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Nucleotide Clusters in Deoxyribonucleic Acids. Separation of Oligonucleotides Released by Deoxyribonuclease I*

Enrique Junowicz† and John H. Spencer

ABSTRACT: Oligonucleotides released from DNA by pancreatic deoxyribonuclease have been separated according to chain length up to the pentanucleotide level, by chromatography on DEAE-Sephadex at 65° using LiCl in 0.005 M Tris-HCl buffer (pH 7.6), 7 M in urea, as the eluting salt. The method is quantitative and good resolution of the longer isostichs has been obtained. All possible nonisomeric dinucleotides and 14 of the 20 possible nonisomeric trinucleotides have been separated quantitatively according to base composition by chromatography on DEAE-cellulose at pH 3.4 and 3.6, respectively, using ammonium formate as the eluting salt.

The 16 possible dinucleotides were resolved into 11 fractions and the proportions of the unresolved pairs of positional isomers determined by degradative analysis. The distribution of oligonucleotides isolated from a manganese-activated pancreatic deoxyribonuclease hydrolysate of *Escherichia coli* DNA was nonrandom. In the total dinucleotide fraction guanine and cytosine predominated at both the 3′ and 5′ ends. In dinucleotides which contained adenine and thymine, adenine and thymine predominated at the 3′ end. These results, compared to similar studies on magnesium-activated hydrolysis, support the possibility of a different specificity of degradation according to the activating ion used.

Studies on oligonucleotides released by enzymatic digestion of DNA have been limited by a lack of separation methods sensitive enough to produce discrete resolution of the complex mixtures of oligonucleotides. Chromatographic resolution is poor unless secondary binding forces, particularly those due to purines, are overcome. Tomlinson and Tener (1963) introduced the use of 7 M urea to reduce secondary binding forces in ion-exchange chromatography of polynucleotides on DEAE-cellulose columns. This modification was extended to DEAE-Sephadex chromatography by Rushizky *et al.* (1964). These methods have been used widely for fractionation of RNase and DNase digests but resolution above the octanucleotide level is poor (Carrara and Bernardi, 1968; Niyogi, 1969; Nestle and Roberts, 1969). Sedat and Sinsheimer (1964) used DEAE-Sephadex and 7 M urea in a heated column in order to fractionate purine oligodeoxynucleotides released by chemical degradation of DNA and achieved a separation according to chain length up to the dodecanucleotide level.

Fractionation of oligonucleotides according to base composition has been attempted by various techniques including ion-exchange (Vanecko and Laskowski, 1961; Becking and Hurst, 1963; Rushizky *et al.*, 1965; Solymosy

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et al., 1965), paper chromatographic (Laskowski, 1967), and electrophoretic techniques (Sanger *et al.*, 1965; Smith, 1967). None of the methods provide satisfactory quantitative separation of all the possible nucleotides in a mixture. Recently Satoh and Inoue (1969) reported the separation of RNA dinucleotides obtained from an alkaline hydrolysate of RNA on a Dowex exchanger.

This report describes a method for the separation according to chain length of oligonucleotides produced from DNase digests with discrete resolution up to the pentadecanucleotide level. Also methods for the separation according to base composition of all the dinucleotides and most of the trinucleotides previously separated according to chain length. The methods have been used to investigate the specificity of DNase I on *Escherichia coli* DNA with manganese as the activating ion.

Experimental Section

Materials

Reagent grade urea was obtained from Baker and Adamson (Allied Chemical Co., Canada Ltd.). To reduce the optical density of concentrated urea solutions, 7 M urea was stirred with 0.25 g of activated charcoal/l. for 30 min. The suspended charcoal was removed by filtration twice through No. 3MM Whatman filter paper then through a 2.5 × 5 cm column of DEAE-cellulose. A 7 M solution of urea in 0.005 M Tris-HCl buffer (pH 7.6) had an A_{260} less than 0.005. DEAE-Sephadex A25, capacity 3.5 mequiv/g, Pharmacia (Canada) Ltd., was prepared according to the instructions of the manufacturer. DEAE-cellulose (Whatman DE11, Reeve Angel, Inc., Clifton, N. J.) was sieved prior to use. The portion passing through a 200 mesh (U. S. standard number) but retained by the 325 mesh size was used in all studies. The DEAE-cellulose was washed according to Peterson and Sober (1956). Charcoal, Norit A (Matheson, Coleman & Bell), was activated by boiling with 2 N HCl followed by washing with ethanol–ammonia–water (50:15:35, v/v) and with water to neutrality.

Enzymes. Pancreatic deoxyribonuclease 1 (DNase I) (EC 3.1.4.5.) once crystallized, code D, *Escherichia coli* alkaline phosphatase (EC 3.1.3.1.) electrophoretically purified, code BAPF, and snake venom phosphodiesterase 1 (EC 3.1.4.1.), code VPH, were obtained from Worthington Biochemical Corp., Freehold, N. J.

