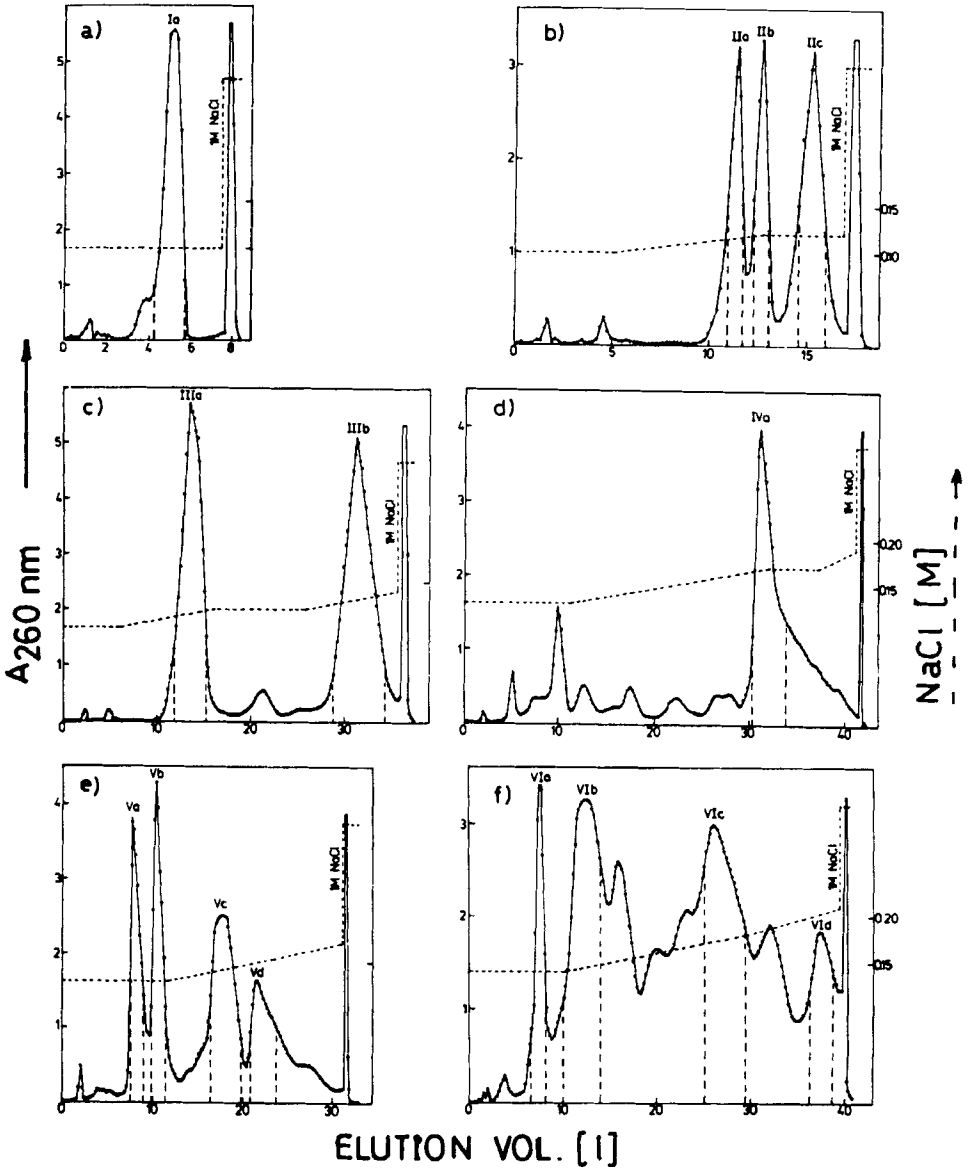


FIG. 2. Elution profiles (a–f) of the rechromatographed lyophilizates (Peaks I–VI, Fig. 1) on QAE-Sephadex A-25. The rechromatography (2nd step, Scheme 2) is performed according to the conditions, mentioned in Table 1, in a NaCl gradient buffered at pH 9.4 by 0.05 *M* Tris-HCl. The fractions of Peaks Va, Vc, VIb are rechromatographed again (see Fig. 3) The fractions of Peak IIIb are dephosphorylated enzymatically and subsequently rechromatographed on QAE-Sephadex (see Fig. 4). Identification and characterization of the peak products are summarized in Table 6.

TABLE 1
Conditions of the Rechromatography of the Lyophilizates (Peaks I-VI, Fig. 1) on QAE-Sephadex Columns Using an Increasing NaCl Gradient Buffered at pH 9.4 by 0.05 *M* Tris-HCl

Applied lyophilizate dissolved in water			Volumes and molar NaCl concentrations of the gradient						
Peak	mg	mL	1st Step	2nd Step. Mixing vessel	Reservoir	3rd Step	4th Step. Mixing vessel	Reservoir	Elution profile, Fig. 2
I	580	10	7.5 L, 0.10	—	—	—	—	—	a
II	720	12	5 L, 0.10	4 L, 0.10	4 L, 0.12	4 L, 0.12	—	—	b
III	3000	50	6 L, 0.10	5 L, 0.10	5 L, 0.12	10 L, 0.12	5 L, 0.12	5 L, 0.14	c
IV	1100	30	11 L, 0.13	10 L, 0.13	10 L, 0.17	5 L, 0.17	2 L, 0.17	2 L, 0.19	d
V	1700	50	11 L, 0.13	10 L, 0.13	10 L, 0.17	—	—	—	e
VI	4530	130	10 L, 0.14	10 L, 0.14	10 L, 0.18	—	5 L, 0.18	5 L, 0.21	f

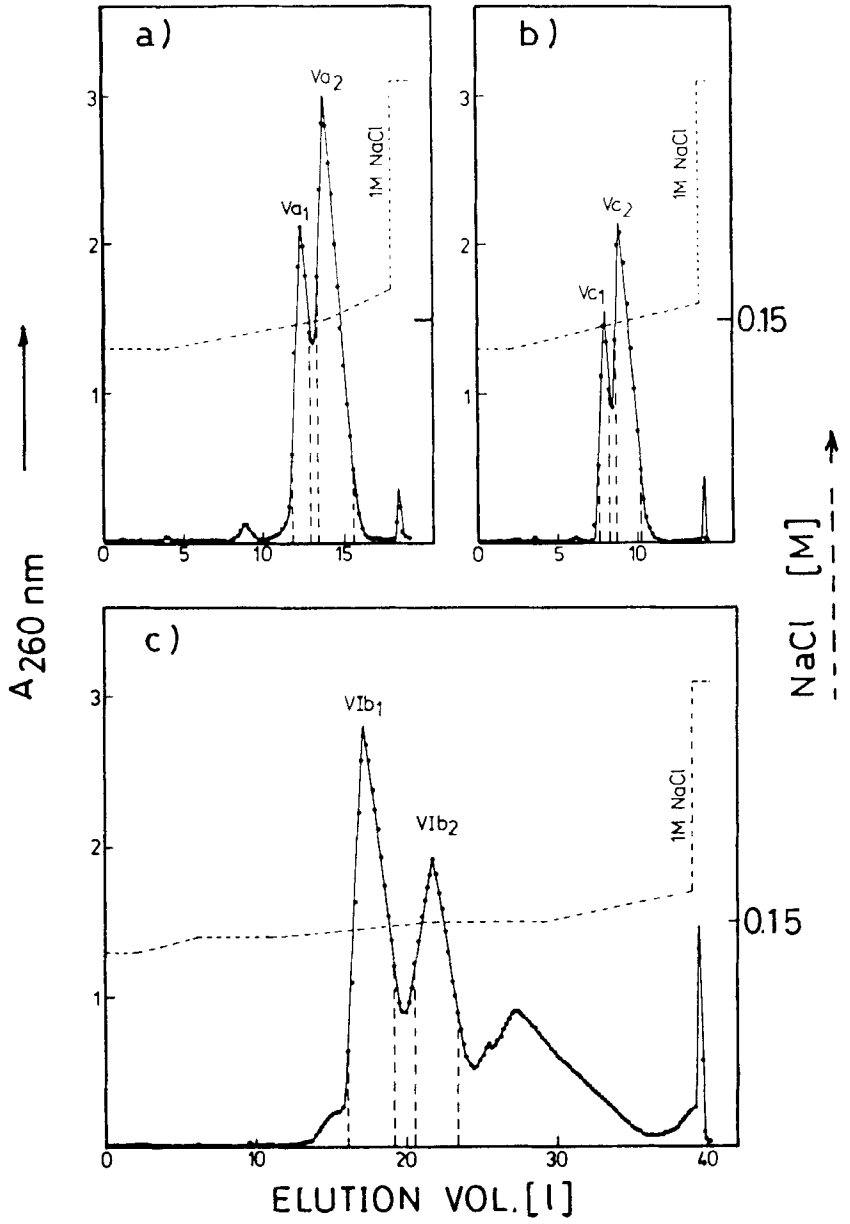


FIG. 3. Elution profiles (a–c) of the rechromatographed lyophilizates (Peaks Va and Vc, Fig. 2e, and Peak VIb, Fig. 2f) on QAE-Sephadex A-25. Rechromatography (3rd step, Scheme 2) is performed using an increasing NaCl gradient in 7 *M* urea, buffered at pH 9.4 by 0.05 *M* Tris-HCl. The peak products are identified in Table 6.

TABLE 2
Conditions of the Column Rechromatography of the Lyophilizates (Peaks Va, Vc, and VIb, Figs. 2e and f) on QAE-Sephadex A-25 Using an Increasing NaCl Gradient in 7 *M* Urea Buffered at pH 9.4 by 0.05 *M* Tris-HCl

Applied lyophilizate dissolved in 7 *M* urea			Volumes and molar NaCl concentrations of the gradient						
Peak	mg	mL	1st Step	2nd Step. Mixing vessel	Reservoir	3rd Step	4th Step. Mixing vessel	Reservoir	Elution profile, Fig. 3
Va	480	15	4 L, 0.13	5 L, 0.13	5 L, 0.15	—	2 L, 0.15	2 L, 0.17	a
Vc	210	6	2 L, 0.13	6 L, 0.13	6 L, 0.16	—	—	—	b
VIb	1370	100	8 L, 0.14	5 L, 0.14	5 L, 0.15	8 L, 0.15	5 L, 0.15	5 L, 0.17	c

