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Composition of the 3′-Phosphate Penultimate Nucleotides Released from Calf-Thymus DNA by Spleen Acid DNAase

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The composition of the 3′-phosphate penultimate nucleotide liberated by spleen acid DNAase was determined using two different experimental approaches involving the use of pancreatic DNAase, in the presence of Mn^{2+}, to degrade the oligonucleotide released by the former enzyme. The results obtained using both approaches indicate that the 3′-phosphate penultimate nucleotide is highly specific. Since previous work had already shown that the 3′-phosphate terminal and the 5′-hydroxy terminal and penultimate nucleotides also have compositions which very significantly deviate from those expected on the basis of a non-specific attack, the conclusion is drawn that spleen DNAase recognizes sequences at least four nucleotides long.

The main purpose of the present work was to determine the composition of the 3′-phosphate penultimate nucleotide (W in the scheme of Fig. 1), in oligonuleotides released by acid DNAase from hog spleen. Previous work from our laboratory led us to the isolation and analysis of the 3′-phosphate terminal nucleotide (X of Fig. 1 [1]) and of the 5′-hydroxy terminal and penultimate nucleotides (Y and Z of Fig. 1 [2]). The composition of these termini[1] was found to be very far from that expected on the basis of a random breakage of DNA [2, 3], a result indicating that spleen DNAase could recognize sequences at least three-nucleotides long in native DNA. Under these circumstances, it appeared interesting to us to investigate the composition of the 3′-phosphate penultimate nucleotides.

The isolation and analysis of this terminus was done using two different approaches. The first one (Fig. 2) was to split the spleen DNAase-produced oligonucleotides with pancreatic DNAase in the presence of Mn^{2+}; the dinucleoside triphosphates, which represent about half of the products originated from the 3′-phosphate ends of the fragments, were dephosphorylated and split with spleen exonuclease; the 3′-phosphate penultimate nucleotides, liberated as nucleotides, were isolated and analyzed. The second approach consisted in splitting with pancreatic DNAase, in the presence of Mn^{2+} (Fig. 3) the tetranucleotides formed by spleen DNAase. The doublets were then isolated, digested with spleen exonuclease and analyzed. The results obtained using both approaches indicate that the 3′-phosphate penultimate nucleotide is highly specific and resembles in composition the 5′-hydroxy penultimate nucleotide, thus leading to the conclusion that spleen DNAase recognizes a sequence of at least four nucleotides.

Abbreviations. $\bar{P}_n$, average degree of polymerization, average size or average chain length of oligonucleotides; QAE-Sephadex, quaternary diethyl-(2-hydroxypropyl)-aminoethyl-Sephadex.

Enzymes. Deoxyribonuclease (EC 3.1.4.5); acid deoxyribonuclease or deoxyribonucleate 3′-nucleotidohydrolase (EC 3.1.4.6); snake venom and spleen acid exonuclease, *E. coli* exonuclease I or orthophosphoric diester phosphohydrolase (EC 3.1.4.1); acid phosphomonoesterase or orthophosphoric monoester phosphohydrolase (EC 3.1.3.2).

Definitions. A_{271} unit, the quantity of material contained in 1 ml of a solution which has an absorbance of 1 at 271 nm, when measured in a 1-cm cell; 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; isostichs are oligonucleotide size groups, regardless of their composition [14].

[1] With this term we designate, conventionally, the 3′-phosphate and the 5′-hydroxy terminal and penultimate nucleotides; these termini will also be indicated as the 3′ and the 5′-terminal doublets, respectively.

MATERIALS AND METHODS

Pancreatic DNAase Digestion of Acid DNAase Hydrolysates

A spleen DNAase hydrolysate of calf thymus DNA (sample V 391 see Soave *et al.* [4]), having an average size, $\bar{P}_n = 14$, was dialyzed against running distilled water [2]. Aliquots of the dialyzed

