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Kinetics of the Middle and Terminal Phases of Degradation of DNA by Spleen Acid DNAase

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The kinetics of the middle and terminal phases of calf thymus DNA degradation by spleen acid DNAase has been investigated in detail with the main purpose of providing a background for a study of the specificity of the enzyme and its use in investigations of nucleotide sequences in DNA.

During the middle phase the ultraviolet absorption, the acid solubility and the reciprocal average degree of polymerization of the hydrolysate, $\bar{P}_n^{-1}$, increase linearly with digestion time, while the elution profile of the DEAE-cellulose-urea chromatogram is progressively shifted to the left. Empirical relationships between $\bar{P}_n^{-1}$ and hyperchromic shift have been established. Extrapolation of $\bar{P}_n^{-1}$ to zero digestion time indicates that bond splitting proceeds at a faster, yet decreasing rate during the early part of digestion.

The beginning of the terminal phase is characterized by a sudden slow down of the reaction rate; this phenomenon has been shown to be essentially due to the melting of double-stranded DNA fragments; the single-stranded fragments so originated are a poor substrate for the enzyme, in spite of the fact that they still contain a large number of sequences which can be split. During the terminal phase, the ultraviolet absorption, the acid solubility and $\bar{P}_n^{-1}$ increase at a progressively slower rate until an end-point is practically reached. Investigations on the liberation of mono- and dinucleotides, the base composition and termini of isostichs and the effects of digestion temperature and of dephosphorylation on the isostich patterns are also reported.

Three different phases have been distinguished in the degradation of native DNA by spleen acid DNAase [1, 2]: (a) The initial phase, in which the macromolecular and biological properties of DNA are dramatically modified, whereas no change can be detected in its spectral properties and no acid-soluble fragments are formed. (b) The middle phase, which is characterized by a hyperchromic shift and the formation of acid-soluble oligonucleotides; both phenomena are linear with digestion time in this phase. (c) The terminal phase which shows a slower and slower further increase in the hyperchromic shift and acid-soluble oligonucleotide formation until both effects reach plateau values.

The initial phase has already been studied in the past [2–4]. We report here results obtained in recent years in investigations on the middle and terminal phases. This work was done with the aims: (a) of clarifying a number of problems existing in these phases; (b) of setting up an improved technology for studying enzymatic DNA digests and (c) of providing a solid background for the use of the enzyme in studies of nucleotide sequences in DNA. Concerning this latter point, we may mention that there are at least two different ways of using DNAases in such studies. The first one, not yet used by us, is based on the analysis of the oligonucleotides produced by the enzymes. The second one is based on the quantitative analysis of the termini[1] released by DNAases; this second method has been developed by us and is outlined in the accompanying paper [5]. In both cases, a good understanding of the events taking place during the middle and terminal

Abbreviations. $\bar{P}_n$, average degree of polymerization, average size or average chain length of oligonucleotides.

Enzymes. Deoxyribonuclease (EC 3.1.4.5); acid deoxyribonuclease (EC 3.1.4.6); snake venom and spleen acid exonuclease (EC 3.1.4.1); micrococcal nuclease (EC 3.1.4.7); acid phosphomonoesterase (EC 3.1.3.2); *E. coli* endonuclease I (EC 3.1.4.–).

Definitions. A_{260} unit, the quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 260 nm, when measured in a 1-cm cell; A_{271} unit, the quantity of material contained in 1 ml of a solution which has an adsorbance of 1 at 271 nm, when measured in a 1-cm cell; isostichs are oligonucleotide size groups, regardless of their composition [34].

[1] With this term we designate, conventionally, the 3′-phosphate terminal nucleotides and the 5′-hydroxy terminal and penultimate nucleotides.

phases is of essential importance. It should be stressed that the information gathered in the present work is relevant for DNAases other than spleen acid DNAase. A final remark is that the spleen enzyme presents a series of problems in its kinetics which are associated to the existence of two different kinds of sites recognized and split by the enzyme and which do not exist in the other three DNAases investigated so far in our laboratory (snail acid DNAase, pancreatic DNAase, *Escherichia coli* endonuclease 1 [6—9]).

MATERIALS AND METHODS

DNA samples CTR 1, CTR 2 (indicated as CTR II in a previous paper, [10]), V 391, were prepared from calf thymus following the detergent procedure of Kay *et al.* [11] according to Bernardi and Sadron's method B [4]. Their sedimentation coefficients were 20 S, 24 S, and 21 S, respectively. Their hyperchromic shifts, upon combined spleen DNAase and spleen exonuclease digestion, were equal to 71 %. All DNA samples were quantitatively recovered from hydroxyapatite column [12] and were free from low eluting materials (mono- and oligonucleotides).

Enzymatic Digestions

DNA samples were dialyzed against 0.05 M ammonium acetate—1 mM EDTA pH 5.5, generally after a few days' storage at 4 °C in the standard solvent of Zubay and Doty [13] saturated with chloroform—isoamyl alcohol (5:1, v/v). The ammonium acetate-EDTA solvent was chosen for the enzymatic digestions for the following reasons: (a) its pH is satisfactory for the three hog spleen enzymes used in the digestions, acid DNAase, acid exonuclease and acid phosphomonoesterase; (b) its ionic strength is such that both the spleen DNAase digest and the final digest (formed by nucleotides and terminal nucleosides) can be loaded as such on DEAE-cellulose columns, thus avoiding concentration steps and (c) its volatility permits concentration of the terminal nucleosides to dryness without accumulation of salt, thereby facilitating their analysis. The use of this solvent, though necessary for some of the experiments reported here, is not strictly needed for most purposes, since spleen DNAase digests may be dialyzed against running distilled water and be adjusted later to other pH and ionic conditions. Dialysis of undephosphorylated spleen DNAase digests was performed at 4 °C [10].

Acid DNAase B [14] digestions were carried out at 0, 22 or 37 °C using DNA solutions having $A_{260} = 8.0$. This is a very largely saturating substrate concentration; at higher DNA concentration, inhibition by excess substrate has been seen when following the reaction by acid solubility [15]. Four enzyme preparations (HS 21a, 22, 24, 25) were used. The enzyme concentration in the incubation mixture was 0.1 unit/ml except where otherwise stated. The enzymatic digestion was followed by determining the hyperchromic shift at 260 nm. Aliquots of the incubation mixture were pipetted off at different digestion times and immediately shaken with 0.1 volume of chloroform—isoamyl alcohol (5:1 or 24:1, v/v) to inactivate the enzyme. After the phase separation had taken place, the supernatants were pipetted off and kept frozen until use.