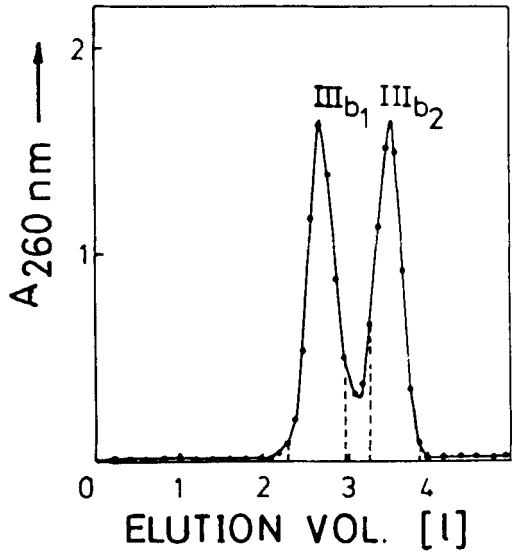
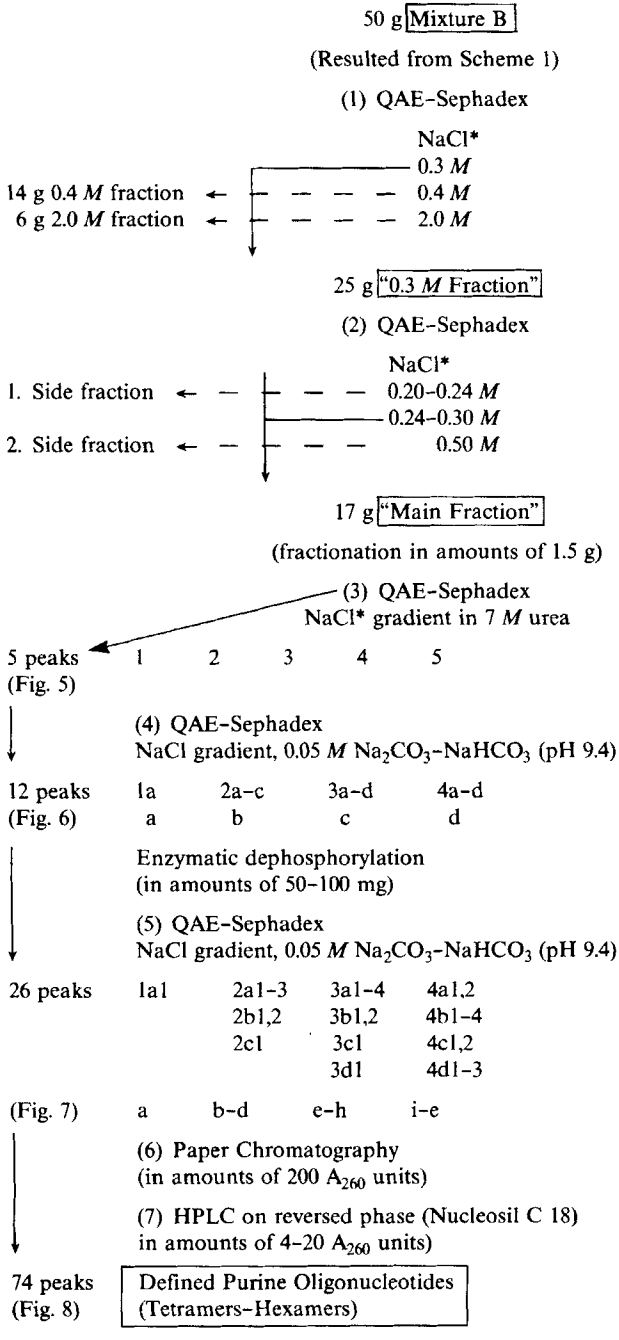


FIG. 4. The separation of a mixture (80 mg) of the two sequence isomers d(A, G) on QAE-Sephadex A-25 (45 × 2 cm). Elution is achieved by 0.05 *M* TEAB (pH 8.5) with a flow rate of about 250 mL/h. Within Peak $IIIb_1$, d(A-G) is leaving the column. Peak $IIIb_2$ contains d(G-A).

see Fig. 4). 80 mg (about 1500 A_{260} units) of the lyophilizate of Peak IIIb of Fig. 2(c) are incubated in 200 mL water, 30 µL 1 *M* Tris-HCl (pH 8.1), together with 100 U alkaline phosphatase for 12 h at 37°C. Afterward the reaction mixture is diluted with water to 10 mL and applied to a QAE-Sephadex A-25 column (45 × 2 cm), previously equilibrated with 0.05 *M* TEAB (pH 8.5). The column is eluted with about 5 L of 0.05 *M* TEAB (pH 8.5) at a flow rate of 250 mL/h. The fractions of Peak $IIIb_1$ and $IIIb_2$ (see Fig. 4) are combined, concentrated by rotary evaporation, and freed from the volatile TEAB by repeated addition and evaporation of small amounts of pyridine. The pyridine is subsequently removed by repeated coevaporation with 2% aqueous ammonia. Then the solutions are lyophilized, yielding 25 mg (about 500 A_{260} units) d(A-G) (31%) from Peak $IIIb_1$ and 30 mg (about 580 A_{260} units) d(G-A) (38%) from Peak $IIIb_2$ in a chromatographically pure form.

V. Fractionation of Mixture B at pH 7.5 (1st step, Scheme 3)

50 g of Mixture B is dissolved in 1.2 L water and pumped on a QAE-Sephadex column (70 × 8 cm), previously equilibrated with 0.2 *M* NaCl



SCHEME 3. * = buffered with 0.05 *M* Tris-HCl to pH 7.5.

and 0.05 *M* Tris-HCl (pH 7.5). Subsequent to the elution of the column with 10 L of the same buffer, a three-step NaCl gradient buffered at pH 7.5 by 0.05 *M* Tris-HCl is applied. After 60 L of 0.3 *M* NaCl and 30 L of 0.4 *M* NaCl are used as eluents, they were followed by 5 L of 2 *M* NaCl. The flow rate amounts to 800 mL/h. The fractions eluted by 0.3, 0.4, and 2 *M* NaCl are concentrated by means of rotary evaporation, desalted by ultrafiltration (UM 2 membrane), and then lyophilized. From the "0.3 *M* fraction" about 25 g (50%), from the "0.4 *M* fraction" about 14 g (28%), and from the "2 *M* fraction" about 6 g (12%) are obtained as a light yellow powder each.

1. Fractionation of the "0.3 M Fraction" at pH 7.5 (2nd step, Scheme 3). 25 g (about 545,000 A_{260} units) of the "0.3 *M* fraction" are dissolved in 500 mL water and pumped on a QAE-Sephadex column (70 × 8 cm), having been equilibrated with 0.2 *M* NaCl and 0.05 *M* Tris-HCl (pH 7.5). The column is eluted with the following five-step NaCl gradient, buffered with 0.05 *M* Tris-HCl at pH 7.5 at a flow rate of 800 mL/h: 1) 10 L of 0.20 *M* NaCl, 2) 50 L of 0.24 *M* NaCl, 3) 20 L of 0.27 *M* NaCl, 4) 10 L of 0.30 *M* NaCl, and 5) 8 L of 0.50 *M* NaCl. The 10 L of the first step together with the first 10 L of the second step (0.24 *M* NaCl) form the "1st side fraction" (30,400 A_{260} units, 5.6%), which has not been examined. The subsequent eluates of the second to the fourth steps, resulting in the "main fraction," are combined, whereas the 8 L eluate of the last step (0.50 *M* NaCl) is abandoned as the "2nd side fraction" (100,500 A_{260} units, 18.4%). The "main fraction" (414,000 A_{260} units, 76%) is concentrated, desalted by ultrafiltration (UM 2 membrane), and lyophilized, yielding about 17 g (68%) of a light yellow powder which is fractionated in the following separations.

2. Fractionation of the "Main Fraction" of V 1 at pH 7.5 with Addition of 7 M Urea (3rd step, Scheme 3; see Fig. 5). 1.5 g (about 36,000 A_{260} units) of the "main fraction" are dissolved in 50 mL of 7 *M* urea and pumped on a QAE-Sephadex column (65 × 5 cm), previously equilibrated with 0.15 *M* NaCl, 0.05 *M* Tris-HCl (pH 7.5), and 7 *M* urea. This column is eluted at a flow rate of 800 mL/h, with a four-step NaCl gradient, buffered at pH 7.5 with 0.05 *M* Tris-HCl, to which 7 *M* urea has been added: 1) 14 L of 0.15 *M* NaCl in the mixing vessel, 14 L of 0.25 *M* NaCl in the reservoir; 2) 3 L of 0.25 *M* NaCl; 3) 3 L of 0.25 *M* NaCl in the mixing vessel, 3 L of 0.32 *M* NaCl in the reservoir; 4) 2 L of 1 *M* NaCl. The fractions of Peaks 1-4 (Fig. 5) are combined and worked up according to the next description, those of Peak 5 being abandoned.

3. Rechromatography of the Fractions of Peaks 1-4 (Fig. 5) on a QAE-Sephadex Column at pH 9.4 (4th step, Scheme 3; see Fig. 6). The combined peak fractions of V 2 are diluted to three times their original volume with water and applied to QAE-Sephadex columns. Thus, urea and part of the NaCl are eluted, the purine oligonucleotides remaining adsorbed. The latter are fractionated according to the conditions listed in Table 3 in an increasing NaCl gradient, buffered with 0.05 *M* Na_2CO_3-$NaHCO_3$ at pH 9.4, with a flow rate of 400 mL/h, respectively. In the end, any material probably adsorbed is eluted with about 200 mL of 1 *M* NaCl. The peak fractions (see Fig. 6) are combined, concentrated by means of rotary evaporation, desalted by ultrafiltration (UM 2 membrane), and lyophilized.

4. Rechromatography of the Enzymatically Dephosphorylated Lyophilizates (Peaks 1a, 2a-c, 3a-d, 4a-d, Fig. 6) on a QAE-Sephadex Column at pH 9.4 (5th step, Scheme 3; see Fig. 7). 50-100 mg of the respective lyophilizates of Peaks 1a, 2a-c, 3a-d, and 4a-d

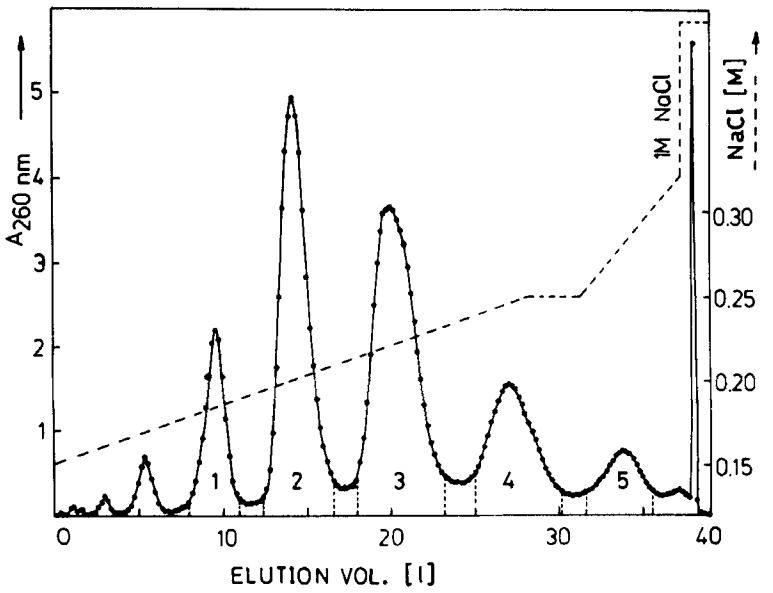


FIG. 5. Elution profile from 1.5 g of the "main fraction" (Scheme 3) on a QAE-Sephadex column (65 × 5 cm). Fractionation (3rd step, Scheme 3) is performed at a flow rate of 800 mL/h in an increasing NaCl gradient, buffered at pH 7.5 with 0.05 *M* Tris-HCl under the addition of 7 *M* urea. The fractions of Peaks 1-4 are combined within the vertical dashed lines and rechromatographed in the 4th step (Scheme 3, see Fig. 6).

