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Enzymatic Hydrolysis of Calf Thymus Deoxyribonucleic Acid Adsorbed on Diethylaminoethylcellulose*

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ABSTRACT: A procedure is described for the enzymatic hydrolysis of calf thymus deoxyribonucleic acid (DNA) adsorbed on DEAE-cellulose. After nuclease inactivation, stepwise elution with solutions of increasing NH_4HCO_3 concentrations yielded large oligomers. These compounds were characterized by base ratio and end-group chain-length determinations, centrifugation through sucrose density gradients, and column chromatography on DEAE Sephadex A-25 in 7 M urea. Applications of the procedure are discussed.

Studies on the determination of nucleotide sequences in DNA have lagged behind those concerned with RNA largely because (a) of the smaller size of RNAs, especially tRNAs, which allowed the elucidation of complete base sequences (Holley *et al.*, 1965; Zachau *et al.*, 1966; Madison *et al.*, 1966), and (b) because of the lack of specific DNases comparable to pancreatic RNase and RNase T_1. Such specific DNases, if available, would facilitate the preparation and isolation of large oligomers ($n = >10$), important for nucleotide sequence characterization. Known DNases such as micrococcal nuclease (EC 3.1.4.7), pancreatic DNase (EC 3.1.4.5), and *E. coli* endonuclease I do not have strict specificities and yield, in limit digests, oligomers of average chain length 2–8 (Roberts *et al.*, 1962; Tomlinson and Tener, 1963; Sinsheimer, 1952; Lehman, 1963) Chemical methods of DNA hydrolysis (Volkin *et al.*, 1951; Tamm *et al.*, 1952; Whitfeld, 1954; Dekker *et al.*, 1953; Burton and Petersen, 1960; Habermann *et al.*, 1963; Chargaff *et al.*, 1963; Hall and Sinsheimer, 1963) or combinations of enzymatic and chemical methods (such as DNA polymerase incorporation of ribonucleotides into newly synthesized DNA followed by alkaline hydrolysis; Berg *et al.*, 1964) yield large oligonucleotides from DNA. However, there is a need for additional methods for the preparation of large oligodeoxynucleotides, especially of those with mixed purine:pyrimidine ratios, because they yield more nucleotide sequence information than oligomers of chain length $n = 2$–8.

Enzymatic hydrolysis of high molecular weight RNA adsorbed on DEAE-cellulose was previously shown to yield large oligomers in good yields. Thus, after RNA of the *E. coli* phage MS 2 was bound to the adsorbent, hydrolyzed *in situ* with ribonucleases, and the enzymes were removed, stepwise elution with solutions of increasing salt concentrations yielded large oligomers in good yield. This was ascertained by end-group chain-length determinations and by column chromatography. With the RNase of *Bacillus subtilis* (Nishimura and Nomura, 1958), nucleotide composition of the oligomers so obtained was significantly different from that of MS 2 RNA itself (Rushizky *et al.*, 1966).

This report describes an extension of the above RNA procedure to the enzymatic hydrolysis of calf thymus DNA adsorbed on DEAE-cellulose. With both pancreatic DNase and micrococcal nuclease, oligomer fractions so obtained had average chain lengths (by end-group determination) ranging from $n = 27$ to 228. The oligomer fractions were also examined by column chromatography in 7 M urea on DEAE-Sephadex, by nucleotide composition, and by sedimentation through sucrose density gradients.

Experimental Procedure

Spectrophotometric measurements were made in cells of 1-cm light path and are expressed as absorbance at 260 mμ (A_{260}).

Enzymes. Micrococcal nuclease (EC 3.1.4.7) of 90% purity was a gift of Drs. H. Taniuchi and C. B. Anfinsen (Heins *et al.*, 1967). Alkaline phosphatase was isolated from *E. coli* C 90 as described by Neu and Heppel (1965). Recrystallized pancreatic DNase (EC 3.1.4.5) was obtained from Sigma.