Acid phosphomonoesterase B [16] (this enzyme was previously referred to as acid phosphomonoesterase II) was used to dephosphorylate the acid DNAase digests. 0.03—0.5 units/ml were used. Digestion times were 3—15 h; incubation temperatures were 22 or 37 °C. No exonuclease activity could be detected under much more stringent conditions. Inactivation of the enzyme was done as for acid DNAase.

Acid exonuclease [17] was used at a concentration of 0.1—0.5 units/ml. Digestion was followed by determining the hyperchromic shift at 260 nm. The mixtures of nucleotides and terminal nucleosides were kept in the frozen state until analyzed.

DEAE-cellulose-urea chromatography was performed according to Tomlinson and Tener [18].

Average chain length or average degree of polymerization, $\bar{P}_n$, of the oligonucleotides obtained by acid DNAase digestion. Dephosphorylated oligonucleotides were digested with acid exonuclease; acid DNAase was also added when the average chain length was higher than 15 in order to decrease the size and cause melting before exonuclease digestion. The digests were then loaded on DEAE-cellulose columns. 150 A_{271} units of late digests (average size lower than 20) were loaded on columns (1.3×15 cm); alternatively, 500 A_{271} units of early digests (average size higher than 20) were loaded on larger columns (2.8×15 cm). Nucleosides were washed through with water; nucleotides were eluted with 1 M ammonium acetate pH 5.5. After the new low-scale analytical methods [19,20] became available, 1.5 to 5 A_{271} units were used along with DEAE-cellulose columns (0.8×7 cm). Fractionated DEAE-cellulose was used in this case [5]. $\bar{P}_n$ was taken as $(A_{271}\ N_p + A_{271}\ N)/(A_{271}\ N)$ where N_p and N indicate nucleotides and (terminal) nucleosides, respectively.

Acid-soluble nucleotides were determined as already described [28].

Melting curves were determined using the automatic equipment of Rump *et al.* [22].

Analysis and termini of isostichs were determined according to Thiery *et al.* [5] on oligonucleotides prepared as described by Devillers-Thiery *et al.* [23].

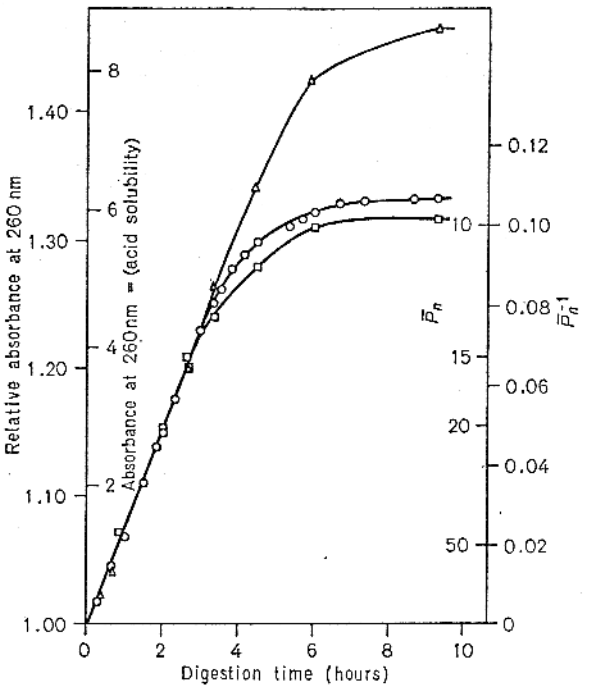

Fig. 1. *Hyperchromic shift (○), acid solubility (△, values corrected for dilution with perchloric acid) and reciprocal average size (□), $\bar{P}_n^{-1}$ of calf-thymus DNA during spleen DNAase digestion.* The values shown in this figure refer to the digestion of 1800 ml of DNA sample V 391, $A_{260} = 8.0$, in 0.05 M ammonium acetate—1 mM EDTA pH 5.5, at 23 °C with 0.3 ml spleen DNAase HS 21 a (1100 units/ml). The average degree of polymerization of oligonucleotides, $\bar{P}_n$, is given on the right-hand inner scale

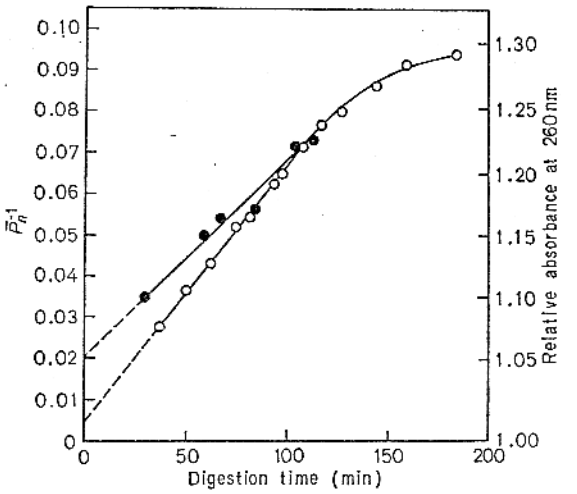

Fig. 2. *Reciprocal average size and hyperchromic shift of calf-thymus DNA during spleen DNAase digestion.* Open circles show $\bar{P}_n^{-1}$ values as obtained when dephosphorylation was done on undialyzed oligonucleotides: 100 ml CTR2 DNA, $A_{260} = 8.0$, in 0.05 M ammonium acetate—1 mM EDTA pH 5.5, was incubated at 22 °C with 0.045 ml spleen DNAase HS 25 (350 units/ml). Filled circles show $\bar{P}_n^{-1}$ values as determined when dephosphorylation was done after dialysis of oligonucleotides: CTR2 DNA, 190 ml, was incubated with 0.1 ml, of spleen DNAase HS 24 (250 units/ml). In this latter case, values were corrected for the small difference in the enzyme activity present in the two incubation mixtures on the basis of the hyperchromic shift caused by the enzyme preparations. Solvent and DNA concentration as in Fig. 1

RESULTS

The Middle and Terminal Phases of Spleen-DNAase Digestion

Fig. 1 shows the time course of hyperchromic shift, acid solubility and reciprocal average degree of polymerization, $\bar{P}_n^{-1}$, during spleen DNAase digestion of calf thymus DNA at 22 °C. After about 3 h of digestion, under the experimental conditions used, the three plots deviated from linearity showing a downward curvature. The intercept on the ordinate axis of the plot of $\bar{P}_n^{-1}$ *versus* digestion time appears to be equal to zero in Fig. 1. A positive intercept of the extrapolation to zero digestion time on the ordinate axis is, however, evident if one uses data obtained in degradations in which more size determinations were done in the early digestion times (Fig. 2).