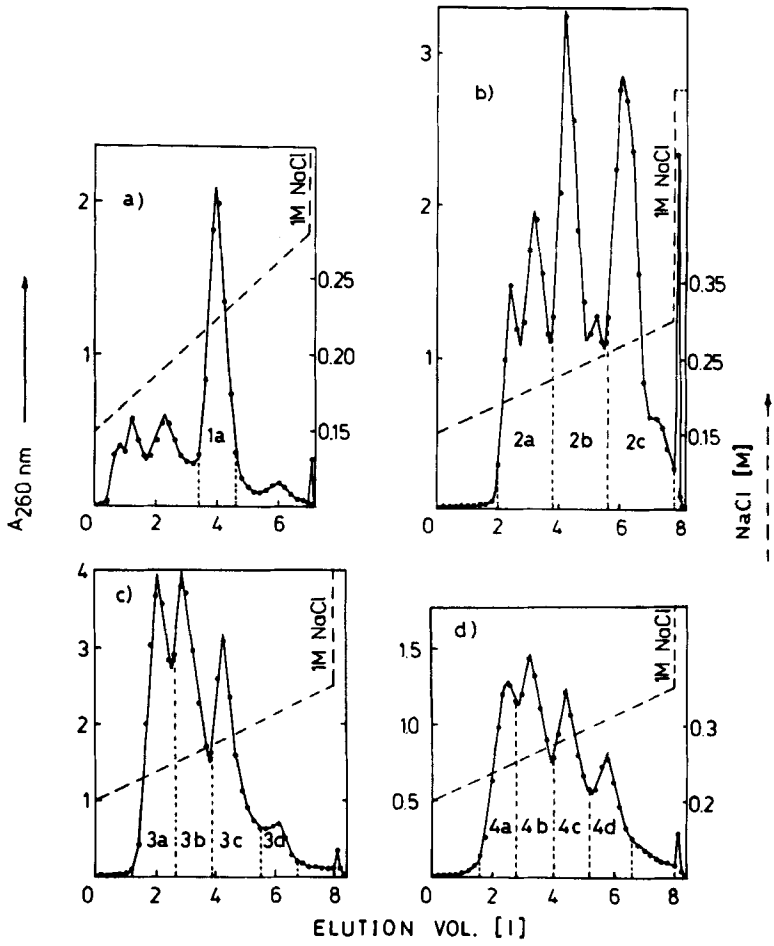


FIG. 6. Elution profiles (a–d) of the rechromatographed lyophilizates (Peaks 1–4, Fig. 5) on QAE-Sephadex A-25. The rechromatography (4th step, Scheme 3) is performed using an increasing NaCl gradient, buffered at pH 9.4 by 0.05 *M* Na_2CO_3–$NaHCO_3$ (see Table 3). The products of Peaks 1a, 2a–c, 3a–d, and 4a–d are isolated. The resulting lyophilizates are dephosphorylated and rechromatographed within the 5th step (Scheme 3, see Fig. 7).

TABLE 3
Conditions of the Rechromatography of Fractions from Peaks 1-4 (Fig. 5) on QAE-Sephadex Columns (i.d. 2 cm) Using an Increasing NaCl Gradient (4th Step, Scheme 3)

		NaCl gradient buffered at pH 9.4 with 0.05 *M* Na_2CO_3-$NaHCO_3$				
		Mixing vessel		Reservoir		
Peak no.	Column length (cm)	Volume (L)	NaCl (*M*)	Volume (L)	NaCl (*M*)	Elution profile, Fig. 6
1	20	3.5	0.15	3.5	0.28	a
2	45	4.0	0.15	4.0	0.30	b
3	50	4.0	0.20	4.0	0.35	c
4	45	4.0	0.20	4.0	0.35	d

obtained in V 3 are dissolved in 0.4 mL water, mixed with 0.05 mL of 1 *M* Tris-HCl (pH 8.1) and 100 U of alkaline phosphatase, and incubated at 37°C for 12 h. Then each reaction mixture is diluted to 10 mL with water and fractionated on a QAE-Sephadex column according to the conditions mentioned in Table 4. Each column has been equilibrated previously with the appropriate elution buffer of the 1st step of the gradient. The flow rate is adjusted at 400 mL/h. The fractions (see Fig. 7) are combined, concentrated in a rotary evaporator, desalted by means of a UM 05 membrane, and lyophilized.

5. Paper Chromatography of the Lyophilizates of V 4 (6th step, Scheme 3). About 200 A_{260} units of the lyophilizates of Peaks 2a1-3; 2b1, 2; 2c1; 3a3, 4; 3b2; 3c1; 3d1; 4a1.2; 4b1-4; 4c1, 2; and 4d1-3 obtained in V 4 are each applied to one sheet of chromatographic paper, and oligonucleotides are afterward eluted from the paper and lyophilized.

6. Reversed-Phase HPLC of the Lyophilizates of V 5 (7th step, Scheme 3; see Fig. 8). About 200 A_{260} units of each of the lyophilizates of V 5 are dissolved in 0.5 mL water. Between 10 and 50 µL of these solutions are fractionated on a Nucleosil C 18 (7.0 µm) column (250 × 4.6 mm) by means of HPLC at room temperature. A mixture of 75% A (A = 0.1 *M* ammonium acetate, pH 7.5) and 25% B (B = methanol-water; 60:40 volume ratio) is used as eluent. The elution is monitored at 254 nm, the flow rate being 1 mL/min and the chart speed 2 mm/min. Fractions are collected 15 cm off the UV detector (outlet: 0.23 mm), brought to

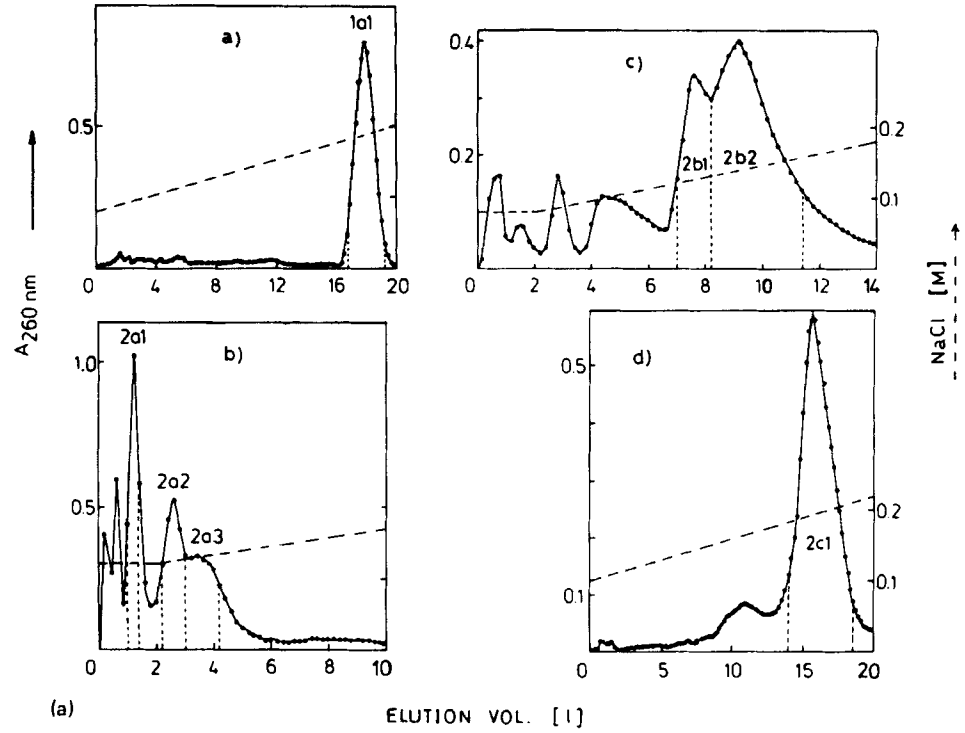
dryness in a speed-vac at 40°C, redissolved in 0.1 mL water, and subsequently lyophilized. Aliquots of the lyophilizates (0.01 A_{260} units) are labeled radioactively and sequenced with the "fingerprint" method (*12*).

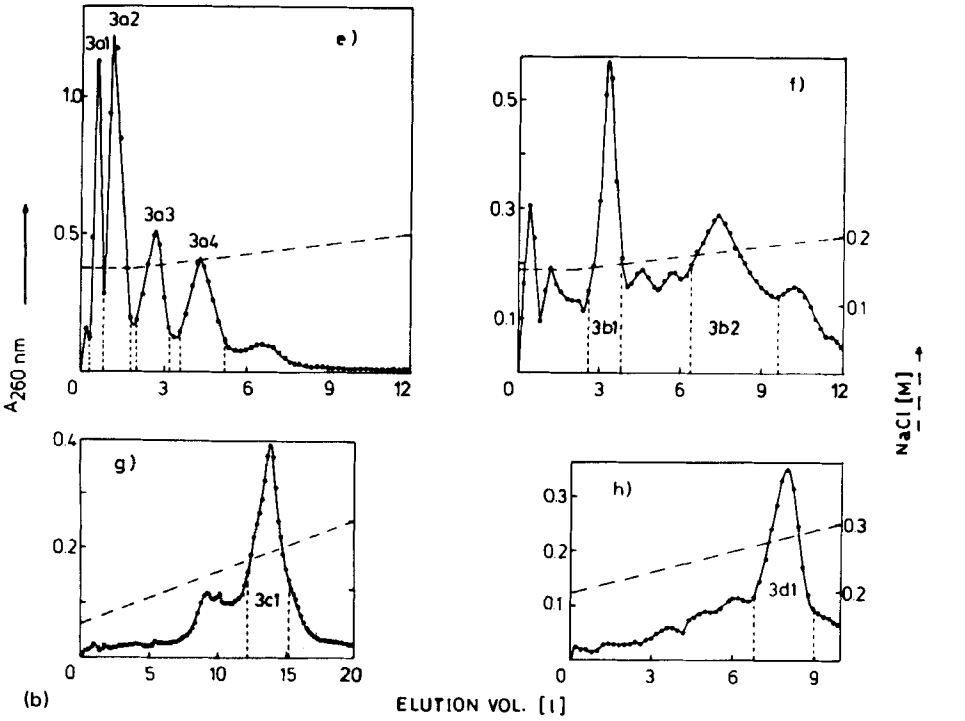
RESULTS

On treating DNA with hydrazine, the pyrimidine bases are destroyed. Under alkaline conditions, intact purine nucleotides are set free from the depyrimidinated DNA. We succeeded in transferring this method of degradation, which has been developed for analytical studies (*1–8*), to a preparative scale (*9*). Working up the hydrolyzate by means of routes of separation stepwise, purine oligonucleotides may be isolated chromatographically in preparative amounts. The routes of separation combine different chromatographic procedures in such a way that the isolation of purine oligonucleotides is not restricted to a special DNA hydrolyzate, but made available for the working up of partial hydrolyzates of depyrimidinated DNA in general.

Partial Hydrolysis of the DNA and Preseparation of the Hydrolyzate (see Scheme 1)

DNA from herring sperm offers the advantage of being available commercially at low cost in sufficient purity. As summarized in Scheme 1, the DNA is depyrimidinated and subsequently hydrolyzated. As a rule, about 40 g of the DNA is degradated at each batch. The mixture of purine nucleotides obtained is dissolved in 5 L water and then crudely fractionated on a weakly basic anion exchanger column (DEAE-cellulose, 50 × 7 cm). At first the column is eluted with water, thus separating the high amount of the uncharged hydrolysis products from the adsorbed purine nucleotides. After this, the column is eluted in a two-step NaCl gradient. Mixture A, which is eluted with 0.1 *M* NaCl, yields about 7 g of mono-, di-, and tripurine nucleotides. In the second step the column is eluted with 1 *M* NaCl. The mixture obtained (B) consisted of about 5 g containing tetra-, penta-, and hexapurine nucleotides as the main products as well as longer chain purine oligonucleotides as side products. Depending on the kind of oligonucleotides to be isolated, the respective mixtures are submitted to further separation. Using the described routes for separating the mixtures, either 20 g of Mixture A (see Scheme 2) or 50 g of Mixture B (see Scheme 3) can be fractionated. The necessary amounts are obtainable through increasing the degradation of DNA or through repetition of the described approach.