Nucleic Acid Materials. Calf thymus DNA (highly polymerized, lot DNA 607) was obtained from Worthington. DNA from calf thymus was also prepared by extraction with 1 M NaCl–phenol (Colter *et al.*, 1962)

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with the following modification. Instead of using a Waring Blendor, small pieces of frozen thymus were passed through a meat grinder and the last remnants of tissue were flushed out with crushed ice. For the first phenol extraction, the collected material was stirred for 3 hr at 23° and about 200 rpm in 1 M NaCl–phenol as described (Colter *et al.*, 1962). The subsequent phenol extractions were carried out by stirring at 23° for 15 min. The final product was stored at −20°.

DNA at 1 mg/ml in H_2O at neutral pH was taken to have an A_{260} of 25, corresponding to a P content (Ames and Dubin, 1960) of 8.8%. For denaturation, DNA at 1–2 mg/ml in H_2O was heated for 30 min at 100°, cooled in ice, and stored at 4° over $CHCl_3$ until used. Upon hydrolysis with alkali or RNase T_2 (Rushizky and Sober, 1963), no Up was found in the prepared DNA under conditions where 0.1% (by A_{260}) could have been detected. Hexa-, hepta-, and octanucleotides of RNase T_1 digests of yeast RNA were isolated by column chromatography in 7 M urea on DEAE Sephadex A-25 at pH 7.5 (Rushizky *et al.*, 1965). tRNA from yeast, used as a marker for column chromatography, was obtained from General Biochemicals, Chagrin Falls, Ohio, as well as from Dr. B. P. Doctor.

Adsorbents. DEAE-cellulose (Schleicher & Schuell Selectacel lot no. 1710, 0.8 mequiv/g. 100–230 mesh) was washed (Peterson and Sober, 1956) and preequilibrated in appropriate buffers (Table I). DEAE Sephadex A-25 (Pharmacia) with a rated capacity of 2.7 mequiv/g was freed of fines and washed as described for CM-cellulose (Peterson and Sober, 1956), suspended in starting buffer, and packed into columns by gravity flow. The exchanger in the columns was then equilibrated until the pH and conductivity of the effluent solution were the same as those of the influent solution. Column chromatography was performed as described in the legend to Figure 1.

Enzymatic Hydrolysis of DNA. DEAE-cellulose, about 17 g by dry weight, was equilibrated in 0.5 M Tris-HCl (pH 8.5) plus 0.001 M $CaCl_2$ (digestion buffer) to the proper pH and conductivity values. The adsorbent was filtered and suspended in a polyethylene flask in 500 ml of digestion buffer. Heat-denatured DNA, 50 mg by P determination (total A_{260} 1820), was added with stirring and the suspension was placed on a shaker at 37° for 4 hr. Micrococcal nuclease (0.05 mg) was then added in 100 ml of digestion buffer. This amount of enzyme, designated as *E*, was sufficient to hydrolyze unprotected DNA to mono- and dinucleotides (Roberts *et al.*, 1962). After shaking at 37° for 20 hr, the suspension was diluted to 2500 ml with 0.001 M EDTA, and stirred for 5 min. The adsorbent was filtered, and after washing on a funnel to an A_{260} of 0.05 or less with several portions to a total of 8 l. of 0.02 M NH_4HCO_3 (pH 8.6), the DEAE-cellulose was packed in the same buffer in a 4 i.d. × 20 cm high column. Stepwise elution was started with 0.1 M NH_4HCO_3 (pH 8.6) and continued with 0.3, 0.5, 0.7, 1, and 2 M NH_4HCO_3 (pH 8.6) at a flow rate of about 100 ml/hr. Eluents were changed after the A_{260} per milliliter fell

TABLE I: Fractionation of Partial Enzymatic Digests of Calf Thymus DNA by Stepwise Elution with NH_4HCO_3 (pH 8.6).