Fig. 3 shows the evolution of hyperchromicity and acid solubility of spleen DNAase digests as obtained at 22 or 0 °C. The hyperchromic shift shows a linear relationship with $\bar{P}_n^{-1}$; a deviation from linearity takes place when the average size reaches the decanucleotide level. When determined after addition of urea to the digests, the hyperchromic shift line is only translated towards higher values of relative absorbance; in this case, however, the linear relationship is not obeyed for average sizes higher than 35. The solubility of oligonucleotides in 2% (final concentration), cold (0 °C) perchloric acid also shows a linear relationship with $\bar{P}_n^{-1}$. An upward curvature becomes evident, however, at the level of 12 nucleotides; at an average size of 10, a plateau is reached indicating that, at this point, the digest is completely acid soluble. The hyperchromic shift and acid-solubility curves have the same origin, at an average size of about 100 nucleotides, under the experimental conditions used. The liberation of tri- to pentanucleotides (not shown in the figure) also is linear with $\bar{P}_n^{-1}$, an upward curvature beginning at $\bar{P}_n = 10$; the origin of this line is at a $\bar{P}_n = 30$.

If digestion is performed at 0 instead of 22 °C, the relationships of hyperchromic shift and acid solubility *versus* reciprocal average size are modified in two respects: (a) the intercept on the abscissa axis of both straight lines is equal to 0.02 (Fig. 3), corresponding therefore to an average size of about 50 instead of 100, as found at 22 °C; (b) the linear region covers a wider $\bar{P}_n$ range (Fig. 3); at a $\bar{P}_n^{-1}$

value equal to 0.12 the slowing down of the reaction is not yet visible.

Fig. 4 shows the melting curves obtained with spleen DNAase digests having average sizes comprised between 46 and 16. These experiments were performed in 0.05 M ammonium acetate—1 mM EDTA pH 5.5, in order to obtain information on the structure of the oligonucleotides under our usual experimental conditions. As expected, the melting temperature, T_m and the hyperchromic shift decrease with decreasing size. The comparison of the melting curves obtained for the starting DNA and for DNA degraded to a $\bar{P}_n = 46$ shows that the enzymic degradation causes a destabilization and a lowering of the T_m before any change in hyperchromicity is apparent. The total hyperchromic shift (enzymatic plus heating) of the oligonucleotides was found to be equal to a constant value for all samples, indicating that the hyperchromic shift caused by the enzyme is essentially due to the melting of the fragments released and that the contribution of phosphodiester bond breakage is negligible in the range explored.

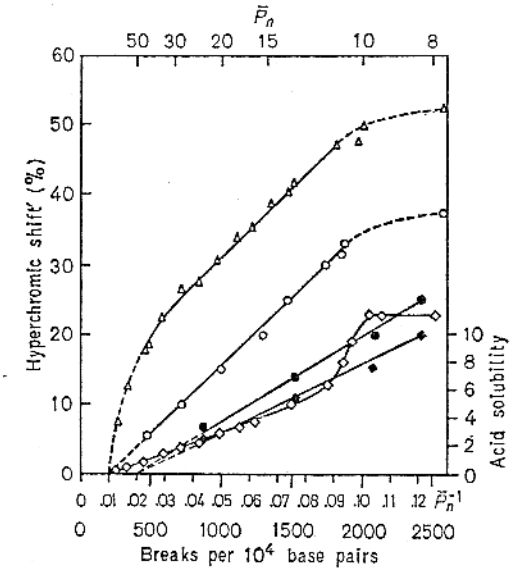

Fig. 3. *Plot of hyperchromic shifts in the absence and in the presence of urea (circles and triangles, respectively), acid solublity (lozenges) during spleen DNAase digestion at 22 °C and 0 °C (open and closed symbols, respectively)* versus *reciprocal average size of calf-thymus DNA*

The Isostich Pattern in the Middle and Early Terminal Phase of Digestion

The distribution of the oligonucleotides at different levels of degradation in the middle and early terminal phase of calf thymus DNA by spleen DNAase is given in Table 1. The chromatographic patterns of the hydrolysates on DEAE-cellulose-

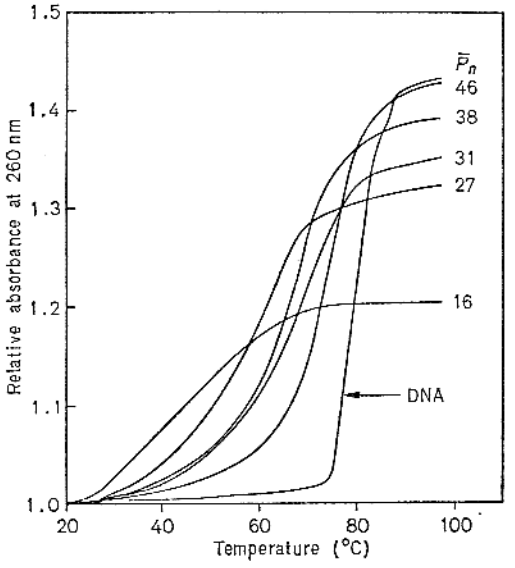

Fig. 4. *Ultraviolet melting curves of spleen DNAase digests of calf-thymus DNA (sample CTR 2) in 0.05 M ammonium acetate—1 mM EDTA pH 5.5.* The average sizes of the samples are shown in the figure. The melting curve of the starting DNA in the same solvent is shown for comparison. The values of absorbance at 260 nm of all samples used in these determinations were close to 0.6. The digest of $\bar{P}_n = 31$ showed the same T_m as that of $\bar{P}_n = 38$, and equal to 66 °C