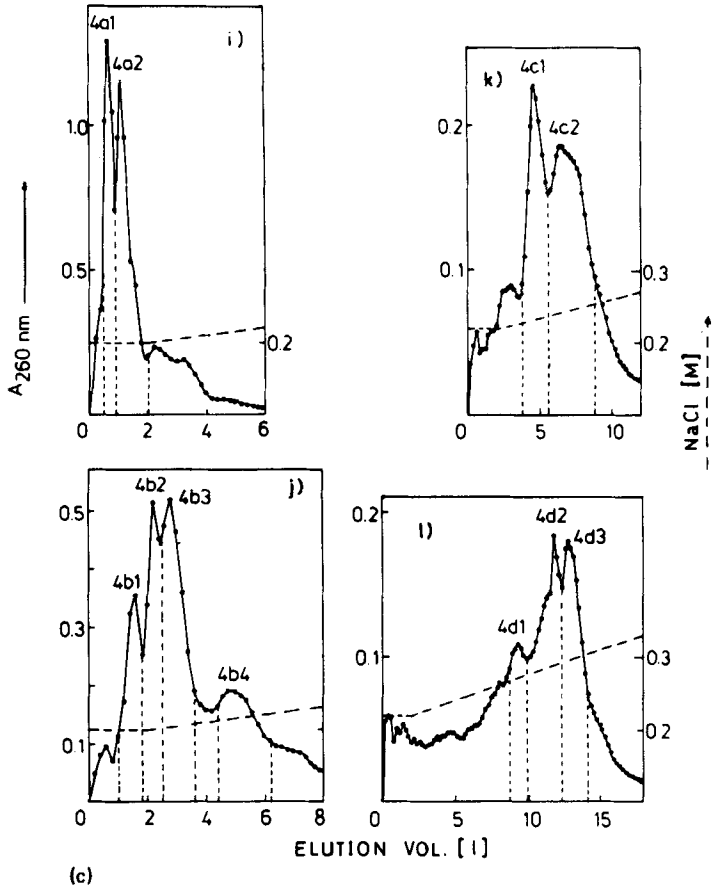


FIG. 7. Elution profiles (a–e) of the rechromatographed lyophilizates (Peaks 1a, 2a–c, 3a–d, and 4a–d, Fig. 6) on QAE-Sephadex A-25. The lyophilizates are rechromatographed in 50–100 mg using an increasing NaCl gradient buffered at pH 9.4 by 0.05 *M* Na_2CO_3–$NaHCO_3$ (see Table 4). The peak fractions are combined within the vertical dashed lines, desalted, and lyophilized. The lyophilizates of Peaks 1a1, 3a1, 3a2, and 3b1 contain pure trimers or defined sequence-isomers (see Tables 7 and 8). The isolated products of the other peaks are purified by paper chromatography and subsequently fractionated by HPLC (7th step, Scheme 3; see Fig. 8).

TABLE 4
Conditions of the Rechromatography (50–100 mg) of the Dephosphorylated Lyophilizate (Peaks 1a, 2a–c, 3a–d, and 4a–d; Fig. 6) on QAE-Sephadex Columns (i.d. 2 cm) Using an Increasing NaCl Gradient Buffered at pH 9.4 by 0.05 *M* Na_2CO_3-$NaHCO_3$

Applied lyophilizate peak	Column length (cm)	Volumes and molar NaCl concentrations of the two-step gradient			Elution profile, Fig. 7
		1st Step	2nd Step. Mixing vessel	Reservoir	
1a	35	—	10 L, 0.08	10 L, 0.20	a
2a	35	2 L, 0.12	4 L, 0.12	4 L, 0.17	b
2b	45	2 L, 0.12	6 L, 0.12	6 L, 0.18	c
2c	45	—	10 L, 0.12	10 L, 0.22	d
3a	45	2 L, 0.15	5 L, 0.15	5 L, 0.20	e
3b	45	2 L, 0.15	5 L, 0.15	5 L, 0.20	f
3c	45	—	10 L, 0.15	10 L, 0.30	g
3d	35	—	5 L, 0.20	5 L, 0.30	h
4a	45	2 L, 0.20	2 L, 0.20	2 L, 0.22	i
4b	45	2 L, 0.20	3 L, 0.20	3 L, 0.23	j
4c	35	2 L, 0.22	5 L, 0.22	5 L, 0.27	k
4d	35	2 L, 0.22	8 L, 0.22	8 L, 0.33	l

Isolation of Mono-, Di-, and Tripurine Nucleotides from Mixture A (see Scheme 2)

At the first step (see Scheme 2), Mixture A is fractionated into components with increasing negative charge on the strongly basic anion exchanger QAE-Sephadex A-25 using a four-step NaCl gradient, buffered at pH 7.5 (see Fig. 1). The products eluted with 0.05 *M* NaCl are rejected. In the 2nd step, Peaks I and II are eluted with 0.1 *M* NaCl, followed by Peaks III_1 and III_2. Peaks IV and V leave the column subsequently, with 0.15 *M* NaCl, in the 3rd step of the separation. In the 4th step, Peak VI is eluted with 1 *M* NaCl. The results of the separation are summarized in Table 5. The route of separation, which was performed five times with 20 g of Mixture A, leads to similar elution profiles. However, Peaks III_1 and III_2, as well as Peaks V and VI, are separated from each other to a different degree. These differences are probably caused by the mixtures resulting from various approaches of partial hydrolysis in which the moieties of certain purine nucleotides may vary significantly. The fractions of Peaks III_1 and III_2 are combined and will be referred to in the following as Peak III. The peak fractions are collected within the vertically dashed lines in the illustrations, desalted

by ultrafiltration, and lyophilized. During the ultrafiltration, losses to different degrees occur. The reasons are differences in the quality of the membranes and the fact that during ultrafiltration, terminal phosphate groups are cleaved. Dephosphorylated oligonucleotides are more likely to pass the membrane than the oligonucleotides phosphorylated at their terminals.

In the 2nd step of separation (see Scheme 2), the lyophilizates of Peaks I–VI are further fractionated at pH 9.4 on QAE-Sephadex A-25 in a NaCl gradient, resulting in the elution profiles shown in Fig. 2. The results of the separation are listed in Table 6. During this rechromatography, differences in the nucleobases of the oligonucleotides are perceivable, resulting in partial separation of adenylate-rich from guanylate-rich oligonucleotides.

The peak fractions are prepared and examined by paper chromatography. If an aliquot of a certain lyophilizate moves uniformly in different chromatographic systems, this lyophilizate is regarded as being "chromatographically pure." In case the lyophilizate shows several spots in the paper chromatogram, all the spots are cut out and eluted. The composition of each lyophilizate is calculated from the measured A_{260} units of each single spot. The paper chromatograms demonstrate that the lyophilizates of Peak Ia–IVa are "chromatographically pure," whereas those of Peaks Va–VId show a different number of components (see Table 7). As evidenced by the R_F values of the nucleotides of Peak VId, the lyophilizate contains tetramers. For this reason the lyophilizate is combined with Mixture B, which is submitted to further separation described in the second part of the present paper.

The lyophilizates of Peaks Va, Vc, and VIb contain such a multitude of components that further column chromatographic separation is necessary (Scheme 2, Step 3). This is performed on QAE-Sephadex A-25 at pH 9.4 with the addition of 7 *M* urea and results in the elution profiles of Fig. 3. The results of the 3rd step of separation are summarized in Table 6. The paper chromatogram demonstrates that the lyophilizates of Peaks Va_1 and Va_2 are 86 and 93% "chromatographically pure." Peaks Vc_1, Vc_2, VIb_1, and VIb_2 contain several components, probably resulting from the main products which have lost their terminal phosphate groups.

The sequence of the isolated purine nucleotides as well as of the main products eluted from the paper chromatograms are determined from the UV absorbance ratios, R_F values, and the results of the enzymatic degradation by alkaline phosphatase and phosphodiesterase from snake venom. This is performed in a similar manner to the examination of the sequence-isomers of pyrimidine nucleotides, which is well described (*10*). The characterization and identification of the isolated purine nucleotides

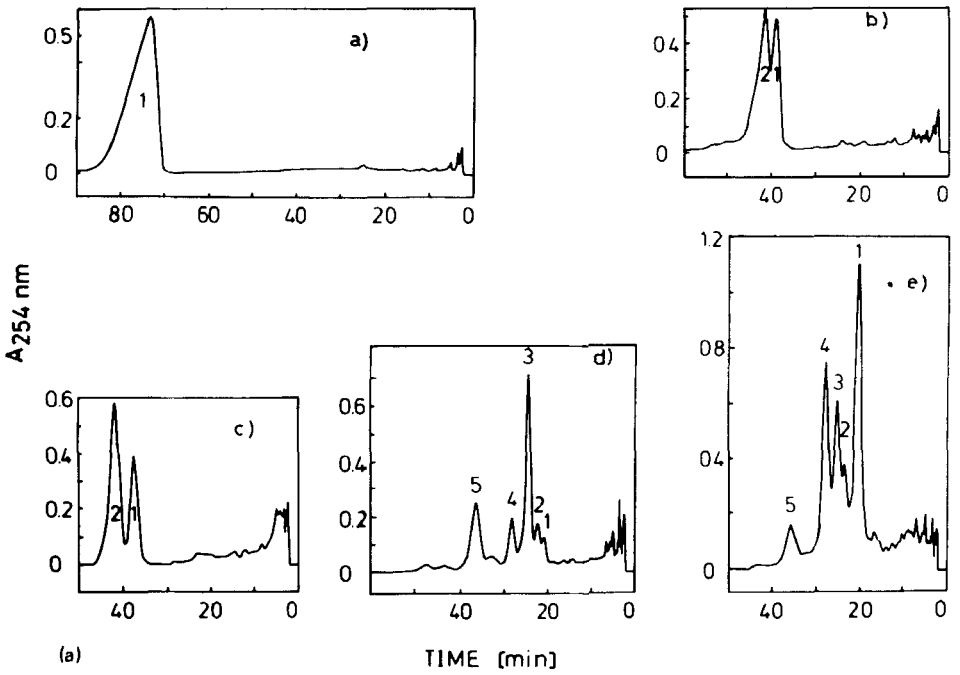


FIG. 8. Elution profiles (a–u) of the rechromatographed lyophilizates (Peaks 2a1–3; 2b1, 2; 3c1; 3a3, 4; 3b2; 3c1; 3d1; 4a1, 2; 4b1–4; 4c1, 2; 4d1–3; Fig. 7) on reversed phase (Nucleosil C 18) column (250 × 4.6 mm) using HPLC. The fractionation is performed using 75% A (A: 0.1 *M* ammonium acetate pH 7.5), 25% B (B: methanol–water, 60:40) at room temperature. Flow rate: 1 mL/min.

are summarized in Tables 7 and 8. Briefly, the tables show the following: Peak Ia (Fig. 2a) contains $d(A)_3$ which is degradated with phosphodiesterase to pdA and dA in a 1.94:1.00 ratio. d(A-G-A) is to be found in the lyophilizate of Peak IIa (Fig. 2b) and degraded with phosphodiesterase to pdA, pdG, and dA in a 1.00:1.09:0.97 ratio. Because dG could not be detected, the sequence d(G-A-A) must be omitted. The distinction between the sequence-isomers d(A-G-A) and d(A-A-G), which in principle results in identical degradation products in the same ratios upon phosphodiesterase treatment, is rendered possible by means of the partial enzymatic degradation, resulting in dimers. The dimer d(A-G) is identified by both its R_F value and absorbance ratio. As is shown by the lack of $d(A)_2$ in the partial hydrolyzate, the sequence d(A-A-G) must be omitted. The lyophilizate of Peak IIb (Fig. 2b) contains d(A-A-G). With phosphodiesterase, pdA, pdG, and dA are obtained in a ratio of