Expt No.	Length of Digestion at 37° (hr)	Amt of Enzyme (*E*)[a]	Digestion Buffer (M), pH	Amt of DNA (% of total A_{260}) Eluted at the Indicated Molarity of NH_4HCO_3						Recov in % of Total A_{260}[d]
				0.02 + 0.1	0.3	0.5	0.7	1.0	2.0	
MN 1[b]	24	2	Tris-HCl (0.5), 8.5	34	1	6	36	9	1	85
MN 2	12	2	Tris-HCl (0.1), 8.5	13	6	9	24	11		63
MN 3	12	2	Tris-HCl (0.5), 8.5	21	3	9	23	10		65
MN 4	18	2	Tris-HCl (0.1), 8.5	18	6	13	27	9		73
MN 5	24	1	NH_4HCO_3 (0.1), 8.5	5	2	5	34	1	5	52
MN 6	24	0.5	NH_4HCO_3 (0.1), 8.5	20	1	4	10	8	2	45
D 1	24	1	NaOAc (0.02), 5.7	27	3	1	24	11	1	68
D 2	24	0.5	NaOAc (0.02), 5.7	24	3	6	16	4	1	54
D 3	16	1	NaOAc (0.02), 5.7	20	5	16	16	10	1	68
D 4[c]	24	1	NaOAc (0.02), 5.7	32	11	18	42	13	2	118[e]

[a] *E* = amount of enzyme needed to digest DNA to completion in the absence of DEAE-cellulose. [b] MN = micrococcal nuclease; D = pancreatic DNase. [c] Heat-denatured DNA was used for all experiments except for D 4, where native DNA was employed. A larger hyperchromic effect would result from enzymatic hydrolysis of native than of denatured DNA. This is probably the reason for the high recovery (118%). [d] Adsorbed on DEAE-cellulose.

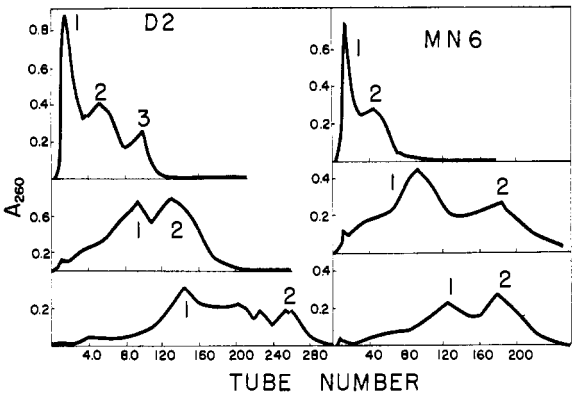

FIGURE 1: Column chromatography on 1.5 i.d. × 75 cm high columns of DEAE Sephadex A-25 in 7 M urea, 0.02 M Tris-Cl (pH 7.5), and 0.3 M NaCl (Rushizky *et al.*, 1965). The columns were developed at 23° with an 8000-ml linear gradient of 0.3–1.0 M NaCl at a flow rate of 85 ml/hr, 19 ml/tube. About 500–1500A_{260} units of oligomer fractions of expt D 2 and MN 6 (Table I), eluted from DEAE-cellulose with 0.5, 0.7, and 1.0 M NH_4HCO_3 (top to bottom) were examined under identical conditions of chromatography where markers of hexa- to octanucleotides and yeast tRNA (not shown) were eluted at tubes 10–20 and 80–100, respectively. Peak fractions as numbered were pooled, desalted, and characterized by base ratio and end-group chain-length determinations (Table III).