Table 1. *Properties of spleen-DNAase digests obtained in the middle and early terminal phase of digestion*
Data refer to the isostich patterns shown in Fig. 5. The average size is as calculated from the hyperchromic shift of digests using the relationship shown in Fig. 3. Values for the isostichs are expressed as percentage A_{260} units recovered from the columns. In the case of digest A, values are given as percentage of A_{260} units of the loaded digest, as measured in 7 M urea. Fractions 1—5 are mono- to pentanucleotides. Fraction R is formed by the material eluted after the pentanucleotide peak

DNA digest	Hyperchromic shift at 260 nm	Average size, $\bar{P}_n$	Isostichs					
			1	2	3	4	5	R
			%	%	%	%	%	%
A	12.0	25.0	0.06	0.16	0.57	0.90	0.40	97.9
B	26.2	13.0	0.14	0.24	2.70	5.0	3.20	88.7
C	31.2	11.2	0.22	0.43	3.40	6.90	4.60	84.45
D	34.0	10.4	0.31	0.69	4.10	7.20	5.20	82.5
E	37.5	6.0	1.26	1.19	7.60	14.40	11.70	63.85

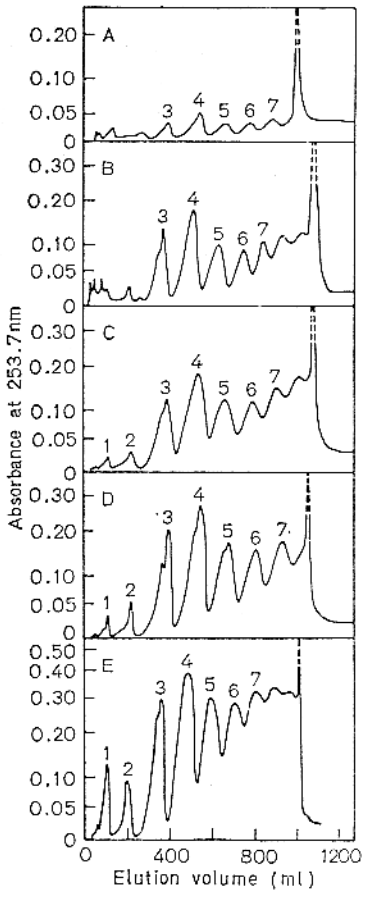

Fig. 5. *DEAE-cellulose-urea chromatography of spleen DNAase digests.* 1180 ml calf-thymus DNA (CTR 1; $A_{260} = 8.0$), were digested at 23 °C with 0.2 ml spleen DNAase (HS 21a) having an activity of 940 units/ml (see also Table 1). A second addition of enzyme (0.05 ml) was made after sample D was taken out (17 h); a third addition (0.1 ml) was made at 28 h of digestion, before sample E was taken out. 20-ml aliquots from the incubation mixture taken at 75 min, 150 min, 4 h, 17 h, 38 h (these are digests A to E shown above), were loaded on acetate-form DEAE-cellulose columns (1.25×20 cm) washed with water. Elution was performed at a flow rate of 52 ml/h with a molarity gradient (0.05—0.675 M) of ammonium acetate pH 5.5 containing 7 M urea. A step of 2 M ammonium acetate pH 5.5, containing 7 M urea was applied to the column as the elution of the octanucleotide peak began. A step of NaOH was made in the case of digest A, since 70% of the material was not eluted by the 2 M ammonium acetate step. The figure shows the absorbance at 253.7 nm of the column effluents as recorded by an LKB-Uvicord equipped with a 1-cm cell. The numbers indicate the size of the isostichs

urea columns are shown in Fig. 5. They are characterized by: (a) a very large fraction formed by unresolved material and poorly resolved peaks; the amount of this fraction regularly decreased from 98% in digest A (average size 25) to 64% in digest E (average size 6.0). Since the hexa- and heptanucleotides increased with digestion time, the decrease of the unresolved fraction occurred at the expenses of the large fragments; (b) a series of peaks formed by tri-, tetra- and pentanucleotides: these fractions increased in amount, as digestion proceeded, from about 2% to about 36% of the total digest; the tetranucleotide peak was predominant at all digestion stages; (c) mono- and dinucleotides formed only 0.2% of the total digest in hydrolysate A and increased to a maximum value of 2.5% in hydrolysate E.

The Liberation of Mono- and Dinucleotides

DEAE-cellulose-urea chromatography of spleen DNAase digests shows that mono- and dinucleotides are liberated at a much lower rate than tri- to pentanucleotides in the middle and early terminal stages of digestion. This prompted further investigations on their origin. The results obtained can be summarized as follows.

a) The report [24] that acid DNAase can split off the 3′-phosphate terminal nucleotide from trinucleotides produced by micrococcal nuclease and our finding that mono- and dinucleotides are present in digests A to D in a roughly stoichiometric ratio induced us to check whether spleen-DNAase-produced trinucleotides could be further split by the enzyme. In spite of the very large spleen DNAase concentration (1 unit/ml) and long digestion time (14 h at 37 °C) used, no degradation of trinucleotides (2.1 A_{271} units) could be detected indicating that only micrococcal-nuclease-produced trinucleotides can be split by acid DNAase, but not those produced by the latter enzyme.

b) The presence of mononucleotides in the digests and the different rates of liberation of mono- and dinucleotides compared to the higher oligonucleotides, prompted an extremely rigorous control of the exonuclease activity present in spleen DNAase. This was done in the following way. A crude nuclease II preparation, obtained from 80 kg of hog spleen according to Bernardi *et al.* [14], was adsorbed batchwise on CM-Sephadex C-50 equilibrated with 0.075 M potassium phosphate pH 6.8 and eluted with 0.4 M potassium phosphate buffer pH 6.8; this procedure removed most of the contaminant enzymes. The spleen DNAase preparation so obtained was submitted, in sequence, to a series of chromatographic purification on (a) carboxymethyl-Sephadex, (b) hydroxyapatite, (c) carboxymethl-Sephadex, (d) hydroxyapatite and (e) hydroxyapatite, using the experimental conditions of Bernardi *et al.* [14]. Fractions from both sides of the acid DNAase B peak obtained in each chromatogram were used to digest calf thymus DNA. The amounts of mono- to pentanucleotides found in each case are shown in Fig. 6. The relative amount of mononucleotides, still quite high in the digest obtained with the