Nucleic Acid Preparations. Nucleotides, nucleosides, and bases were obtained from Calbiochem, Los Angeles, Calif. Calf thymus DNA was prepared by the method of Kay *et al.* (1952). The DNA contained 0.4% protein (Lowry *et al.*, 1951), no RNA (Dische, 1955), and the $\epsilon(P)_{260}$ was 6875. *E. coli* K12 DNA was prepared by the method of Saito and Miura (1963) from late-log-phase cells grown on high peptone medium obtained from General Biochemicals, Chagrin Falls, Ohio. Following the 2-propanol precipitation step the DNA was washed with increasing concentrations of ethanol from 70 to 100%, redissolved in 0.1 M EDTA (pH 7.0), and dialyzed against five changes of 0.005 M Tris-HCl buffer (pH 7.6). The DNA contained 0.4% protein (Lowry *et al.*, 1951), 0.7% RNA (Dische, 1955), and the $\epsilon(P)_{260}$ was 6900. The base composition was adenine, 24.5%; thymine, 24.9%; guanine, 25.7%; and cytosine, 24.8%. The T_m of the DNA in standard saline citrate (pH 7.0) (0.15 M NaCl–0.015 M sodium citrate), was 90.0°. The DNA was completely excluded from Sephadex G-100.

DNase I Assay. DNase I was assayed by a modification of the method of Kunitz (1950). The substrate stock solution contained 0.05 mg/ml of DNA dissolved in 0.005 M Tris-HCl buffer (pH 7.6) containing 0.001 M $MnCl_2$ for *E. coli* DNA and 0.005 M $MgCl_2$ for calf thymus DNA. A 3.0-ml sample of substrate solution in a stoppered cuvet was placed in a Gilford 2000 spectrophotometer fitted with thermospacers and a Haacke constant-temperature circulating bath, equilibrated to 25°, and 10–100 μl of DNase I solution containing 0.02 mg/ml in 0.005 M Tris-HCl buffer (pH 7.6) was added. The cuvet contents were mixed by stirring with a plastic rod and the absorbance at 260 nm was recorded. The slope of the linear portion of the curve was proportional to the amount of enzyme over this concentration range. One unit of enzyme is defined as the activity which caused an increase in absorbance of 1.0 optical density unit/min per 3 ml under the conditions described above. Protein concentration was measured by the method of Lowry *et al.* (1951) and specific activities of 76 and 29 units per mg were obtained from the manganese- and magnesium-activated reaction mixtures, respectively.

DNase I Hydrolysis of DNA. Calf thymus DNA, 2 mg/ml in 0.005 M $MgCl_2$–0.005 M Tris-HCl buffer (pH 7.6), was incubated with 0.080 unit/ml of DNase I at 25° for 8 min. *E. coli* DNA, 0.584 mg/ml in 0.005 M $MnCl_2$–0.005 M Tris-HCl buffer (pH 7.6), was incubated with 0.176 unit/ml of DNase I at 37° for 30 hr. The hydrolyses were followed by increase in absorbance at 260 nm and the reaction was stopped completely by addition of EDTA of a concentration 40% higher than that of the activating ion.

Chromatographic Separations. FRACTIONATION OF OLIGONUCLEOTIDES ACCORDING TO CHAIN LENGTH. DNase digests were fractionated according to chain length on DEAE-Sephadex A25 at 65° in 7 M urea. Chromatography columns were jacketed for circulation of water and fitted with internally threaded ends (Ace Glass Inc., Vineland, N. J.), a sintered-glass disk, Teflon flow control stopcock, and Chromatronix Teflon tubing adaptors. DEAE-Sephadex A25 was suspended in 0.005 M Tris-HCl buffer (pH 7.6), 7 M in urea, and degassed at 80° under vacuum. The exchanger was packed into the column, containing 10 ml of starting buffer, by gravity flow at 65°. The DNase digest was diluted 1:4 with 0.005 M Tris-HCl–7 M urea buffer (pH 7.6) degassed at room temperature and applied to the hot column at a flow rate of approximately 100 ml/hr. Approximately 5 ml of buffer was maintained on top of the exchanger to allow time for temperature equilibration of the entering solution to 65° and to avoid bubbles in the column during the chromatographic procedure. The column was eluted with a linear gradient of 0 to 0.4 M LiCl in 0.005 M Tris-HCl–7 M urea buffer (pH 7.6). Column effluents were monitored at 254 nm using a LKB Uvicord or at 260 nm using a Gilford Model 2000 spectrophotometer. Flow rate was controlled with a Büchler polystaltic pump. Column temperature was controlled with a Haacke constant-temperature circulating bath.

FRACTIONATION OF OLIGONUCLEOTIDES ACCORDING TO BASE COMPOSITION. Isostich fractions from DEAE-Sephadex chromatography were pooled and desalted using DEAE-cellulose columns according to the method of Rushizky and Sober (1962). (a) *Mononucleotides* were analyzed