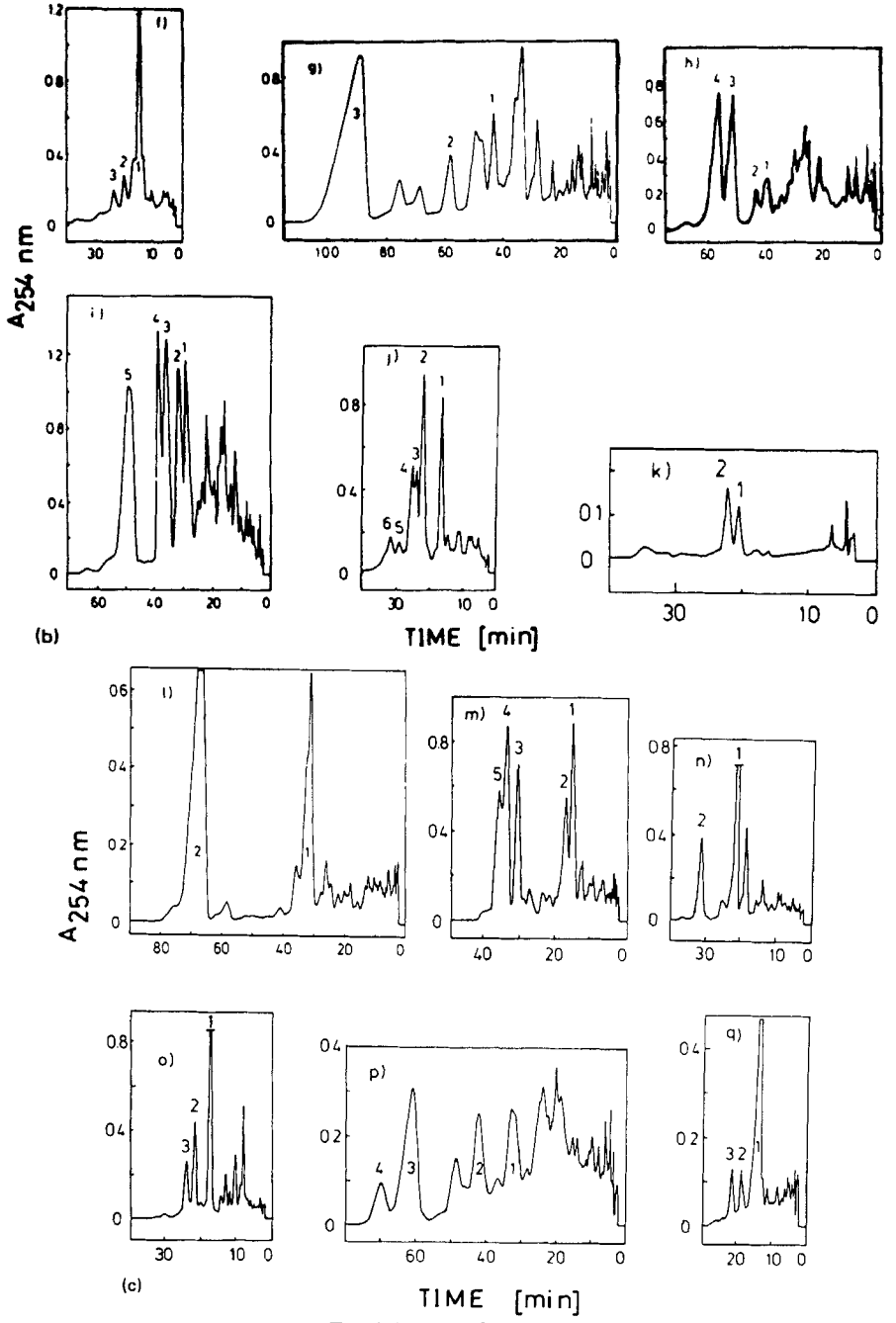


FIG. 8 (*continued*).

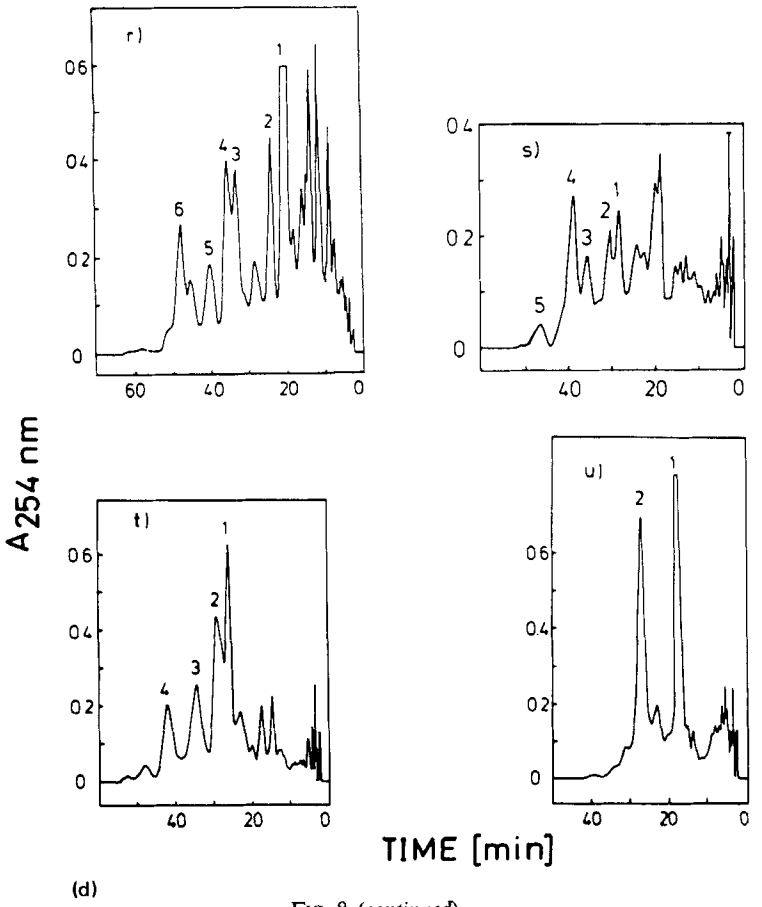


FIG. 8 (*continued*).

1.00:1.05:0.94. By enzymatic partial hydrolyzation, $d(A)_2$ is formed. The lyophilizate of Peak IIc (Fig. 2b) contains d(G-A-A), which is identical in both R_F value and absorbance ratios to the lyophilizate of Peak IIb. Phosphodiesterase degradation results in pdA and dG in a ratio of 1.86:1.00, thus confirming the sequence d(G-A-A).

The lyophilizate of Peak IIIa (Fig. 2c) consists of about 91% $pd(A)_2$ and about 9% $d(A)_2p$, which both move uniformly in the chromatographic systems A and B. After phosphatase treatment, the product again moves as a single spot. The degradation of the dephosphorylated product with phosphodiesterase gives pdA and dA in a ratio of 1.00:1.09. The presence of differently phosphorylated isomers in uniformly moving mixtures is evidenced through direct degradation with phosphodiesterase. Phos-

TABLE 5
Results of the Column Chromatography (1st Step, Scheme 2; see Fig. 1) of Mixture A (20 g) on a QAE-Sephadex A-25 Column Using an Increasing NaCl Gradient Buffered at pH 7.5 by 0.05 *M* Tris-HCl

Peak		Eluted at NaCl (*M*)	Amounts eluted			Amounts isolated		
			A_{260} units	%	%[a]	mg	%	%[a]
I		0.10	9,800	2.8	4.1	100	0.5	0.8
II		0.10	12,200	3.5	3.5	120	0.6	0.9
III_1	III	0.10	31,700	9.2	23.7	1,330	6.7	10.1
III_2		0.10	49,200	14.3		980	4.9	
IV		0.15	40,900	11.9	11.9	1,100	5.6	4.1
V		0.15	49,300	14.3	6.0	1,700	8.6	4.0
VI		1.00	113,900	33.0	36.1	4,600	3.0	26.0
Intermediate fractions		—	38,000	11.0	14.7	—	—	—

[a]Average value of five separations.

phodiesterase from snake venom degradates only 5′-phosphorylated oligonucleotides from the 3′- to the 5′-terminal, whereas 3′-phosphorylated oligonucleotides are not hydrolyzed.

In Peak IIIb (Fig. 2c) the mixture of the sequence isomers pd(A,G) and d(A,G)p is eluted in a pure form. The dephosphorylated mixture is separated on QAE-Sephadex A-25 with 0.05 *M* TEAB (pH 8.5) into d(A-G) and d(G-A) (see Fig. 4). Peak $IIIb_1$, in which about 34% of the mixture applied is eluted, consists of d(A-G), whereas d(G-A) being eluted in Peak $IIIb_2$ represents 39% of the mixture applied. The sequence of the dimers is confirmed by phosphodiesterase degradation. The column chromatographical fractionation of the sequence-isomers may be improved upon eluting the column with 0.03 *M* Tris-HCl (pH 9.0) instead of TEAB. This advantage is diminished considerably in view to the yield of material isolated; large losses occur during the removal of Tris-HCl buffer by ultrafiltration. Applying the elution conditions (DEAE-Sephadex A-25, ammonium formate pH 3.4) reported by other authors (*11*), surprisingly we did not succeed in separating this mixture of sequence-isomers.

In the lyophilizate of Peak IVa (Fig. 2d), 82% $pd(G)_2$ and 18% $d(G)_2p$ are contained. In the chromatographic systems A and B, these dinucleotides move uniformly but remarkably slower than pd(A,G) and d(A,G)p. After phosphatase treatment there is only one spot that moves uniformly in both chromatographic systems. The R_F values are less in reference to

TABLE 6

Results of the Rechromatography of the Lyophilizates (Peaks I–VI, Fig. 1; Peaks Va and c, Fig. 2e; VIb, Fig. 2f) on a QAE-Sephadex A-25 Column (The lyophilizates of peaks I–VI are rechromatographed using an increasing NaCl gradient buffered at pH 9.4. The other lyophilizates are rechromatographed using the same gradient with addition of 7 *M* urea)

Lyophilizate		Amounts applied, A_{260} units	Purine nucleotide eluted					Purine nucleotides isolated			
								Amounts			
Peak	Fig.		NaCl (mM)	Peak	Fig.	Amounts, A_{260} units	%	mg	%	Designation	Purity, %[a]
I	1	11,000	100	Ia	2a	4,690	42.6	20	3.5	$d(A)_3$	100
II	1	14,400	110–120	IIa	2b	1,890	13.1	10	1.4	d(A-G-A)	100
			120	IIb	2b	2,200	15.3	80	11.1	d(A-G-A)	100
			120	IIc	2b	3,000	20.9	70	9.7	d(G-A-A)	100
III	1	47,800	110–120	IIIa	2c	13,300	27.9	130	4.3	$pd(A)_2$[b]	100
			130–140	IIIb	2c	16,800	35.2	230	7.7	pd(A,G)[b]	100
IV	1	24,900	170	IVa	2d	9,470	38.0	160	14.5	pd(G)[b]	100
			130	Va	2e	3,570	42.6	130	7.6	—	—
			130	Vb	2e	4,720	13.7	110	6.5	$pd(A)_3$[b]	92
			140–150	Vc	2e	6,980	20.2	210	12.3	—	—
			150	Vd	2e	3,770	10.9	120	7.1	pd(G)p	76
VI	1	81,700	140	VIa	2f	3,810	4.7	150	3.3	$pd(A)_3$[b]	95
			140–150	VIb	2f	11,140	13.6	530	11.7	—	—
			170–180	VIc	2f	11,240	13.8	520	11.5	pd(G-A-G)[b]	86
			200–210	VId	2f	4,080	5.0	190	4.2	$pd(G_2,A_2)$[b]	53
Va	2e	7,990	140–150	Va_1	3a	2,010	25.2	80	16.7	$pd(A_2)$[b]	86
			150–160	Va_2	3a	4,410	55.2	170	35.4	pd(A)p	93
Vc	2e	3,960	145	Vc_1	3b	860	21.8	40	19.0	pd(A-G-A)[b]	85
			150	Vc_2	3b	2,270	57.4	110	52.4	pd(G-A-A)[b]	46
VIb	2f	21,500	140–150	VIb_1	3c	5,620	26.1	310	22.6	pd(A-G-A)[b]	73
			150	VIb_2	3c	4,280	19.9	230	16.8	pd(G-A-A)[b]	66

[a]Calculated from the A_{260} units which were obtained from the spots eluted from the paper chromatograms.