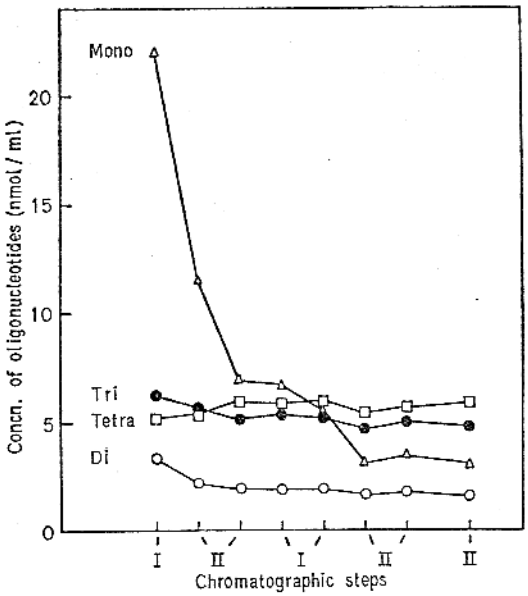

Fig. 6. *Concentrations of mono- to tetranucleotides found in calf-thymus DNA digests obtained by using spleen DNAase fractions from the subsequent chromatographic purifications indicated on the abscissa axis.* When two values are given for the same chromatography, they refer to fractions taken symmetrically at the left and the right-hand side of the chromatographic DNAase activity peak, respectively. In each case, 20-ml aliquots of DNA ($A_{260} = 8.0$) in 0.05 M ammonium acetate–1 mM EDTA pH 5.5, were incubated with 120 units spleen DNAase at 0 °C for 72 h, therefore under conditions allowing the terminal plateau to be reached. The hydrolysates were subsequently chromatographed on DEAE-cellulose-urea columns (1.25×20 cm). (I) Carboxymethyl-Sephadex and (II) hydroxyapatite columns

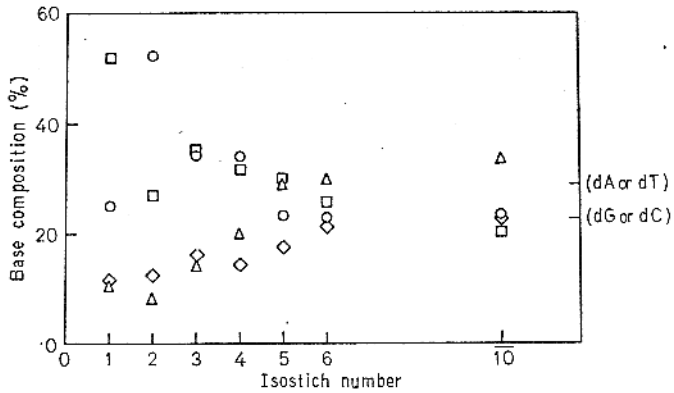

Fig. 7. *Analysis of base composition of spleen DNAase isostichs.* (See Materials and Methods for the experimental conditions used.) (△) Thymidine, (○) deoxyadenosine, (□) deoxyguanosine and (◇) deoxycytidine; the (dA + dT) and (dG + dC) levels, respectively, of calf-thymus DNA are shown on the righthand ordinate

Table 2. *Analysis of mononucleotides present in spleen DNAase digests*
The results are given in molar fractions. For digests D and E, see Table 1; results for 3′-phosphate terminals are as obtained in the middle digestion phase [5]

DNAase digests	Deoxy-guanosine	Deoxy-adenosine	Thymidine	Deoxy-cytidine
Digest D	41.4	34.3	15.6	8.5
Digest E	46.0	38.0	8.5	6.9
3′-Phosphate terminals	45.0	28.0	18.5	8.5

enzyme from the first carboxymethyl-Sephadex step, strongly decreased in the hydrolysate obtained with the enzyme from the first hydroxyapatite column; in this case more mononucleotides were produced by early-eluting fractions compared to the late-eluting ones, a finding which may be correlated with the lower eluting molarity of spleen exonuclease compared to spleen DNAase; the decrease of mononucleotides liberated by enzymatic fractions from subsequent chromatograms leveled off to reach a constant ratio to the other isostichs. This result suggests that the residual, extremely weak, exonucleolytic activity is an intrinsic activity of the enzyme. Additional evidence in favor of this conclusion will be presented below. Incidentally, this DNAase preparation, which had gone through so many purification steps, was found to be active on bis(*p*-nitrophenyl) phosphate, as expected on the basis of the fact that this is also an intrinsic activity of the enzyme [15, 21]. A ratio of 0.04 phosphodiesterase units/DNAase unit was found fo this DNAase preparation (the activity on the synthetic substrate was measured on 10 mM bis-(*p*-nitrophenyl) phosphate; see [25] for the definition of the phosphodiesterase unit).

b) The composition of mononucleotides was found to be rather similar to that of the 3′-phosphate terminal nucleotides released during the middle phase (Table 2).

Base Composition and Termini of the Isostichs

Fig. 7 shows the base composition of the different oligonucleotide classes obtained from an acid DNAase hydrolysate of average size 6.5. Small fragments (mono to tetranucleotides) show a very large scatter in their composition. This tends to become more uniform as the size of isostichs increases. The base composition of fragments having a size higher than 6, which form 64 % of the total digest and have an average size of 10, are higher in thymine and lower in adenine compared to the base composition of calf thymus DNA.

Fig. 8 shows the 3′-phosphate terminal (Fig. 8 A), the 5′-hydroxy terminal (Fig. 8 B) and penultimate nucleotides (Fig. 8 C), as determined on the spleen DNAase isostichs. In contrast with the termini of

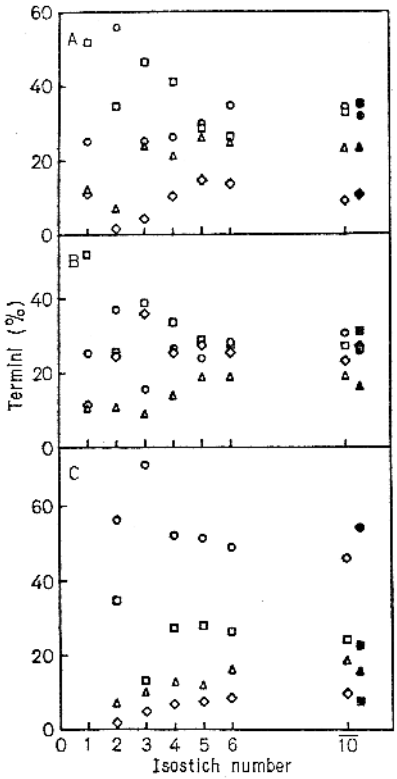

Fig. 8. *Analysis of termini in isostichs of calf-thymus DNA obtained after spleen DNAase digestion.* (A) 3′-P terminals, (B) 5′-OH terminals and (C) 5′-OH penultimates. Symbols used are those of Fig. 7. Filled-in symbols show the composition of the unfractionated hydrolysate. Mononucleotides are presented in (A) and (B). 3′-Terminals of dinucleoside monophosphate are presented in (A) and (C), 5′-terminals are presented in (B)