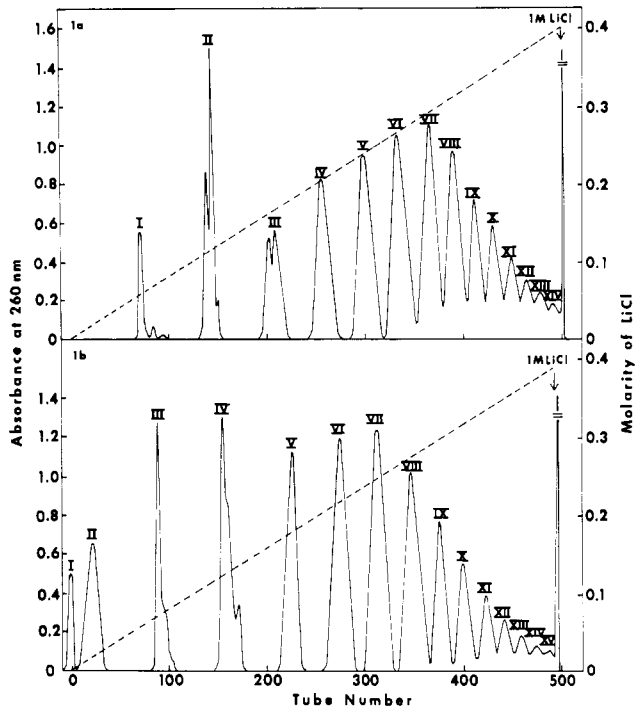

FIGURE 1: Chromatography of a DNase 1 digest of calf thymus DNA. (a) On a column of DEAE-Sephadex (75 × 0.9 cm, chloride form) at 65°. The digest (86.5 mg of calf thymus DNA, 3.46 units of DNase I, 25°, 8 min) was applied to the column and eluted with a linear gradient of 0–0.4 M LiCl in 0.005 M Tris-HCl–7 M urea buffer (pH 7.6) at 65°. Total volume of eluent was 8 l.; 16-ml fractions were collected. After the gradient elution the column was washed with 1.0 M LiCl. (————) Absorbance at 260 nm. (---------) Molarity of LiCl. (b) Dephosphorylated with *E. coli* phosphomonoesterase, on a column of DEAE-Sephadex (75 × 0.9 cm, Cl^- form) at 65°. The digest (see part a) treated with 15 units of phosphomonoesterase, 37°, 6 hr, was applied to the column and eluted as described in part a.

according to the method of Blattner and Erickson (1967). (b) *Dinucleotides.* Dinucleotide fractions were not desalted separately. The dinucleotide isostich fractions from DEAE-Sephadex chromatography were pooled, diluted with five volumes of distilled water, and applied to a DEAE-cellulose column (4.0 g, 325 mesh, 0.9 × 25 cm), formate form. The column was washed with 0.05 M formic acid to remove urea and eluted with a linear gradient of 0–0.1 M ammonium formate (pH 3.4). (c) *Trinucleotides.* A portion of the trinucleotide isostich fraction (see legend, Figure 6) from DEAE-Sephadex chromatography, desalted as described above, was diluted with five volumes of distilled water and applied to a DEAE-cellulose column (20 g, 325 mesh, 0.9 × 130 cm), formate form. The column was washed with 0.05 M formic acid and eluted with a linear gradient of 0–0.3 M ammonium formate (pH 3.6). For the trinucleotide fractionation it is essential to remove fines from the DEAE-cellulose as much as possible. In the present experiments 20 g of DEAE-cellulose was suspended in 2 l. of water, allowed to settle for 15 min, and the solution was decanted. This was repeated six times.

Characterization of Oligonucleotides. Chain-length determination was based on the elution position from DEAE-Sephadex, Dowex 50 chromatography for the separation of mononucleotides, DEAE-cellulose chromatography for the separation of dinucleotides and trinucleotides, and ratios of total and monoesterified phosphorus. Monoesterified phosphorus was released by *E. coli* alkaline phosphatase digestion and the inorganic and total phosphorus assayed by the method of King (1932).

Dinucleotides were characterized by hydrolysis with snake venom phosphodiesterase (Koerner and Sinsheimer, 1957) and analysis of the mononucleotides released (Blattner and Erickson, 1967) and by *E. coli* alkaline phosphatase hydrolysis (Garen and Levinthal, 1960; Khorana and Vizsolyi, 1961) followed by snake venom phosphodiesterase hydrolysis and analysis of the nucleosides and nucleotides (Junowicz and Spencer, 1969). The various conditions for enzymatic hydrolysis have been described previously (Černý *et al.*, 1968).

Trinucleotides were characterized by base ratio analysis according to the method of Wyatt (1955) using the 2-propanol–HCl solvent system.

Spectral Analysis of Dinucleotides. Dinucleotides separated according to base composition were desalted by sublimation of the ammonium formate *in vacuo* at 65° and dissolved in 1.0 ml of 0.001 M sodium phosphate buffer (pH 7.0)–0.005 M in $MgCl_2$. For measurement of hyperchromicity, a 0.4-ml sample of this solution was adjusted to pH 9.2 with 1 μl of 0.28 N NaOH, 1 μl of snake venom phosphodiesterase (3.8 μg of enzyme, potency 0.33) was added, and the solution was incubated for 2 hr at 37°. A sample without enzyme diluted in the same way was used as control. The hydrolyzed sample and control were adjusted to pH 3.4 by addition of 1 μl of 0.56 N HCl, the absorbances at maximum wavelength and 260 nm measured, and the hyperchromicities calculated. the extinction coefficients of the dinucleotides were calculated from the per cent hyperchromicity at 260 nm, pH 3.4, and the extinction coefficients of the equimolar mixtures of the mononucleotides corresponding to the respective dinucleotide according to the expression

$$\epsilon_{260}\ \text{dinucleotides} = \frac{\epsilon_{260}\ \text{mononucleotide mixture} \times 2 \times 100}{100 + \%\ \text{hyperchromicity}}$$

Results

Separation of Deoxyoligonucleotides According to Chain Length. The chromatographic fractionation of a DNase I digest of calf thymus DNA according to chain length on DEAE-Sephadex at 65° in 7 M urea (pH 7.6) is shown in Figure 1a. The absorbance profile shows a separation of 14 peaks corresponding to oligonucleotides of chain length 1–14. A peak of longer oligonucleotides was eluted from the column by 1 M LiCl. Variations in column length, column diameter, pH of the eluting buffer, and temperature were all investigated. Separation deteriorated below pH 6 and 45° and with shorter and broader columns.