[b]The 5′-phosphorylated oligonucleotides are contaminated with 3′-phosphorylated oligonucleotides in amounts between 6 and 29%.

TABLE 7
Results of the Paper Chromatography of the Lyophilized Peak Fractions (paper: Macherey, Nagel & Co., MN 260)

Lyophilizate		Spot		R_F values relative to pdA chromatographic systems			
Peak	Fig.	No.	Amount (%)	B	A	A[a]	Purine nucleotide identified
Ia	2a	1	100	1.08	0.60	0.60	$d(A)_3$
IIa	2b	1	100	0.95	0.51	0.51	d(A-G-A)
IIb	2b	1	100	0.94	0.52	0.52	d(A-A-G)
IIc	2b	1	100	0.94	0.52	0.52	d(G-A-A)
IIIa	2c	1	100	0.90	0.36	1.80	$pd(A)_2$[b]
IIIb	2c	1	100	0.70	0.23	1.52	pd(A,G)[b]
IVa	2d	1	100	0.40	0.19	1.11	$pd(G)_2$[b]
Va_1	3a	1	6	1.11			—
		2	86	0.90	0.36	1.80	$pd(A)_2$[b]
		3	8	0.77			—
Va_2	3a	1	5	0.92			—
		2	2	0.76			—
		3	93	0.60	0.14	3.70	pdAp
Vb	2e	1	4	0.99			—
		2	3	0.87			—
		3	92	0.70	0.13	0.60	$pd(A)_3$[b]
Vc_1	3b	1	10	0.95			—
		2	5	0.70			—
		3	85	0.57	0.09	0.51	pd(A-G-A)[b]
Vc_2	3b	1	16	0.93			—
		2	14	0.77			—
		3	23	0.70	0.26	1.54	pd(G-A)[b]
		4	46	0.55	0.08	0.52	pd(G-A-A)[b]
Vd	2e	1	7	0.59			—
		2	16	0.52			—
		3	76	0.44	—	3.51	pdGp
VIa	2f	1	5	0.90			—
		2	95	0.70	0.13	0.60	$pd(A)_3$[b]
VIb_1	3c	1	16	0.95	—	0.51	—
		2	11	0.70	—	1.50	—
		3	73	0.57	0.09	0.51	pd(A-G-A)[b]
VIb_2	3c	1	8	0.93	—	0.53	—
		2	26	0.70	—	1.54	—
		3	66	0.55	0.08	0.52	pd(G-A-A)[b]
VIc	2f	1	8	0.80			—
		2	6	0.71			—
		3	86	0.51	0.07	0.40	pd(G-A-G)[b]
Ia_1	7a	1	100		0.30	0.30	$d(G)_3$
$3a_1$	7e	1	100		0.60	0.60	$d(A)_3$
$3a_2$	7e	1	100		0.51	0.51	$d(A_2,G)$
$3b_1$	7f	1	100		0.40	0.40	$d(A,G_2)$

[a] R_F values of nucleotides treated with alkaline phosphatase.
[b] See Table 6.

TABLE 8
UV Absorbance Ratios and Results of the Enzymatic Degradation of the Dephosphorylated Purine Nucleotides Using Snake Venom Phosphodiesterase

Purine nucleotide obtained			Absorbance ratios (pH 7.0)				Degradation with phosphodiesterase		
			250/260		280/260			Ratios of the degradation products	
	Peak	Fig.	calcd.	obsd.	calcd.	obsd.	Degradation products	Expected	obsd.
$d(A)_3$	Ia	2a	0.79	0.84	0.14	0.24	pdA, dA	2:1	1.94:1.00
d(A-G-A)	IIa	2b	0.88	0.92	0.29	0.33	pdA, pdG, dA	1:1:1	1.00:1.09:0.97
d(A-A-G)	IIb	2b	0.88	0.95	0.29	0.39	pdA, pdG, dA	1:1:1	1.00:1.05:0.94
d(G-A-A)	IIc	2b	0.88	0.95	0.29	0.39	pdA, dG	2:1	1.86:1.00
$d(A)_2$	IIIa	2c	0.79	0.83	0.14	0.23	pdA, dA	1:1	1.00:1.09
d(A-G)	$IIIb_1$	4	0.93	0.97	0.38	0.42	pdG, dA	1:1	1.00:1.05
d(G-A)	$IIIb_2$	4	0.93	0.96	0.38	0.41	pdA, dG	1:1	1.02:1.00
$d(G)_2$	IVa	2d	1.11	1.15	0.68	0.63	pdG, dG	1:1	1.00:0.98
$d(A)_2$	Va_1	3a	0.79	0.81	0.14	0.22	pdA, dA	1:1	1.00:1.03
dA	Va_2	3a	0.79	0.78	0.14	0.16	—	—	—
$d(A)_3$	Vb	2e	0.79	0.84	0.14	0.24	pdA, dA	2:1	1.90:1.00
d(A-G-A)	Vc_1	3b	0.88	0.94	0.29	0.34	pdA, pdG, dA	1:1:1	0.91:1.00:1.05
d(G-A-A)	Vc_2	3b	0.88	0.94	0.29	0.40	pdA, dG	2:1	2.00:1.18
dG	Vd	2e	1.11	1.15	0.68	0.68	—	—	—
$d(A)_3$	VIa	2f	0.79	0.78	0.14	0.23	pdA, dA	2:1	1.90:1.00
d(A-G-A)	VIb_1	3c	0.88	0.92	0.29	0.33	pdA, pdG, dA	1:1:1	0.89:1.01:1.00
d(G-A-A)	VIb_2	3c	0.88	0.95	0.29	0.39	pdA, dG	2:1	2.00:1.14
d(G-A-G)	VIc	2f	0.98	1.07	0.47	0.50	pdA, pdG, dG	1:1:1	0.97:1.00:1.07
$d(G)_3$	$1a_1$	7a	1.11	1.10	0.68	0.63	pdG, dG	2:1	2.05:1.00
$d(A)_3$	$3a_1$	7e	0.79	0.84	0.14	0.23	pdA, dA	2:1	1.94:1.00
$d(A_2,G)$	$3a_2$	7e	0.88	0.92	0.29	0.33	$\frac{pdA + dA}{pdG + dG}$	2:1	1.86:1.00
$d(A,G_2)$	$3b_1$	7f	0.98	1.03	0.47	0.50	$\frac{pdA + dA}{pdG + dG}$	1:2	1.00:1.97

those of d(A,G). The phosphodiesterase degradation of the dephosphorylated dimers yields pdG and dG in a ratio of 1.00:0.98. The presence of both $pd(G)_2$ and $d(G)_2p$ is proved by the direct degradation with phosphodiesterase.

The lyophilizates of Peaks Va_1–VId are each separated into a main spot and one or even more side spots. In the following only the main spots are submitted to further examination. The lyophilizate of Peak Va_1 (Fig. 3a) is a mixture of $pd(A)_2$ and $d(A)_2p$ contained in an 86% amount. This mixture closely resembles the lyophilizate of Peak IIIa in the absorbance ratios as well as in the R_F values and products of enzymatic degradation.

The lyophilizates of peaks Va_2 (Fig. 3a) and Vd (Fig. 2e) are composed of 93% pdAp and 76% pdGp. These substances are degraded by alkaline phosphatase and can be identified as dA and dG. With phosphodiesterase no degradation could be observed. In the lyophilizates of Peaks Vb (Fig. 2e) and VIa (Fig. 2f) a mixture of $pd(A)_3$ and $d(A)_3p$ is present in yields of 92 and 95%, respectively. After phosphatase treatment the product moves as a single spot in System A, showing the same R_F value as $d(A)_3$ isolated from Peak Ia. Degradation by phosphodiesterase yields pdA and dA in a ratio of 1.90:1.00. The amount of different phosphorylated trinucleotides in both peaks is calculated as 91% $pd(A)_3$ and 9% $d(A)_3p$. Examination of the lyophilizates of Peaks Vc_1, Vc_2, VIb_1, and VIb_2 leads to the conclusion that the main product of Peak Vc_1 is the same as that of Peak VIb_1. Peak Vc_2 is identical to Peak VIb_2. For this reason, only Peaks VIb_1 and VIb_2 are characterized. The lyophilizate of Peak VIb_1 (Fig. 3c) contains a mixture of pd(A-G-A) and d(A-G-A)p in 73% yield, as well as Peak Vc_1 (Fig. 3b) in 85% yield. In both the peaks there are in total 94% pd(A-G-A) and 6% d(A-G-A)p. In the chromatographic Systems A and B the main product of Peak VIb_1 moves uniformly. After treatment with phosphatase, the dephosphorylated product is pure, as determined by paper chromatography. The product closely resembles $d(A_2,G)$, eluted in Peak II, in both R_F value and absorbance ratios. By degrading the dephosphorylated product with phosphodiesterase, pdA, pdG, and dA are obtained in a ratio of 0.89:1.01:1.00. The partially degraded product (dimer) from Peak VIb_1 is cleaved by phosphodiesterase to pdG and dA in a ratio of 1.01:1.00. It has been identified as d(A-G). This is further confirmed by R_F value and absorbance ratios. The total degradation with phosphodiesterase eliminates the presence of d(G-A-A), because dG is missing in the total hydrolyzate. Partial hydrolysis, on the other hand, shows evidence of the existence of the structure d(A-G-A) because d(A-A-G) should contain $d(A)_2$.

Peak VIb_2 (Fig. 3c) contains a 66% mixture of pd(G-A-A) and d(G-A-A)p. The mixture is also present in Peak Vc_2 (Fig. 3b) in the amount of 46%. The mixture of the trinucleotides in both peaks is composed of 89% pd(G-A-A) and 11% d(G-A-A)p. The main product of the lyophilizate from Peak VIb_2 resembles in its chromatographic, spectral, and enzymatic behavior pd(A-G-A) eluted in Peak VIb_1. On degrading the dephosphorylated product with phosphodiesterase, pdA and dG occur in a ratio of 2.00:1.14. Only the structure of d(G-A-A) coincides with this result. Additional proof is given by partial degradation. The dimer found in the partial hydrolyzate is identified as d(G-A) by its R_F value, absorbance ratios, and total degradation with phosphodiesterase. Degradation yields pdA and dG in a ratio of 1.00:1.17.