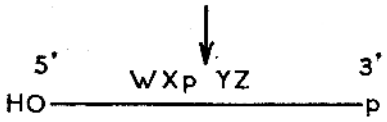

Fig. 1. *Splitting of DNA by acid DNAase.* See text for details

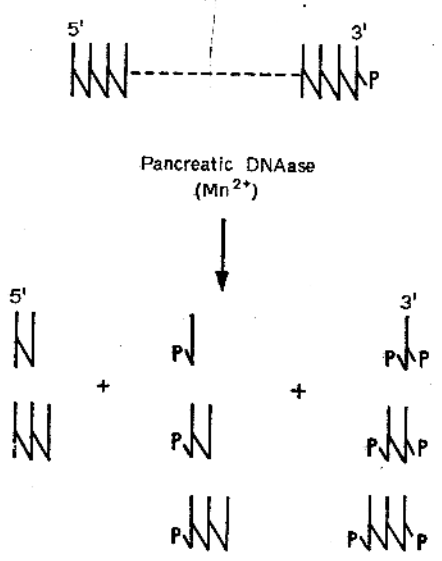

Fig. 2. *Products formed by digestion of acid DNAase oligonucleotides with pancreatic DNAase in the presence of Mn^{2+}.* The amount of mononucleotides formed from the inner sequences of the fragments is very small (0.7% of the total digest)

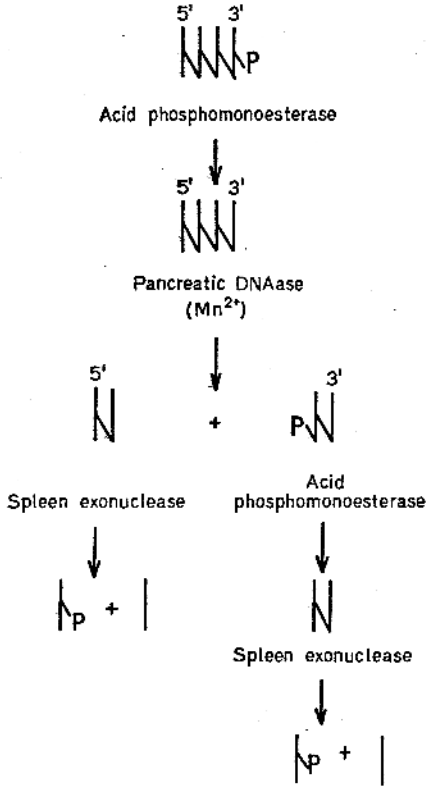

Fig. 3. *Determination of the 5'-hydroxy and the 3'-phosphate terminal doublets of tetranucleotides formed by acid DNAase*

oligonucleotides were adjusted with 0.1 N $(NH_4)OH$ to five different values in the pH 6—8 range and made 5 mM in $MgCl_2$ or $MnCl_2$; alternatively, both $MgCl_2$ and $MnCl_2$ were added, the concentration of each cation being 2.5 or 12.5 mM (in some cases up to 50 mM ammonium acetate was also present to increase the ionic strength). Each sample (11 A_{271} units in 2 ml) was digested at 37 °C with 0.6 mg/ml pancreatic DNAase (code D, Worthington, Freehold, N.J.); in one case, temperature was 45 °C, the pH being 7.0 and the concentration of $MgCl_2$ and $MnCl_2$ being 2.5 mM each. The pH was kept constant during digestions by addition of 0.1 N $(NH_4)OH$. After 1 h of digestion, samples were emulsified with chloroform—isoamyl alcohol mixture (24:1, v/v) to inactivate the enzyme. The average size of each sample was determined as described by Soave *et al.* [4]. The $\bar{P}_n$ value was found to be close to 4 in the hydrolysates obtained in the presence of 5 mM Mg^{2+}, in agreement with previous results [5]. In contrast, $\bar{P}_n$ values of only 2 ± 0.3 (or close to 2.7 when the ionic strength was higher) were found in all other hydrolysates, which were obtained in the presence of Mn^{2+}, a result also in agreement with those already reported [6].

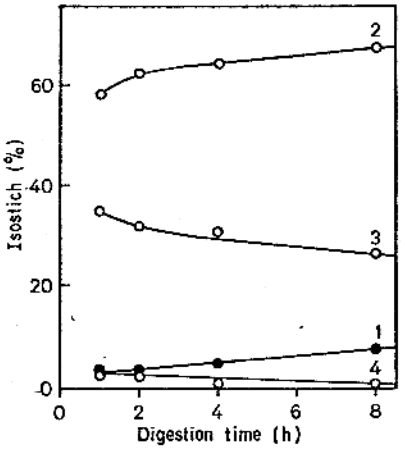

Fig. 4. *Kinetics of liberation of oligonucleotides by pancreatic DNAase.* 1—4 indicate mono- to tetranucleotides, respectively

The kinetics of pancreatic DNAase degradation in the presence of Mn^{2+} was investigated (Fig. 4). A water-dialyzed spleen DNAase digest (20 ml, 154 A_{260} units and $\bar{P}_n = 30$) was incubated with 0.6 mg/ml pancreatic DNAase at pH 7.0, in the presence of 2.5 mM $MnCl_2$; 5-ml aliquots were taken