Removal of the 5′-terminal phosphate groups from the oligonucleotides by digestion with *E. coli* phosphomonoesterase prior to chromatography resulted in an improved resolution of the longer oligonucleotides and separation of 14 peaks as shown in Figure 1b. The experiments in Figure 1a,b are both from the same initial DNase I digest. In Figure 1b the first peak is nucleosides which are not adsorbed by DEAE-Sephadex and elute during application of the sample. The

TABLE I: Distribution of Oligonucleotide Isostichs Released from Calf Thymus DNA by DNase I before and after Hydrolysis with *E. coli* Phosphomonoesterase.

	Oligonucleotide: General Formula N_np_n				Oligonucleotide: General Formula N_np_{n-1}	
Peak No.	Oligonucleotide Isostich	A_{260}[a]	% of Total A_{260}	Total P: Term P	A_{260}[a]	% of Total A_{260}
I	1	43.34, 39.85	2.1, 2.0		50.81, 50.09	2.5, 2.4
II	2	149.45, 158.20	7.1, 7.8	2.0	139.20, 145.74	6.9, 7.0
III	3	133.15, 132.63	6.3, 6.5		125.65, 136.51	6.2, 6.6
IV	4	197.80, 204.54	9.4, 10.1		202.53, 211.99	10.0, 10.3
V	5	254.28, 245.87	12.1, 12.1	4.9	245.12, 243.95	12.1, 11.8
VI	6	263.13, 268.71	12.5, 13.3		271.83, 266.96	13.4, 12.9
VII	7	246.54, 251.12	11.7, 12.4		249.83, 258.23	12.3, 12.5
VIII	8	181.44, 184.23	8.6, 9.1	7.9	181.64, 186.61	9.0, 9.0
IX	9	144.90, 141.98	6.9, 7.0		143.00, 144.42	7.0, 7.0
X	10	114.28, 107.91	5.4, 5.3		112.50, 105.00	5.6, 5.1
XI	11	79.99, 76.40	3.8, 3.6		72.77, 76.80	3.6, 3.7
XII	12	53.76, 51.80	2.6, 2.6	12.2	50.22, 50.70	2.5, 2.5
XIII	13	44.81, 40.26	2.1, 2.0		33.88, 36.55	1.7, 1.8
XIV	14	26.56, 28.07	1.3, 1.4		23.20, 26.78	1.1, 1.3
XV	15				18.82, 22.27	0.9, 1.1
1.0 M		168.09, 93.47	8.0, 4.6		104.40, 102.87	5.2, 5.0
Total A_{260}		2101.22, 2025.04			2025.40, 2065.47	100.0, 99.8
% recovery[b]		99.1, 95.5			96.4, 98.3	

[a] The values are from two identical separate experiments. [b] Determined from the A_{260} of the material applied to the column in 7 M urea.

corresponding peak in Figure 1a is the mononucleotide fraction (peak I). To test the efficacy of the separation peak XII from Figure 1b was rechromatographed on an identical column eluted with a 4-l. linear gradient of 0.3–0.4 M LiCl in 0.005 M Tris-HCl–7 M urea buffer (pH 7.6) at 65°. No traces of contamination from peaks XI and XIII were observed. The distribution of the oligonucleotides separated according to chain length, based on optical density analyses, and chain-length determinations based on phosphate analyses, are shown in Table I. Total recovery was better than 95%. The very good agreement between the results for oligonucleotides of general formula N_np_n[1] (Figure 1a) and N_np_{n-1} (Figure 1b) confirms the chain-length homogeneity of each peak.

To examine the theoretical basis of the fractionation and investigate the possible application of the separation technique to longer oligonucleotides a plot of log chain length *vs.* molarity of the eluting salt at peak maximum for each isostich in Figure 1a,b was made (Figure 2). Included is data from a DNase I digest of calf thymus DNA chromatographed exactly as described in Figure 1a except that elution was at 25°. The graph shows the superior separation obtained with the 65° chromatograms compared with the 25° chromatogram, particularly of the longer oligonucleotides, and the improved separation at 65° when 5′-terminal phosphates are removed.

The chromatographic fractionation of a 30-hr DNase I digest of *E. coli* DNA according to chain length is shown in Figure 3. The absorbance profile shows a separation of eight peaks. Peaks Ia and Ib are mononucleotides, peaks II–VII corresponding to isostichs 2 to 7. Washing of the column with 1 M LiCl did not elute any ultraviolet-absorbing material. Recoveries were better than 98%. The distribution of the oligonucleotides based on optical density and total phosphorus, and chain-length analysis data are shown in Table II. The material from this and other similar chromatograms was used for all further analyses.

Separation of Oligonucleotides According to Base Composition. MONONUCLEOTIDES. The components of the mononucleotide peaks (Ia and Ib, Figure 3), representing only 3% of the total DNase I digest, were analyzed according to Blattner and Erickson (1967). The results are shown in Table III. Neither peak contained cytidylic acid and the total adenylic acid from both peaks was 6.5%.

DINUCLEOTIDES. To determine the optimum pH for separation of dinucleotides a plot of net charge *vs.* pH was made (*cf.* Cohn, 1955). This is shown in Figure 4. Net charge was calculated from the p*K*'s of the component mononucleotides.