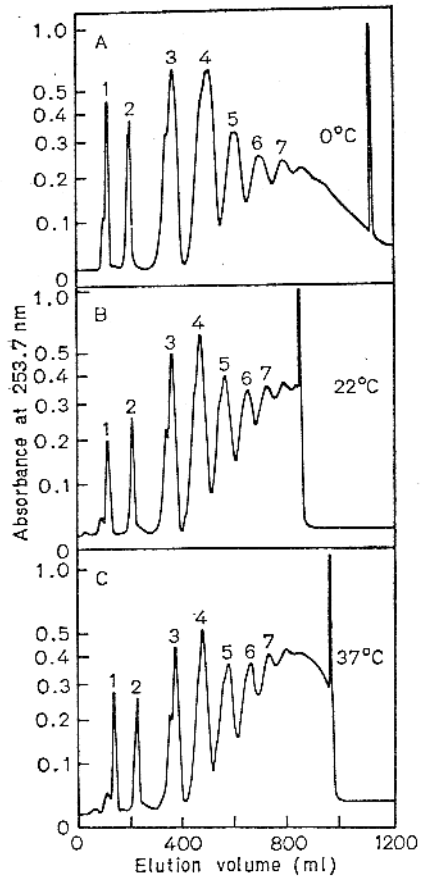

Fig. 9. *DEAE-cellulose-urea chromatograms of acid DNAase digests obtained at 0 °C (A), 22 °C (B) and 37 °C (C), at the level of the terminal plateau.* See footnotes of Table 3 for the experimental conditions used

tetranucleotides and higher fragments, the termini of di- and trinucleotides are quite different in their composition from those of the unfractionated hydrolysate.

The Isostich Pattern in the Late Terminal Phase of Digestion

The distribution of the oligonucleotides was also investigated after very prolonged digestion with larger amounts of enzyme, in the late terminal phase in which no further hyperchromic shift can be detected. The results obtained are shown in Table 3; they indicate that an extremely slow decrease of the average size of the oligonucleotides and a correspondingly small increase in the amounts of mono- to pentanucleotides takes place. Because of the practical invariance of its isostich pattern, it is convenient to use the late terminal phase of the digestion in order to check several effects on acid DNAase digestion.

The Effect of Digestion Temperature on the Isostich Pattern

The DEAE-cellulose-urea chromatograms of acid DNAase digests obtained at 0, 22 and 37 °C at the level of the terminal plateau of hyperchromic shift show that the relative amounts of tri- and tetranucleotides strongly increase when incubation temperature is decreased; pentanucleotides practically do not vary in amount and higher isostichs actually decrease in quantity. Mononucleotides and dinucleotides do not vary at 22 and 37 °C, but they increase at 0 °C (Fig. 9).

Effect of Dephosphorylation on the Isostich Pattern

Dephosphorylation of the digest during spleen DNAase degradation leads to increased amounts of di- to pentanucleotides and to a decrease of mononucleotides and of the higher isostichs. In digests obtained at 0 °C, the percentage of tri- plus tetranucleotides reaches 42% of the digest

Table 3. *Properties of spleen-DNAase digests obtained at 0, 22 and 37 °C in the terminal phase of digestion*
The digests were obtained as follows: Digests A_{1-4}: 100 ml DNA sample CTR 2 in 0.05 M ammonium acetate—1 mM EDTA pH 5.5, were digested at 22 °C with 50 units spleen DNAase HS 25. At the times indicated 25-ml aliquots were taken; 20-ml aliquots were used for DEAE-cellulose-urea column chromatography; 5-ml aliquots were used for chain length and 3′-*P*-terminal nucleotide determination. Digests B_{1-4}: 140 ml DNA sample CTR 2 in 0.05 M ammonium acetate—1 mM EDTA pH 5.5, were digested at 0 °C with 875 units spleen DNAase HS 24. 25-ml aliquots were taken at times indicated. 20-ml aliquots were used for DEAE-cellulose-urea column chromatography; 5-ml aliquots were used for chain and 3′-*P*-terminal nucleotide determination. In digest B_1* the digestion was stopped with chloroform-isoamylalcohol; the sample was further kept in its solvent for another 38 h. Digest C: 0.2 ml DNA sample CTR 2, in 0.05 M ammonium acetate—1 mM EDTA pH 5.5, (8.0 A_{230} units) were digested at 0 °C with 4.8 units acid DNAase HS 24 and 0.2 units acid phosphomonoesterase B. The sample was then diluted twice and loaded on a DEAE-cellulose-urea column. The average chain length was not determined. Digest D: 21-ml DNA sample CTR 2 were digested at 37 °C with 37 units spleen DNAase HS 25

DNA digests	Digestion time	Hyperchromic shift at 260 nm	Average size	Isostichs					
				1	2	3	4	5	R
	h			%	%	%	%	%	%
A_1	32	39	5.95	1.20	1.36	7.28	13.02	10.58	66.54
A_2	45	39	5.68	1.34	1.47	7.68	14.75	10.53	64.15
A_3	58	39	5.60	1.79	1.57	8.30	14.17	11.63	62.68
A_4	72	39	5.36	1.68	1.63	8.02	14.36	10.92	63.39
B_1	36	35	4.84	2.23	2.75	13.13	19.40	11.78	50.55
B_2	48	35	4.69	3.09	3.0	13.14	20.79	12.61	47.46
B_3	60	35	4.57	2.89	3.56	14.84	20.79	13.07	44.65
B_4	74	35	4.41	3.41	3.35	15.52	21.37	13.0	43.34
B_1*	36	35	—	2.40	3.04	13.13	20.10	12.96	48.37
C	72	—	—	2.2	4.4	17.3	25.0	15.8	35.6
D	9	40	—	1.78	1.32	6.10	10.51	11.28	69.0

(the tetranucleotides representing 25%), as opposed to 33% in the absence of dephosphorylation (Table 3).