TABLE II: Base Ratios of Oligonucleotide Fractions Derived from Partial Enzymatic Digests of DNA.[a]

		Guanine	Cytosine	Adenine	Thymine
Calf thymus DNA (Worthington)		21	22	28	28
Calf thymus DNA (Colter)		22	21	28	29
Expt	Fractions eluted with NH_4HCO_3 (M)				
D 3	0.3	29	22	28	21
	0.5	29	23	28	20
	0.7	27	23	28	22
	1.0	23	23	28	26
D 4	0.3	25	22	30	22
	0.5	28	22	30	20
	0.7	24	23	28	25
	1.0	23	22	28	27
MN 5	0.5	27	21	30	22
	0.7	25	22	29	25
	1.0	25	21	29	24
MN 6	0.5	29	21	29	20
	0.7	27	21	29	23
	1.0	23	23	29	25

[a] For a description of the procedure, see text. Oligonucleotide samples were tested in quadruplicate and at two or more different DNA concentrations. Variation between different assays was ±1%. Recovery of the material after paper chromatography was better than 85%. [b] See Table I.

below 0.05. Effluents obtained at the same salt concentration were pooled, the total A_{260} was determined, and the solutions were hydrolyzed. When the volume of pooled effluents was large, the solution was diluted 1:5 with H_2O and concentrated by passage through DEAE-cellulose (Rushizky and Sober, 1962). The DNA fractions so obtained were stored at 23° over $CaCl_2$ as lyophilized powders.

Digestion of calf thymus DNA with pancreatic DNase was carried out as above but in a digestion buffer of 0.02 M sodium acetate (pH 5.5) and 0.015 M magnesium acetate (Sinsheimer, 1952). Because commercial DNase preparations vary with respect to their enzymatic activity, enzyme:DNA (by phosphorus) ratios of 1:20 and 1:40 were used. Under comparable conditions but in the absence of DEAE-cellulose, hydrolysis of calf thymus DNA to mono- to octanucleotides has been obtained at enzyme:substrate ratios of 1:1000 (Tomlinson and Tener, 1963).

Characterization of Isolated Oligonucleotide Fractions. The lyophilized oligonucleotide fractions were characterized with respect to base composition (Table II) after hydrolysis with 60% $HClO_4$ (Marshak and Vogel, 1951) and paper chromatography on Whatman No. 3 paper with *t*-butyl alcohol–concentrated ammonium hydroxide–water (85:5:10, v/v). When applied to the papers in a band 4 cm wide, DNA hydrolysates containing 50 μg of guanine were resolved without trailing. The amounts of the bases were determined by spectrophotometry (Vischer and Chargaff, 1948).

The oligonucleotide fractions were also examined by column chromatography on DEAE Sephadex A-25 in 7 M urea. Tubes containing major peaks were pooled, desalted, and further characterized by base ratio and end-group chain-length determinations with alkaline phosphatase. These procedures are described in the legends to Figure 1 and Table III.

Results

Calf thymus DNA (0.1–1.0 g), prepared according to Colter *et al.* (1962), was denatured by heating, cooled in ice, and adsorbed on DEAE-cellulose at 23° at the pH and metal concentration optimum of pancreatic (EC 3.1.4.5) or micrococcal DNase (EC 3.1.4.7). After centrifugation of the suspension, complete adsorption of the DNA was ascertained by the virtual absence of A_{260} material from the supernatant solution. Sufficient nuclease was added in amounts (E) equal to or larger than those required to hydrolyze the DNA to

TABLE III: Characterization of Oligonucleotide Fractions Obtained by Chromatography on DEAE Sephadex A-25 in 7 M Urea.[a]

Expt No.[b]	Fraction Eluted with NH_4HCO_3 (M)	Peak No.[c]	Base Ratios of Oligomer Fractions from Peaks[d]				Av Chain Length of Oligomer Fractions in Peaks[e]
			Guanine	Cytosine	Adenine	Thymine	
MN 6	0.5	1	28	17	37	18	16
		2	30	20	29	21	42
	0.7	1	34	20	29	17	67
		2	25	24	24	27	191
	1.0	1	27	20	27	26	109
		2	21	24	26	29	228
D 2	0.5	1	30	22	32	16	15
		2	31	23	30	16	41
		3	27	27	25	22	63
	0.7	1	29	20	31	20	53
		2	27	26	26	21	98
	1.0	1	28	23	28	21	146