[1] The following abbreviations have been used: DNase I, pancreatic deoxyribonuclease I; A, deoxyadenosine; G, deoxyguanosine; C, deoxycytidine; T, thymidine; p, esterified phosphoric acid. To indicate nucleotides and oligonucleotides of known sequence p is placed at the left of the symbol if the phosphoric acid is linked to the 5′-hydroxyl of the nucleoside; at the right if it is on the 3′-hydroxyl. The term "isostich" refers to oligonucleotides of identical chain length, disregarding their base composition (Shapiro and Chargaff, 1964).

TABLE II: Distribution of Oligonucleotide Isostichs Released from *E. coli* K12 DNA by DNase I.

Peak No.	Oligonucleotide Isostich	A_{260}[a]	% of Total A_{260}	μg of P	% of Total P	Total P: Term P
Ia	1	75.20 ± 5.16	1.88 ± 0.13	376.2	3.0	1.1
Ib	1	54.62 ± 2.09	1.35 ± 0.04			
II	2	1173.37 ± 7.08	29.32 ± 0.10	3360.7	26.8	2.0
III	3	1210.20 ± 5.56	30.25 ± 0.12	3686.8	29.4	2.9
IV	4	984.80 ± 5.14	24.62 ± 0.13	3222.8	25.7	4.2
V	5	408.52 ± 1.25	10.20 ± 0.05	1479.7	11.8	5.1
VI	6	89.15 ± 3.26	2.23 ± 0.09	338.6	2.7	6.3
VII	7	15.73[b]	0.4	72.6	0.5	7.2

[a] The values are the means of three separate analyses plus and/or minus standard error of the mean. [b] This fraction was discarded in two experiments.

In the pH range 1.0–4.0 the terminal monoesterified phosphate has only one ionizable group. The largest net charge difference between the various dinucleotides is in the pH range 3.4–3.7. Initial attempts to fractionate dinucleotides in this pH range on DEAE-Sephadex at 25 and 65° and DEAE-cellulose at 25° in 7 M urea buffers were unsuccessful. Only six of the ten possible dinucleotides were partially separated. The elution positions of the purine-containing dinucleotides were not related to their net charge. This indicated preferential adsorption to the column matrix. Bartos *et al.* (1963) have shown a relationship between the composition of dinucleotides in a mixture and elution position which is also partially dependent on the purine:pyrimidine ratio of the dinucleotides. To delay the elution of the purine rich dinucleotides, fractionation was attempted on DEAE-cellulose with buffers at pH's from 3.2 to 3.7 in the absence of 7 M urea. Optimal separation of peak II from Figure 3 into 11 discrete peaks was obtained at pH 3.4 as shown in Figure 5. Larger amounts of dinucleotides can be successfully fractionated provided the volume of the gradient is increased. Each peak was identified by spectrophotometric and base analysis. The positional isomers pCpA and pApC were completely resolved by the procedure. The distribution of the dinucleotides and hyperchromicity and extinction data at 260 nm, pH 3.4, are shown in Table IV. The order of elution shows the purine-containing dinucleotides are not eluted according to their net charge. The spectral properties

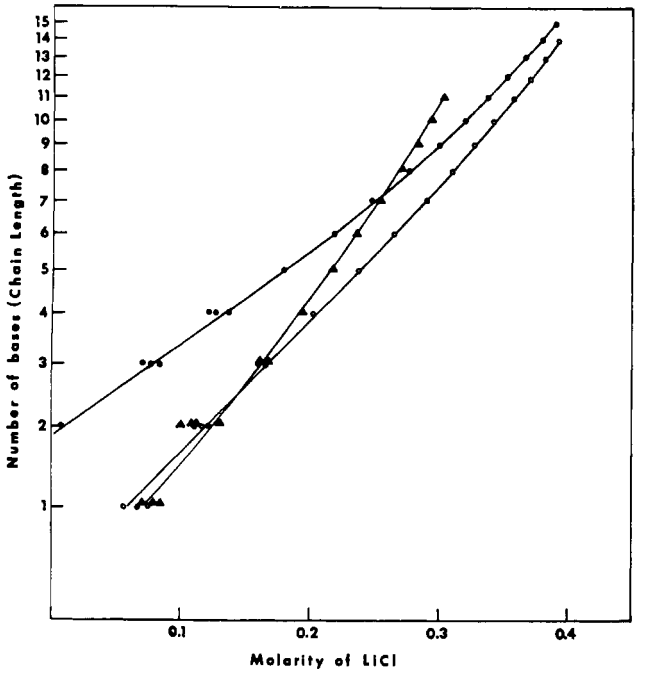

FIGURE 2: Relationship between chain length and molarity of eluting salt of isostich fractionations on DEAE-Sephadex. Salt molarities were taken at peak maximum of the isostich elution profiles. (○—○) DNase I digest on DEAE-Sephadex at 65°. (●—●) DNase I digest dephosphorylated with phosphomonoesterase, on DEAE-Sephadex at 65°. (▲—▲) DNase I digest on DEAE-Sephadex at 25°.