DISCUSSION

The Kinetics of Spleen-DNAase Digestion

Initial Phase. The results of Fig. 2 indicate that the extrapolation of the $\bar{P}_n^{-1}$ plot of DNA hydrolysates *versus* digestion time has a positive intercept on the ordinate axis having a value of about 0.02, corresponding to an average size of 50. Since the $\bar{P}_n^{-1}$ value of the initial DNA is very close to zero, it should be concluded that phosphodiester bond breakage proceeds at a faster rate at the beginning of the reaction than later on. These data may be interpreted as follows: initially a particular class of sequences is split at a faster rate than the sequences split during most of the degradation; since the rate in the initial phase is decreasing, one should think that these "preferred" sequences are progressively exhausted in this phase. The intercept on the ordinate axis gives an estimate of the amount of these preferred sequences; assuming a $\bar{P}_n^{-1}$ value of 0.25 as that of the final hydrolysate (Table 3) an intercept of 0.02 indicates that the preferred sequences correspond to about 8% of all susceptible sequences. Now, it is known [1—4] that spleen DNAase degrades DNA according to both a diplotomic (double breakage) and a haplotomic (single breakage) mechanism. Since it is known that the ratio of diplotomic to haplotomic splitting rates is decreasing in the initial phase [26, 27] and since it seems that the initial rate of diplotomic mechanism is higher than that of the haplotomic one [28], it is tempting to identify the preferred sequences as those which are split by the diplotomic mechanism. Further comments on this point will be presented in a following paper [5].

Middle Phase. The linear relationship found to hold between $\bar{P}_n^{-1}$ and digestion time is due to the fact that, during this phase the enzyme is splitting, at a linear rate, the substrate present in the incubation mixture in a largely saturating concentration. The linearity of the hyperchromic shift and acid solubility of the fragments with time is a consequence of their linear relationships with $\bar{P}_n^{-1}$ (Fig. 3). Concerning the conventional limit between the middle and the terminal phase, if this is taken, as indicated above, at the end of the linear phase, it corresponds, under the experimental conditions of Fig. 1, to an average size of 15.

Terminal Phase. It has already been shown [29] that the characteristic slowing down of the reaction rate at the beginning of the terminal phase is not due to enzyme inactivation, nor to end-product inhibition, the latter being very weak, though certainly playing an increasing role as digestion proceeds through the terminal phase (see below). Another explanation which can be ruled out now is a true substrate exhaustion, since a large number of susceptible sequences are still available at the beginning of this phase; very interestingly, the splitting of these sequences during the terminal

phase releases termini having a composition which does not vary with digestion time [5].

The essential reason for the decrease in the reaction rate early in the terminal phase appears to be the progressive melting of the double-stranded DNA fragments, which takes place when the average size of hydrolysate reaches a threshold range of values (Fig. 4). The resulting single-stranded fragments, though still containing a very large number of susceptible sequences, as just mentioned, are a poor substrate for an enzyme which shows a marked preference for native *versus* denatured DNA [21] and which binds inhibitory double-stranded polyribonucleotides with antiparallel strands, much better than single-stranded ones [30]. The finding that the slowing down of the reaction takes place at lower $\overline{P}_n$ values when digestion is performed at 0 °C instead of 22 °C (Fig. 3), and the effect of temperatures on the isostichs pattern (see below) are in agreement with the explanation given above. A finding in apparent contradiction with it is the linearity of the $\overline{P}_n^{-1}$ *versus* time plot in a size range (30 to 15; Fig. 1) where double-stranded fragments already melt. In fact, at a size level of 16, no more than 50% of the double-stranded fragments have melted (Fig. 4), whereas, at enzyme concentrations like that of the experiment of Fig. 1, initial velocities for DNA concentrations ranging from 8.0 to 0.2 A_{260} units were found to be identical, indicating that a concentration equal to only 5% of that normally used is still saturating for the enzyme. This observation underlines the rapidity of the decrease in the concentration of double-stranded fragments in the average size range just below 16.

The present interpretation of the well-known sharp decrease in the reaction rate of DNAases [31] is radically different from that given by Laskowski [32], who considers that "autoretardation", (as he calls this rate decrease) is due to the fact that the smaller and smaller fragments formed are "progressively more resistant substrates", thus missing the transitional character of this phenomenon and the difference existing between a true substrate exhaustion and the exhaustion of a preferred substrate.

Relationships between Hyperchromic Shift, Acid Solubility and $\overline{P}_n^{-1}$

a) The data of Fig. 3 show that hyperchromic shift and acid solubility of the digest are linear functions of the reciprocal average size of the oligonucleotides, or, in other words, of the number of end groups released by the enzyme. In the case of calf thymus DNA the empirical equation permitting to calculate the reciprocal average size, $\overline{P}_n^{-1}$, from the measured hyperchromic shift (as a percentage) is the following:

$$\overline{P}_n^{-1} = 0.24 \times \text{shift} + 0.019.$$

Similar relationships were determined for bacterial DNAs:

$$\overline{P}_n^{-1} = 0.25 \times \text{shift} + 0.013 \ (\textit{H. influenzae} \text{ DNA})$$

$$\overline{P}_n^{-1} = 0.30 \times \text{shift} + 0.013 \ (\textit{E. coli} \text{ DNA})$$

$$\overline{P}_n^{-1} = 0.48 \times \text{shift} + 0.008 \ (\textit{M. luteus} \text{ DNA}).$$

A more general equation, valid in all above cases, is the following:

$$\overline{P}_n^{-1} = 0.65 \times \text{shift} \times \%\,(\text{dG} + \text{dC}) + 0.01.$$

All equations apply to hyperchromic shifts measured under the experimental conditions used in the present work. From a practical point of view, these equations are extremely useful in that they permit estimates of the average size of the digests without end-group determination to be obtained. As will be shown later [6], these equations also apply to another enzyme, the acid DNAase from snail hepatopancreas.