[a] See Figure 1 for a description of the column chromatography. [b] See Table I. [c] See Figure 1 for peaks. [d] The determination of base ratios is given in the Methods section. [e] Determined as ratio of total to terminal phosphate released by alkaline phosphatase of *E. coli*. Several aliquots of each oligomer (0.5–1.0 mg) were hydrolyzed with 200–400 units of enzyme (Neu and Heppel, 1965; Heppel *et al.*, 1961), in 0.25 M Tris-HCl (pH 8.5) for 16–20 hr at 38°. There was no significant increase in release of inorganic phosphate between 2 and 16 hr of digestion.

completion in the absence of DEAE-cellulose. The suspension was shaken for 12–24 hr at 37°, and then diluted 1:5 with 0.001 M EDTA. The supernatant solution was filtered, and the adsorbent was washed extensively with 0.02 and 0.1 M NH_4HCO_3 (pH 8.6). The material so eluted consisted of mono- and dinucleotides. Elution of adsorbent was continued with 0.3, 0.5, 0.7, 1.0, 2 M NH_4HCO_3 of the same pH. The total yield of A_{260} and material recovered with 0.02–2 M NH_4HCO_3 ranged from 54 to 85%. Better than 95% recovery could be obtained by washing of the adsorbent with 0.1 N NaOH. As shown in Table I, with both nucleases about 40–50% of the total A_{260} adsorbed on the DEAE-cellulose was thus recovered in the oligomer fractions eluted with 0.5–1.0 M NH_4HCO_3 (pH 8.6).

Pancreatic DNase hydrolyzes native and denatured DNA at similar rates (Tamm *et al.*, 1952), and it was therefore possible to study the hydrolysis of native DNA adsorbed to DEAE-cellulose. The elution pattern was quite similar to that obtained with heat-denatured DNA (Table I, D 4). The apparent recovery was greater in the case of native DNA since it was measured in per cent of A_{260} applied to the DEAE-cellulose and no correction was made for hyperchromic effects.

The oligomer fractions obtained by stepwise elution from DEAE-cellulose (Table I) were examined in various ways. Base ratio determinations after hydrolysis with $HClO_4$ gave values many of which differed significantly from those of the starting material. Base ratio variations were also observed between oligomers prepared with pancreatic or with micrococcal nuclease. These differences may be due, in part, to the higher affinity of DEAE-cellulose for purines than for pyrimidines (Staehelin *et al.*, 1959; Bartos *et al.*, 1963).

In addition to characterization by base ratios, the oligomer fractions obtained by stepwise elution from DEAE-cellulose (Table I) were also run on analytical columns of DEAE Sephadex where the elution position depends largely on chain length (Figure 1). Markers of hexa-, hepta-, and octanucleotides were found not to be adsorbed to DEAE Sephadex under the conditions used for chromatography, and there was very little of such material in the DEAE-cellulose fractions listed in Table I.

The major peaks of A_{260} material from the DEAE Sephadex columns were further characterized by determination of base ratio and average chain length (Table III). It is apparent that these fractions were composed of oligomers of considerable size. Furthermore, oligomer fractions eluted by higher salt concentrations from DEAE-cellulose (Table I) were also more difficult to elute from DEAE Sephadex and were of larger average chain length (Figure 1 and Table III).

The chain-length determinations with alkaline phosphatase gave values for the oligomer fractions similar to those obtained with oligoribonucleotides prepared with micrococcal nuclease by the same procedure (Rushizky *et al.*, 1966). Alkaline phosphatase is known to function best with small oligonucleotides, while at chain lengths over that of tRNA (70–80), stoichiometric re-

lease of phosphate is only achieved over narrow ranges of enzyme:substrate ratios (Neu and Heppel, 1965). However, there was general agreement between the elution positions of markers of tRNA and that of oligomer peak fractions of similar size (expt D 2, 0.7 M NH_4HCO_3 fraction, peaks 1 and 2; see Table III and Figure 1).