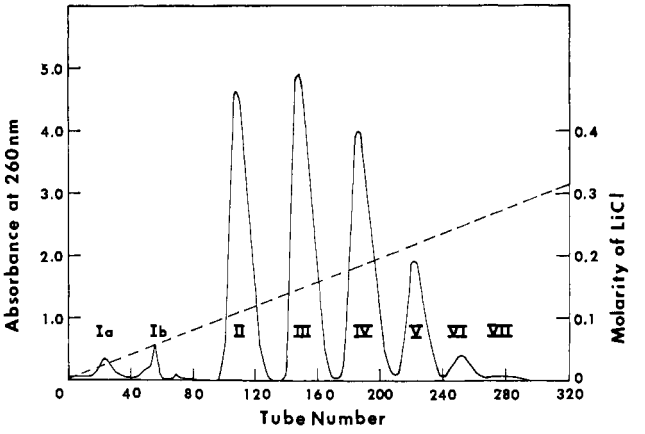

FIGURE 3: Chromatography of a DNase I digest of *E. coli* K12 DNA on a column of DEAE-Sephadex (25 × 0.9 cm, chloride form) at 65°. The digest (133.0 mg of *E. coli* DNA, 40.5 units of DNase I, 37°, 30 hr was applied to the column and eluted with a linear gradient of 0–0.4 M LiCl in 0.005 M Tris-HCl–7 M urea buffer (pH 7.6) at 65°. The total volume of eluent was 6 l.; 15-ml fractions were collected. (————) Absorbance at 260 nm. (--------) Molarity of LiCl.

TABLE III: Distribution of Mononucleotides Released from *E. coli* K12 DNA by DNase I.[a]

	Fraction Ia		Fraction Ib		Total (Fractions Ia and Ib)	
Mononucleotide	μmole	mole %	μmole	mole %	μmole	mole %
pT	0.539	54.1	0.050	14.5	0.589	34.3
pG	0.373	37.4	0.280	80.9	0.653	59.2
pA	0.085	8.5	0.016	4.6	0.101	6.5
pC	0	0	0	0	0	0

[a] Fractions Ia and Ib refer to Figure 3 and Table II.

of the dinucleotides at pH's 1, 7, and 12 when compared with equimolar mixtures of the corresponding mononucleotides confirmed the purity of the isolated dinucleotides and their identity. The properties of the positional isomers pCpA and pApC were identical. Absence of differences in the spectral properties of ribodinucleotide and trinucleotide positional isomers have been reported by Toal *et al.* (1968). Thus in calculations of the extinction coefficients and molar proportions of the dinucleotides (Tables IV and V) it has been assumed that the spectral properties of the positional isomers are identical. The hyperchromicity of dinucleotides has been reported previously (Michelson, 1968). The distribution of each positional isomer, determined by end-group analysis, is shown in Table V. The total end-group analysis of the dinucleotide fraction is presented in Table VI. There is a preferential hydrolysis of DNA by DNase I adjacent to G and C residues indicated by the larger proportion of pGpC, pCpG, pCpT, pCpC, and pGpG present in the dinucleotide fraction (Table V) and the high content of G + C at the 5′ and 3′ ends of the total dinucleotide fraction (Table VI).

TRINUCLEOTIDES. Chromatographic separation of the trinucleotide peak III from Figure 3 on DEAE-cellulose at pH 3.6 is shown in Figure 6. Variations in column size and pH of the eluting buffer from 3.3 to 3.7 did not improve the separation. Recoveries were better than 92%. The distribution and base composition of the separated peaks is shown in Table VII. Peaks 1 and 2 did not contain nucleotide material; 16 of the 22 separated trinucleotide peaks were identified from their base ratios and accounted for 14 of the 20 possible trinucleotides, 2 pairs of peaks having the same composition. The 4 peaks with base ratios which did not allow easy identification of trinucleotide content comprise mixtures of trinucleotides not resolved by the chromatographic conditions. As in the case of the dinucleotides, purine-rich trinucleotides are retained longer on the column.

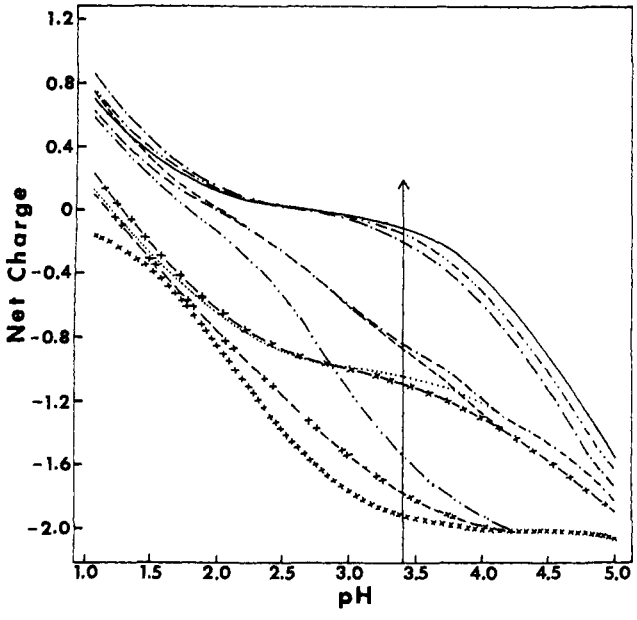

FIGURE 4: Net charge per molecule of dinucleotides as a function of pH, calculated from the pK values of the mononucleotide components. (————) pCpC, (-- · -- · --) pApC, (- · - · - · -) pApA, (- · - · - · -) pCpG, (-------) pApG, (- · - · - · - · -) pGpG, (-x-x-x-) pApT, (· · · · · · · · · · · · · · ·) pCpT, (--xx--xx--) pGpT, and, (xxxxxxxxxx) pTpT.