The lyophilizate of Peak VIc (Fig. 2f) consists of an 86% mixture of pd(G-A-G) and d(G-A-G)p, which moves uniformly in both the chromatographic Systems A and B. The R_F values are slightly lower in respect to the previously eluted trinucleotides pd(A_2,G). After phosphatase treatment, the degraded product moves uniformly in System A but again more slowly than the dephosphorylated main products from Peaks VIb_1 and VIb_2. From the dephosphorylated product pdA, pdG and dG are obtained in a ratio of 0.97:1.00:1.07 by phosphodiesterase. Because dA cannot be detected, the presence of d(A-G-G) can be excluded. In the partial degradation, d(G-A) occurs, thus eliminating the presence of the sequence d(G-G-A). The direct degradation with phosphodiesterase results in a mixture having the composition of 71% pd(G-A-G) and 29% d(G-A-G)p.

Isolation of Tetra-, Penta-, and Hexapurine Nucleotides from Mixture B (see Scheme 3)

400 g of partially hydrolyzated DNA is fractionated in portions of 40 g (see Scheme 1). The yield is about 50 g of Mixture B, which is subsequently separated stepwise (see Scheme 3). In the first step these purine nucleotides are fractionated on a strong basic anion exchanger QAE-Sephadex column by using a three-step NaCl gradient, buffered at pH 7.5. By using 0.3 *M* NaCl, about 50% of the mixture applied is eluted in the first elution range. About 28% of the mixture leaves the column with 0.4 *M* NaCl and another 12% is eluted with 2 *M* NaCl. The so-called "0.3 *M* fraction" (about 25 g) of the first elution range is subsequently rechromatographed, whereas the fractions eluted with 0.4 and 2 *M* NaCl are not submitted to further examination.

Rechromatography of the "0.3 *M* fraction" is performed in the second

step by using a five-step NaCl gradient buffered at pH 7.5. About 76% of the purine nucleotides is eluted from the column as a "main fraction" by using 0.24–0.30 *M* NaCl. Two minor fractions are obtained which are not separated. In the first of these fractions, purine nucleotides of up to a chain length of three monomer units are eluted. They are not completely removable from the higher-molecular weight nucleotides during prechromatography (Scheme 1) on DEAE-cellulose. The second minor fraction is combined with the products of the 0.40 *M* fraction of the 1st step. The products of the "main fraction" are desalted by ultrafiltration and lyophilized. During the ultrafiltration about 8% of the oligonucleotides is lost, thus only about 17 g result from the 25 g of the lyophilizate of the "0.3 *M* fraction." In the 3rd step the "main fraction" is rechromatographed in portions of 1.5 g on QAE-Sephadex, adding 7 *M* urea to the NaCl gradient. 7 *M* urea can diminish unspecific interactions, and the negative charge of the phosphate groups mainly rules the nature of the adsorption of the purine nucleotides on the anion exchanger in natural media. The elution profile from this step of separation (see Fig. 5) shows five main peaks which are well separated from each other. They include about 88% of the purine nucleotide in the mixture applied. The fractions that leave the column between the respective peaks and with 1 *M* NaCl are referred to as side fractions and are rejected. The combined fractions of Peaks 1–4 are diluted to three times their volume and subsequently fractionated. Peak 5 fractions are not examined further.

In the 4th step the diluted peak fractions are applied to a QAE-Sephadex column. Urea and most of the NaCl elute early, while the oligonucleotides adsorb to the column. Then the column is eluted in an increasing NaCl gradient at pH 9.4. The adenylate-rich purine oligonucleotides are eluted prior to the guanylate-rich ones. The results are summarized in Table 9 and the elution profiles are shown in Fig. 6(a–d). While the rechromatography of Peak 1 (Fig. 5) results in a main peak (1a, Fig. 6a), Peak 2 (Fig. 5) is separated into three different peaks (2a–c, Fig. 6b). Peaks 3 and 4 (Fig. 5) yield four main peaks each during their rechromatography (3a–d, Fig. 6c; 4a–d, Fig. 6d). The fractions are combined, desalted by ultrafiltration, and then lyophilized. During the ultrafiltration an average 10% share of the purine oligonucleotide is lost.

Terminal phosphate groups are removed enzymatically before the next step of separation by treatment with alkaline phosphatase. In the following separation steps the purine oligonucleotides which are fractionated have free hydroxyl groups at their terminals. 50–100 mg of the 12 lyophilizates of Peaks 1a, 2a–c, 3a–d, and 4a–d are incubated with alkaline phosphatase and then chromatographed in the 5th step on a

TABLE 9
Results of the Rechromatography (50–100 mg) of the Dephosphorylated Lyophilizate (Peaks 1a, 2a–c, 3a–d, and 4a–d; Fig. 6) on QAE-Sephadex Columns

Lyophilizate		Amounts applied		Purine nucleotide eluted					Purine nucleotide isolated	
							Amounts			
Peak	Fig.	A_{260} units	mg	NaCl (*M*)	Peak	Fig.	A_{260} units	%	Amounts (mg)	%
1a	6a	1630	80	0.18–0.19	1a1	7a	1230	75.5	54	68
2a	6b	1750	80	0.12	2a1	7b	310	17.7	11	14
				0.12–0.13	2a2	7b	370	21.1	14	18
				0.13	2a3	7b	370	21.1	14	18
				Intermediate fractions			700	40.0	—	—
2b	6b	2090	90	0.12–0.13	2b1	7c	360	17.2	12	13
				0.13–0.16	2b2	7c	990	47.4	38	42
				Intermediate fractions			740	35.4	—	—
2c	6b	2250	100	0.18–0.20	2c1	7d	1580	70.2	62	62
				Intermediate fractions			670	29.8	—	—
3a	6c	2290	100	0.15	3a1	7e	470	20.5	19	19
				0.15	3a2	7e	650	28.4	23	23
				0.15–0.16	3a3	7e	410	17.9	15	15
				0.16–0.17	3a4	7e	440	19.2	15	15
				Intermediate fractions			320	14.0	—	—
3b	6c	2280	100	0.15–0.16	3b1	7f	420	18.4	16	16
				0.17–0.19	3b2	7f	720	31.6	29	29
				Intermediate fractions			1140	50.0	—	—

3c	6c	1540	70	0.24–0.26	3c1	7g	740	48.1	31	44
				Intermediate fractions			800	51.9	—	—
3d	6c	990	50	0.27–0.29	3d1	7h	470	47.5	20	40
				Intermediate fractions			520	52.5	—	—
4a	6d	1490	70	0.20	4a1	7i	400	26.8	17	24
				0.20	4a2	7i	640	43.0	26	37
				Intermediate fractions			450	30.2	—	—
4b	6d	1540	70	0.20	4b1	7j	210	13.6	8	11
				0.20	4b2	7j	290	18.8	10	14
				0.21	4b3	7j	410	26.6	16	23
				0.21–0.22	4b4	7j	240	15.6	9	13
				Intermediate fractions			390	25.3	—	—
4c	6d	1210	60	0.21–0.22	4c1	7k	300	24.8	12	20
				0.22–0.25	4c2	7k	490	40.5	22	37
				Intermediate fractions			420	34.7	—	—
4d	6d	1260	60	0.27	4d1	7l	120	9.5	5	8
				0.27–0.29	4d2	7l	310	24.6	14	23
				0.29–0.30	4d3	7l	240	19.0	9	15
				Intermediate fractions			590	46.8	—	—

QAE-Sephadex column in an increasing NaCl gradient buffered at pH 9.4 (see Fig. 7) according to the conditions listed in Table 4. Rechromatography of the 12 dephosphorylated lyophilizates results in 26 main peaks, all of which are eluted at lower NaCl concentrations than the respective phosphorylated oligonucleotides. This proves that the original mixtures contained oligonucleotides having terminal phosphate groups. The results of the separation are summarized in Table 9. During the fractionation an average of 15% is lost. In agreement with the resolution of the respective elution profiles, the peak fractions isolated contain from 40 to 70% of the A_{260} units of the lyophilizates applied. Peaks $4a_2$ and $4b_1$ are combined and will be referred to in the following as Peak $4a_2/b_1$.

Characterizing the 25 lyophilizates of the isolated main peaks showed that 4 main peaks contained trimers. The remaining 21 lyophilizates contained mixtures of purine nucleotides with greater chain lengths. According to the UV absorbance ratios, the R_F values as well as the results of the enzymatic degradation (see Table 8), Peak $1a_1$ contains $d(G_3)$, Peak $3a_1$ $d(A_3)$, Peak $3a_2$ $d(A_2,G)$, and Peak $3b_1$ $d(A,G_2)$. Mixtures of the sequence isomeric trimers are not separated further.

The lyophilizates of the remaining 21 peaks are purified by paper chromatography and then fractionated in the 7th and last step of separation, using HPLC. Traces of salt as well as any remaining side products from sample preparation are removed by paper chromatography. The lyophilizates move uniformly, resulting in single but broad spots. From the paper chromatogram it can be concluded that the lyophilizates examined contained a mixture of products with small differences in their R_F values.

The purine oligonucleotides mixtures are eluted from the paper and are fractionated with analytical HPLC using a Nucleosil C 18 (7.0 μm) column (see Fig. 8). On separating the 21 samples under identical conditions (see Table 10) numerous minor peaks occurred which were not isolated except for some special ones. The major peaks (see Fig. 8) which are numbered were isolated and lyophilized in order to remove the elution buffer. These lyophilizates were then characterized by the "fingerprint" method for which, at the time, only 1/100 to 1/1000 of the amounts isolated are consumed (*12*).

The examination of 74 peaks isolated after HPLC proves that, using the route described, 35 defined purine nucleotides with four to six monomer units are obtainable in preparative amounts from the partial hydrolyzate of depyrimidinated herring sperm DNA. Some oligonucleotides of the same sequence appear in different peaks. In addition, 14 mixtures of sequence-isomers of definite composition and two oligonucleotides with destroyed 3'-terminal monomer units are isolated (see Table 11).