The oligomer fractions obtained with the two DNases did not contain any of the starting DNA. This was determined in three ways. (1) Heat-denatured DNA (Colter DNA) is not eluted from DEAE-cellulose (<1%) under the conditions used, even by 2 M salt. (2) Similarly, the DNA is not eluted from DEAE Sephadex under the conditions of these experiments. (3) Oligomer fractions obtained with 1 M NH_4HCO_3 (Table I) were sedimented for 16–20 hr at 3° and 25,000 rpm through linear density gradients of 5–25% sucrose, 0.05 N in NaCl, and 0.02 M in Tris-Cl (pH 7.5). Since under these conditions intact DNA moved into the gradient while the oligomer fractions did not, the latter were considered to be free of original DNA.

Discussion

With high molecular weight nucleic acids, hydrolysis to oligomers of tRNA size (n = 70–100) may be a useful first step toward determination of their nucleotide sequences. Since biological activity of nucleic acids is lost upon breakage of phosphodiester bonds, oligomers derived from DNA would have no biological activity as do the tRNAs. There is thus a need for many methods for the preparation, fractionation, and characterization of large oligomers without biological activity, even if they are obtained by random cleavage of the starting nucleic acid. Thus, work with tRNAs shows that cleavage need not be base specific to give useful fragments for interlocking smaller pieces.

The procedure described here yields sizable amounts of large oligodeoxynucleotides suitable for studies as outlined above, because they are free of small oligonucleotides (n = <9) and original DNA. The preparation of large oligomers is quite reproducible since it does not depend on an interaction of substrate with limited amounts of enzyme, but on the partial protection of DEAE-cellulose bound DNA against large amounts of nuclease.

It is clear that since the nucleases employed here hydrolyze DNA mostly to mono-, di-, octanucleotides in the absence of DEAE-cellulose, the restricted size distribution of the larger oligomers obtained in the presence of DEAE-cellulose must depend on the interactions of DNA, enzyme, and ion exchanger. DEAE-cellulose and heat-denatured DNA have little or no unique three-dimensional charge configurations, so that enzymatic hydrolysis of the adsorbed DNA would proceed by random protection and random cleavage, rather than by specific hydrolysis of the DNA at only a few sites.

Such specific hydrolysis, even if obtained, would be difficult to ascertain by methods now available. Sucrose density gradient centrifugation, chromatography on MAK columns (Sueoka and Yamane, 1962), or electrophoresis on polyacrylamide gels (McPhie *et al.*, 1966) is of limited use with oligomers of the size of tRNA but without biological activity. With single-stranded DNA labeled at the ends (for a review, see RajBhandary and Stuart, 1966) information about the terminal nucleotide sequences might be obtained from the base composition and chain lengths of the isolated and labeled oligomers. However, even specific hydrolysis at the ends, *per se*, would not suffice to establish specific hydrolysis throughout the remaining portion of the molecule. Thus, the availability of the large oligomers described should aid the development of additional procedures for their fractionation and characterization.

In addition to the methods described here, other procedures could also be developed in order to obtain large oligonucleotide fragments from DNA and RNA. Various polybasic protecting groups might be used to confer partial resistance of nucleic acids to nucleases. Thus, TMV protein reconstitutes with TMV–RNA to yield a virus resistant to RNase (Fraenkel-Conrat and Singer, 1959) while ϕX 174 DNA complexes with ϕX 174 coat protein in the presence of calcium and becomes resistant to DNase (Takai, 1966). Similarly, methylated albumin was found to preserve the infectivity of the RNA of an encephalitis virus against micrococcal nuclease (Norrell and Costlow, 1967). Partial protection of nucleic acids against nucleases has been observed with DEAE dextrans (Maes *et al.*, 1967), and with polylysines, nuclease-resistant polylysine:RNA complexes with high (Gp + Cp) content have been obtained (Sober *et al.*, 1966). It may thus be feasible to prepare a variety of oligonucleotides of different sizes and/or base ratios by varying conditions of protection and enzymatic hydrolysis.

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