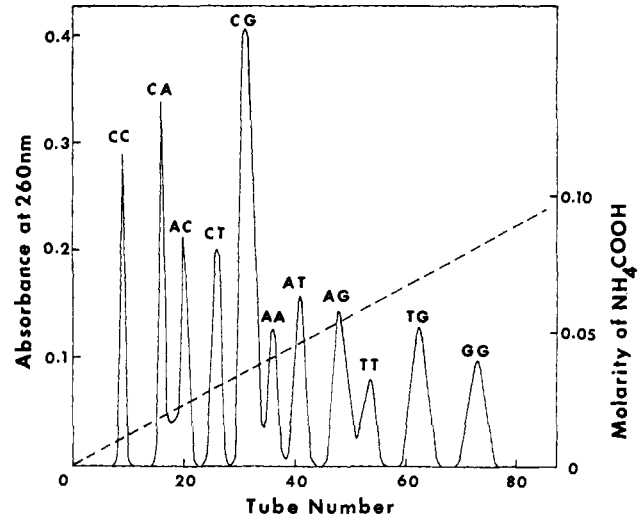

FIGURE 5: Chromatography of dinucleotides from *E. coli* K12 DNA on a column of DEAE-cellulose. The pooled dinucleotide fractions (110 A_{260} units) from Figure 3 were applied to the column (see Experimental Section). The total volume of the eluent was 2 l.; 21-ml fractions were collected. (————) Absorbance at 260 nm. (---------) Molarity of ammonium formate.

TABLE IV: Distribution of Dinucleotides Released from *E. coli* K12 DNA by DNase I and Separated According to Base Composition.

Dinucleotide	Net Charge	A_{260}, pH 3.4	% Hyperchromicity at 260 nm, pH 3.4	$\epsilon_{260} \times 10^{-3}$	μmoles	mole %
CC	0.12	33.8 ± 0.68	5.8	12.5	2.70	8.4
CA[a] + AC	0.16	63.66 ± 2.16	2.2	20.9	3.05	9.5
CT + TC	1.04	64.54 ± 2.06	2.7	14.8	4.36	13.6
CG + GC	0.84	145.36 ± 3.02	2.2	19.0	7.65	23.9
AA	0.20	28.73 ± 0.66	12.7	26.6	1.08	3.4
AT + TA	1.09	61.84 ± 2.28	4.3	22.6	2.74	8.5
AG + GA	0.88	61.41 ± 1.55	3.6	26.8	2.29	7.1
TT	1.90	35.15 ± 1.72	2.4	16.6	2.12	6.6
TG + GT	1.78	79.71 ± 0.78	1.4	21.5	3.71	11.6
GG	1.56	59.36 ± 2.83	3.0	25.2	2.36	7.4

[a] CA and AC were pooled for these analyses. Dinucleotides were from fraction II (Figure 3 and Table II). Net charge is from Figure 4. Values for A_{260} are the means of four separate analyses plus and/or minus standard error of the mean.

Discussion

The method described for chain-length fractionation of deoxyribonucleotides has the advantage over previous methods (Tomlinson and Tener, 1963; Carrara and Bernardi, 1968) of discrete separation of isostichs up to the pentadecanucleotide level. Recoveries are better than 95% and there is no cross contamination of the isostich fractions, at least up to the dodecanucleotide level.

Investigation of the possibility of extending the method for separation of even longer oligonucleotides by plotting chain length *vs.* eluting salt concentration in Figure 2 showed a progressively smaller difference in the salt concentration increase necessary to elute fractions of increasing chain length. Thus with longer oligonucleotides an asymptotic limit will be reached and resolution will disappear. This is contrary to the suggestion of Matthews (1968) of a possibly limitless chain-length fractionation of long oligonucleotides on DEAE-Sephadex. The nonlinearity of the relationships in Figure 2 obviate linear extrapolation of eluting salt concentration to estimate chain length (Petersen and Reeves, 1969). Since the relationship between eluting salt concentration and chain length is not linear some secondary binding forces of the oligonucleotides to the column are not overcome by 7 M urea at 65°. Nevertheless, the higher temperature reduces drastically secondary binding forces which interfere in separations at 25° and increases the dependence of the fractionation on the eluting salt concentration, reducing cooperative displacement where the longer oligonucleotides displace the shorter oligonucleotides which have lower affinity for the DEAE groups (Brownlee *et al.*, 1968). Removal of the 5′-terminal phosphates from the oligonucleotides

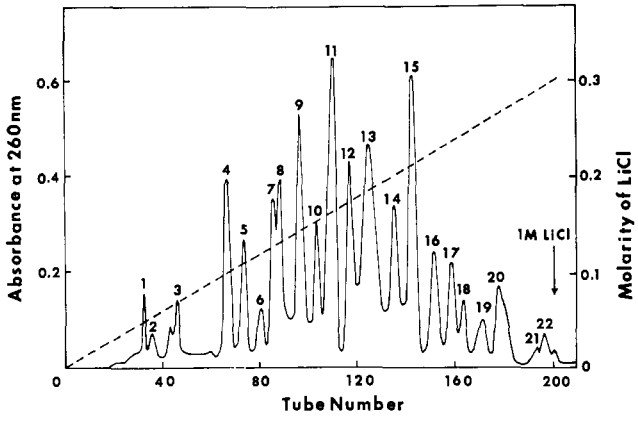

FIGURE 6: Chromatography of trinucleotides from *E. coli* K12 DNA on a column of DEAE-cellulose. The pooled trinucleotide fractions (150 A_{260} units) from Figure 3 were applied to the column (see Experimental Section). The total volume of the eluent was 2 l.; 10-ml fractions were collected. (————) Absorbance at 260 nm. (·····) Molarity of ammonium formate.

TABLE V: Distribution of Isomeric Dinucleotides Released from *E. coli* K12 DNA by DNase I.