b) The straight lines representing the hyperchromicity and acid solubility of calf thymus DNA, respectively, as functions of $\overline{P}_n^{-1}$ cut the abscissa axis at a $\overline{P}_n^{-1}$ value corresponding to a chain length of 100 when digestion is performed at 22 °C. Clearly, under the experimental conditions used, this chain length value corresponds to the limit between the initial and the middle phase. It may be interesting to remark here that the sedimentation constant of calf thymus DNA degraded with spleen DNAase to a hyperchromic shift of 1%, therefore to a point where the average (single-stranded) degree of polymerization is still very close to 100 (corresponding to a single-stranded number-average molecular weight of about 30000), was found to be equal to 6 S; this value corresponds to a (double-stranded) weight-average molecular weight close to 200000 [28], in fair agreement with a previous estimate [33], and indicates the presence of nicks in these fragments. It should also be mentioned here that the limit between the initial and middle phase, as defined above, can also be detected by chromatography on hydroxyapatite columns. DNA samples showing the initial small percentage hyperchromicity or acid solubility, also show a small percentage of material eluting at a lower phosphate molarity than the main DNA peak.

c) The extrapolation of the tri- to pentanucleotide liberation to a single-stranded size of about 30 indicates that small fragments are not released from large fragments, but only set free when the average size of the hydrolysate is quite low. Similar conclusions may be drawn by considering the evolution of the isostich distribution during the enzymatic digestion (see below).

d) A deviation from the linear relationships with reciprocal average size takes place at average sizes of 10 and 12 for hyperchromic shift and acid solubility, respectively. When the average nucleotide

size becomes lower than 10, the hyperchromic shift caused by phosphodiester bond breakage decreases, apparently because hyperchromicity increasingly arises from the breakdown of single-stranded instead of double-stranded structures (see the melting curves of Fig. 4). On the other hand, an average size of 12 seems to represent a value beyond which additional breaks are more effective in causing the oligonucleotides to become acid soluble, a fact which is understandable if one thinks that acid solubility begins at a certain size threshold; in addition, at this digestion level the enzyme accumulates hits on the remaining large, double-stranded fragments. At an average size of 10, the digest becomes completely acid soluble under the conditions used and a final plateau of acid solubility is reached.

The data of Fig. 3 show that, in the linear phase, 100 breaks per 10^4 base pairs cause a hyperchromic shift of 2% at 22 °C and of only 1.25% at 0 °C. This is in agreement with the stabilizing effect of low temperature on the double helix and confirms the conclusion derived from the melting curves that hyperchromicity during enzymatic degradation arises from fragment melting rather than from base unstacking due to bond splitting. The destabilization effect of urea explains the larger hyperchromic shift seen with large fragments. Also in agreement with the expectations is the fact that the absorbance of the acid-soluble fragments is the same at both temperatures, 0.44 A_{260} units per 100 bonds broken, under the experimental conditions used.

If one compares now the intercepts on the abscissa axis of hyperchromic shifts and acid solubilities, as determined at the two temperatures, one finds that the intercept of hyperchromic shift corresponds to a $\overline{P}_n$ value of 100 at 22 °C, but only of 50 at 0 °C (Fig. 3). This means that the hyperchromic shift begins at a lower average size at the lower temperature, again as expected. In contrast, a surprising finding is the fact that the intercept of acid solubility also corresponds to a $\overline{P}_n$ value of 100 at 22 °C and of 50 at 0 °C. The only explanation we can see for this phenomenon is that the ratio of diplotomic over haplotomic breaks is larger at 0 °C compared to 22 °C; in this case, at the same level of total bonds broken, the number of fragments formed having a size below the acid-insolubility limit, would be lower at 0 °C than at 22 °C.

Evolution of the Oligonucleotide Distribution during Spleen-DNAase Degradation

The time course of the isostich pattern of a spleen DNAase hydrolysate is characterized by a regular shift to the left of the DEAE-cellulose-urea chromatogram. This takes place during the middle and early terminal phases of the digestion. In the terminal phase a further evolution of the digest still occurs so that an end point is not reached, but the increase in the amounts of resolved isostichs is extremely small. Expectedly, no further increase in ultraviolet absorption of the digest can be detected at the end of this phase.

The results concerning the mono- and dinucleotides call for several comments. Mononucleotides appear to be formed by a very weak, intrinsic, exonucleolytic activity of spleen DNAase. Very interestingly, their liberation is increased when digestion is performed at 0 °C, under conditions of better preservation of double strandedness of fragments and decreased when the spleen DNAase hydrolysate is submitted to simultaneous digestion with spleen phosphomonoesterase, two phenomena putting this activity in a class different from both spleen and venom exonucleases. The similarity of the composition of mononucleotides with that of 3′-phosphate terminal nucleotides released during the middle phase suggests that they derive from the 3′-ends of double-stranded DNA fragments. Like mononucleotides, dinucleotides are released faster when digestion is performed at 0 °C; they are, however, very little affected by terminal dephosphorylation.

The effect of temperature on the distribution of the oligonucleotides in the late terminal phase is very interesting since it shows that digestion can proceed further if temperature is lowered. It is very likely that this temperature effect is due to the fact that the double-stranded structure of DNA fragments is better preserved at low temperature than at a high temperature thus permitting a higher concentration of "good" substrate late in the digestion.

The effect of dephosphorylation is very interesting in two respects: (a) it shows that phosphorylated oligonucleotides exert a weak yet measurable inhibition, probably of the competitive type, on the enzyme; (b) it leads to a decrease in liberation of mononucleotides.

Composition and Termini of Oligonucleotide Isostichs Produced by Spleen DNAase

The finding that the composition of the smaller fragments shows a great scatter whereas that of the larger ones becomes closer to the composition of the DNA as their sizes increase can be understood in terms of the decreasing influence of the termini (whose composition deviates from the average DNA composition) on the composition of the fragments as their sizes increase. The observed higher level of thymidine in the large fragments liberated by spleen DNAase is not surprising in view of the fact that this nucleoside is relatively low in all termini.

The termini found in different isostichs are expected to show some fluctuations just on statistical grounds. The termini of fragments smaller than tetranucleotides show much larger fluctuations in their composition than those of larger isostichs. A possible explanation for this phenomenon is the influence of the terminal phosphate or hydroxyl groups on spleen DNAase, so that only particular nucleotide sequences can be split. The 3′-terminal nucleotides of fragments show a constant decrease in deoxyguanosine, with increasing size; this is accompanied by a slight increase of the other three bases. The composition of mononucleotides fits well with these trends, further supporting the idea that their origin may be the 3′-terminal ends of oligonucleotides. The 3′-terminal nucleotides of dinucleotides, on the contrary, shows a rather important deviation from these trends and fit well with the trend of the 5′-penultimate nucleotides, suggesting that their origin is the 5′-terminal end of oligonucleotides.

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