TABLE 10
Results of the HPLC of the Lyophilizates from the 7th Step (Scheme 3) on a Nucleosil C_{18} Column (250 × 4.6 mm)

Lyophilizate applied, <20 A_{260} units		Oligonucleotide eluted			
Peak	Fig. 7	Time (min)	Peak no.	Fig. 8	Purine oligonucleotides isolated
2a1	b	70	1	a	d(A-A-A-A)
2a2	b	38	1	b	d(A-A-G-A)
		41	2		d(A-G-A-A)
2a3	b	35	1	c	d(G-A-A-A)
		38	2		d(A-A-A-G)
2b1	c	20	1	d	d(G-A-G-A)
		22	2		$d(A_2,G_2)$
		23	3		d(A-G-A-G)
		27	4		Impurity
		34	5		d(A-G-G-A)
2b2	c	20	1	e	d(G-A-G-A) + d(G-A-A-G)
		23	2		$d(A_2,G_2)$
		24	3		d(G-G-A-A)
		27	4		d(A-A-G-G)
		34	5		d(A-G-G-A)
2c1	d	14	1	f	d(G-G-A-G)
		19	2		Impurity
		23	3		d(A-G-G-G)
3a3	e	43	1	g	Impurity
		58	2		Impurity
		88	3		d(A-A-A-A-A)
3a4	e	39	1	h	d(A-A-G-A)-R^a
		42	2		d(A-G-A-A)-R^a
		50	3		d(A-A-G-A-A)
		55	4		d(A-G-A-A-A)
3b2	f	28	1	i	d(G-A-G-A-A)
		31	2		d(A-A-G-A-G)
		35	3		d(A-G-A-A-G)
		37	4		d(A-G-A-G-A)
		48	5		d(A-G-G-A-A)
3c1	g	17	1	j	d(G-A-G-A-G)
		21	2		d(G-G-A-A-G) + d(G-G-A-G-A)
		23	3		d(G-A-G-G-A)
		25	4		d(A-G-A-G-G)
		30	5		d(A-G-G-A-G)
		39	6		Impurity
3d1	h	17	1	k	d(G-A-G-G-G)
		19	2		d(G-G-G-A-G)

(*continued*)

TABLE 10
(*continued*)

Lyophilizate applied, <20 A_{260} units		Oligonucleotide eluted			
Peak	Fig. 7	Time (min)	Peak no.	Fig. 8	Purine oligonucleotides isolated
4a1	i	31	1	l	Impurity
		70	2		d(A-A-A-A)
4a2/b1	i/j		1	m	Impurity
			2		Impurity
		35	3		d(G-A-A-A)
		38	4		d(A-A-G-A) } d(A-A-A-G) }
		41	5		d(A-G-A-A)
4b2	j	23	1	n	d(A-G-A-G)
		34	2		d(A-G-G-A)
4b3	j	20	1	o	d(G-A-G-A)
		24	2		d(G-G-A-A)
			3		Impurity
4b4	j	32	1	p	Impurity
		42	2		d(A-G-A-G-A-A)
		61	3		d(G-A-A-A-A-A) } d(A-G-A-A-A-A) }
		69	4		d(A-A-A-A-A-G)
4c1	k	14	1	q	d(G-G-A-G)
		19	2		d(G-G-G-A) } d(G-G-A-G) }
		23	3		d(A-G-G-G)
4c2	k	21	1	r	d(G-A-G-A-G-A)
		24	2		$d(G_3,A_3)$
		32	3		$d(G_3,A_3)$
		35	4		d(A-G-A-G-G-A)
		40	5		d(A-G-G-A-G-A)
		47	6		d(A-A-A-A-G-G)
4d1	l	29	1	s	d(G-G-A-A-G-A)
		31	2		$d(G_3,A_3)$
		37	3		Impurity
		40	4		d(A-A-A-G-G-G)
		48	5		$d(G_3,A_3)$
4d2	l	26	1	t	Impurity
		29	2		$d(G_4,A_2)$
		35	3		$d(G_4,A_2)$
		42	4		Impurity
4d3	l	18	1	u	d(G-G-A-G-A-G)
		27	2		$d(G_4,A_2)$

[a]R = group unknown.

TABLE 11
Purine Oligonucleotides Isolated on a Preparative Scale from the Partial Hydrolyzate of Chemically Depyrimidinated Herring Sperm DNA

Oligomeres (number found, expected)	Sequence of the purine oligonucleotides isolated
Dimers (4, 4)	d(A-A), d(G-G), d(A-G), d(G-A)
Trimers (6, 8)	d(G-G-G), d(A-A-A), d(A-G-A), d(A-A-G), d(G-A-A), d(G-A-G)
Tetramers (12, 16)	d(A-A-A-A), d(A-A-G-A), d(A-G-A-A), d(G-A-A-A), d(G-A-G-A), d(A-G-A-G), d(A-G-G-A), d(G-G-A-A), d(A-A-G-G), d(G-G-A-G), d(A-G-G-G), d(A-A-A-G)
Pentamers (14, 32)	d(A-A-A-A-A), d(A-A-G-A-A), d(A-G-A-A-A), d(G-A-G-A-A), d(A-A-G-A-G), d(A-G-A-A-G), d(A-G-A-G-A), d(A-G-G-A-A), d(G-A-G-A-G), d(G-A-G-G-A), d(A-G-A-G-G), d(A-G-G-A-G), d(G-A-G-G-G), d(G-G-G-A-G)
Hexamers (9, 64)	d(A-G-A-G-A-A), d(A-A-A-A-A-G), d(G-A-G-A-G-A), d(A-G-A-G-G-A), d(A-G-G-A-G-A), d(A-A-A-A-G-G), d(G-G-A-A-G-A), d(A-A-A-G-G-G), d(G-G-A-G-A-G)

DISCUSSION

The preparative isolation of 45 defined purine oligonucleotides with two to six monomer units from the hydrolyzate of a depyrimidinated herring sperm DNA is described. The isolation is made possible by performing the fractionation of the whole hydrolyzate, in contrast to the one-step procedure used in analytical separation, using a multistep route of separation. The purine oligonucleotides obtained are identified and characterized plainly. These separation routes are generally applicable and are not restricted to the fractionation of the partial hydrolyzate of a special DNA. It is quite possible to use the DNA that occurs in high amounts as a side product of little or no value in biotechnical processes instead of the herring sperm DNA.

The route of separation makes possible the fractionation of the highly complex partial hydrolyzate in the following steps. At first the hydrolyzate is halved more or less crudely by prechromatography with DEAE-cellulose (see Scheme 1). One-half of the amount (Mixture A) contains the higher share of the monomers to trimers. The second half (Mixture B) is composed of purine nucleotides with four and more monomer units. The fractionation of both halves is achieved according to routes of separation which, though different, follow the same principles.

In the beginning of the separation process, the mixtures are separated into compounds of identical net charge using natural pHs with anion

exchangers. According to the number of phosphate groups, purine nucleotides of different lengths may be in the same fraction. Oligonucleotides of the same length but differing in their terminal phosphate groups may occur in different fractions.

In the partial hydrolyzates of depyrimidinated analytical amount of DNA described previously, mostly oligonucleotides with two terminal phosphate groups were found. In only a few cases could partially dephosphorylated products be detected. Upon separation, on the other hand, oligonucleotides with mainly one terminal group, especially in the 5′ position, are found. Probably the depyrimidination of DNA, with subsequent alkaline hydrolysis, first results in oligonucleotides with two terminal phosphate groups which lose their 3′ terminal phosphate groups during the following chromatographic procedures.

The routes of separation might be simplified significantly by dephosphorylating the partial hydrolyzate prior to the chromatographic steps, thus fractionating only dephosphorylated oligonucleotides in the later parts of the procedure. At the present time this is unrealizable due to the high cost of the large amounts of alkaline phosphatase necessary.

In the second part of the separation procedure, the preseparated fractions are submitted to further rechromatographic analysis under alkaline conditions. Accordingly, purine nucleotide mixtures are separated in such a way that adenylate-rich oligonucleotides are eluted prior to those with mainly guanylate units. By fractionating at different pH values, Mixture A is separable into defined single substances, on a preparative scale, quite easily. This result is remarkable because analytic-scale separations described previously never resulted in single substances.

In the case of Peak 3a-d (see Fig. 6c), fractionating Mixture B under alkaline conditions results in fractions of adenylate-rich and guanylate-rich components only. Peak 3a, containing $pd(A_3)p$, $pd(A_2,G)p$, $pd(A_5)$, and $pd(A_4,G)$, is eluted prior to Peak 3b, which is composed of $pd(A,G_2)p$ and $pd(A_3,G_2)$. Peak 3c is a mixture of sequence isomers $pd(A_2,G_3)$. The mixture $pd(A,G_4)$, eluting in Peak 3d, contains oligonucleotides with four guanylic acid units. A further separation is then achieved by rechromatographing the mixtures, which have previously been dephosphorylated, on anion exchangers. The fractions obtained are finally separated to their respective components by means of reversed-phase HPLC. The amounts, which in the present work were fractionated on an analytical column by HPLC, can be increased considerably if preparative columns are used.

Since the HPLCs were preformed under identical conditions, the results from all the separations can be correlated. By comparing the retention times of oligonucleotides of identical chain length, it is obvious

that with increasing guanylic acid units, the retention is decreased. For instance, $d(A_4)$ has a retention time of about 70 min. Sequence isomers of the composition $d(A_3,G)$ elute between 35 and 41 min. Oligonucleotides of the composition $d(A_2,G_2)$ are eluted between 20 and 34 min. Purine nucleotides of the composition $d(A,G_3)$ have retention times between 14 and 23 min.

In contrast to the effect of the nature of the monomer unit, the chain length of oligonucleotides has only a slight effect on the retention time. The retention time of purine oligonucleotides containing an additional adenylic acid unit increases. If a guanylic acid monomer unit is added, the elution times decrease. For example, the tetranucleotides $d(A_2,G_2)$ elute between 20 and 34 min while pentamers of the composition $d(A_3,G_2)$ leave the column between 28 and 48 min. An additional adenylic acid monomer unit in the 3′ position increases the retention times. An additional guanylic acid unit in the 3′ position, however, results in shorter retention times. For example, d(A-A-G-A) elutes at 38 min while d(A-A-G-A-A) and d(A-A-G-A-G) elute at 50 and 31 min, respectively. According to the present results, the 5′-terminal monomer units have no effect on the retention. A principle allowing the prediction of the elution sequence of different components is not yet apparent.

All the single substances isolated from the partial hydrolyzate of the depyrimidinated herring sperm DNA are summarized in Table 11. The purity of all the purine nucleotides isolated fulfills the criteria for synthetic oligonucleotides, e.g., the observed absorbance ratios correspond to the values calculated. The nucleotides move uniformly on paper chromatograms in different chromatographic systems both before and after treatment with alkaline phosphatase. The oligonucleotides are totally cleaved by snake venom phosphodiesterase. The molar ratios of the cleavage products agree with the values expected. Nearly all the dimeric to tetrameric purine nucleotides theoretically possible are obtained from the partial hydrolyzate. On the contrary, only a few of the penta- and hexamers could be isolated. Presumably some of the missing purine nucleotides were lost during purification as "side products." They can be found, most likely, in those peaks of the HPLC that have not been examined. The missing guanylate-rich hexamers are supposed to be contained in the 0.4 *M* fraction (1st step, Scheme 3), which was not investigated. This assumption is confirmed by the guanylate-rich oligonucleotides; they are strongly retarded and they differ significantly from other oligonucleotides in their chromatographic behavior (*13*).

The assumption that all the possible purine oligonucleotides are contained in the hydrolyzate makes sense according to the following reasoning. All the theoretically possible pyrimidine oligonucleotides with

two to five monomer units have been isolated, with the single exception of the partial hydrolyzate of depurinated herring sperm DNA (*14*). Based on the complementarity of both the single strands of DNA, the partial hydrolyzate of depyrimidinated herring sperm DNA should contain all the corresponding purine oligonucleotides. Of course, it is possible that part of the purine segments is destroyed during the technical isolation or the chemical depyrimidination of herring sperm DNA. Because the undetected purine oligonucleotides occur either in low concentrations (side peak) or as a part of a mixture (main peak with shoulder, double peak), the purification and the examination of these peaks is tedious. It is regarded as unreasonable to follow isolation procedures preparatively. The same reasons are responsible for not rechromatographing any further, by means of HPLC, the isolated oligonucleotide mixtures.

The preparative isolation of oligonucleotides available from the partial hydrolyzates of DNA offers a simple alternative to its chemical synthesis. Especially for the preparation of larger quantities of short-chain oligonucleotides, this method exhibits distinct advantages. Chromatographic purification is necessary at the end of any kind of chemical synthesis. In this respect, steps for the preparative separation of DNA hydrolyzates, described above, may contribute information to the chromatographic purification of synthesized oligonucleotides.

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