Dinucleotide	% of Isomer in Dinucleotide	Mole %
pCpC	100.0	8.4
pCpA	63.8	6.1
pApC	36.2	3.4
pCpT	67.0	9.1
pTpC	33.0	4.5
pCpG	42.7	10.2
pGpC	57.3	13.7
pApA	100.0	3.4
pTpA	56.6	4.8
pApT	43.4	3.7
pApG	30.4	2.2
pGpA	69.6	4.9
pTpT	100.0	6.6
pTpG	44.8	5.2
pGpT	55.2	6.4
pGpG	100.0	7.4

TABLE VI: Base Composition of the 5′ and 3′ Termini of Dinucleotides Released from *E. coli* K12 DNA by DNase I.

Base	C	T	A	G
Mole per cent at 5′ end	33.8	21.1	12.7	32.4
Mole per cent at 3′ end	30.0	25.8	19.2	25.0

resulted in an improved separation, confirming previous observations (Tomlinson and Tener, 1963; Ishikura *et al.*, 1966).

Assuming random cleavage of DNA by DNase I each isostich fraction should contain $(n + 3)!/n!3!$ oligonucleotide components where n = chain length. This formula does not take into account positional isomers. Thus 4 mononucleotides, 10 nonisomeric dinucleotides, and 20 nonisomeric trinucleotides should be present in isostichs 1, 2, and 3. In the *E. coli* K12 DNA manganese digest only 3 mononucleotides were present (Figure 3, Table III). The absence of pC indicates a nonrandom degradation by DNase I under the conditions used. The chromatographic separation of these mononucleotides into two peaks Ia and Ib, Figure 3, each of which contains all the mononucleotide components (Table III) is also unusual but has not been further investigated. All 16 possible dinucleotides were shown to be present (Table V) but the nonrandom distribution supports the previous evidence from the mononucleotide fraction of nonrandom degradation by DNase I. Previous studies of the dinucleotides present in DNase digests (Becking and Hurst, 1963; Sinsheimer, 1955; Privat de Garilhe *et al.*, 1957; Lehman, 1960) have failed to observe all possible dinucleotides and positional isomers. Unfortunately, variations in digestion conditions, source of DNA, and methods of separation do not allow valid comparison of all the results. However, the quantitative distribution of the dinucleotide positional isomers pApC, pCpA; pCpT, pTpC; pCpG, pGpC; and pApT, pTpA is similar to results reported by Becking and Hurst (1963) from calf thymus DNA using manganese as cofactor. Sinsheimer (1955), Privat de Garilhe *et al.* (1957), and Lehman (1960) found predominantly pPy-pPu sequences in the positional dinucleotide isomers they were able to examine. In the present study examination of all 12 positional isomers shows this is true when A is the purine base but pPu-pPy predominates when G is the purine base.

In the total dinucleotide fraction from the *E. coli* K12 DNA manganese digest G and C predominates at both the 3′ and 5′ ends (Table VI). However, in those dinucleotides containing A and T, A and T predominate at the 3′ end. Sinsheimer (1955) using magnesium as cofactor showed T and C predominate at the 5′ end and A and G at the 3′ end of the dinucleotides. Scheffler *et al.* (1968) examined the dinucleotides released from the alternating copolymer d(A-T) by magnesium-activated DNase I and showed T predominates at the 5′ end and A at the 3′ end. Bollum (1965) has also shown differences in the rate of degradation by DNase I of the different strands of the double-stranded homopolymer complexes dA:dT, dG:dC, and dI:dC with magnesium and manganese as cofactors. Thus the present study using manganese as cofactor supports a possibility

TABLE VII: Distribution of Trinucleotides Released from *E. coli* K12 DNA by DNase I and Separated According to Base Composition.[a]

Peak No.	Mole %	Base Ratio G	A	C	T	Trinucleotides Present
3	0.5			1.0		CCC
4	8.0		1.0	2.2		CCA
5	6.8			1.9	1.0	CCT
6	1.5	1.0		6.6	2.3	Mix[b]
7	5.2	1.0		2.2		CCG
8	4.1		1.8	1.0		AAC
9	12.3		1.0	1.2	1.2	ACT
10	4.1	1.4	1.9	1.0	1.1	Mix
11	14.7	1.0	1.0	1.2		GAC
12	3.2	1.0	1.9	1.3	1.3	Mix
13	13.6	1.0		1.3	1.1	CTG
14	3.1		1.0		2.0	TTA
15	6.5	1.2	1.0		1.1	GAT
16	4.3	1.0	1.0		3.1	Mix
17	4.4	2.0	1.0			GGA
18	1.5	1.0			2.3	TTG
19	2.0	1.0			2.0	TTG
20	3.7	1.8			1.0	GGT
21	0.2	1.9			1.0	GGT
22	0.3	1.0				GGG
Total		23.0	22.5	31.3	23.3	

[a] Trinucleotides were from fraction III, Figure 3, and Table II. Values are the means of two separate analyses. [b] Probably CCG and CCT.

previously suggested (Becking and Hurst, 1963; Melgar and Goldthwait, 1968) of a different specificity of degradation by DNase I according to whether magnesium or manganese is used as the cofactor.

The availability of methods for discrete chain-length fractionation of deoxyoligonucleotides up to the pentadecanucleotide level and quantitative separation of all possible dinucleotides and most trinucleotides extends the possibilities of obtaining new information on nucleotide sequences in DNA molecules. The methods are currently being applied to investigation of the base specificity of DNase I degradation with various divalent ions as cofactors.

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