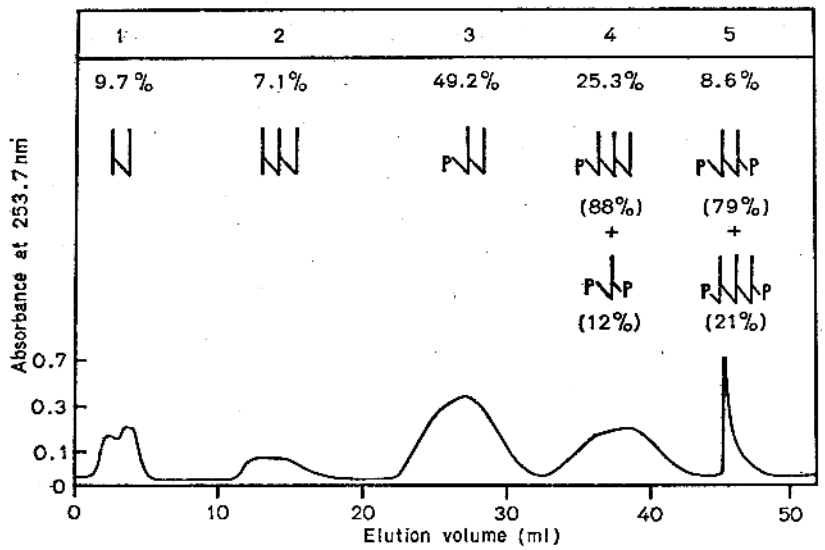

Fig. 5. *Elution profile of an acid DNAase hydrolysate digested by pancreatic DNAase.* The pancreatic DNAase digest (25 A_{260} units) was resolved on a DEAE-cellulose-urea column (0.5×8 cm) using a 0—0.3 M linear gradient of ammonium acetate in 7 M urea (total volume: 70 ml). Flow-rate was 6 ml/h. After the elution of peak 4, column was washed with water; 1 M ammonium acetate was subsequently applied in order to elute the peak 5. Numbers show the percentage A_{271} units recovered in each peak. The percentages of components in each peak are given in parentheses

at different digestion times between 1 and 8 h. After inactivation of the enzyme with chloroform-isoamyl alcohol, oligonucleotides were dephosphorylated and chromatographed on a DEAE-cellulose-urea column in order to resolve mono- to tetranucleotides.

On the basis of the results obtained, a water-dialyzed spleen DNAase hydrolysate ($\overline{P}_n = 14$), was digested for 3 h at 37 °C in the presence of 2.5 mM $MnCl_2$ and 1 mM ammonium acetate pH 7, with 0.6 mg/ml pancreatic DNAase. The products of pancreatic DNAase digestion were resolved on a DEAE-cellulose-urea column (see Results and Fig. 5) and treated as follows: (a) dinucleoside monophosphates (peak 1 of the chromatogram) were desalted and analyzed as described by Ehrlich *et al.* [2] for their hydrolysate II; (b) the identification of components present in peaks 2 and 3 were done on the basis of their elution position and their relative amounts in the digest; (c) trinucleotides and nucleoside diphosphates (peak 4 of the chromatogram) were diluted 10 times and desalted by readsorption on DEAE-cellulose, elution with 1.0 M ammonium acetate [7] and freeze-drying. After dephosphorylation, nucleosides were separated from trinucleoside diphosphates on a DEAE-cellulose column (0.4×4 cm) and analyzed on a BioGel P-2 column [8]. (d) dinucleoside triphosphates and trinucleoside tetraphosphates (peak 5 of the chromatogram) were freeze-dried, dephosphorylated and loaded on a DEAE-cellulose column (0.5×8 cm). The resulting dinucleoside monophosphates were eluted with a linear ammonium acetate gradient (0—0.2 M; total volume: 20 ml; flow rate: 6 ml/h) and the rest was eluted with a 1-M-ammonium-acetate step. Dinucleoside monophosphates were analyzed as indicated above.

Preparation of Oligonucleotides Produced by Spleen-DNAase Digestion

112 ml calf thymus DNA CTR 2 were digested at 23 °C with spleen acid DNAase B, using the experimental conditions described by Soave *et al.* [4], up to a hyperchromic shift of 37.5% (the average degree of polymerization, $\overline{P}_n$, of the digest was 6.5). After inactivation of the enzyme, the digest was dephosphorylated with spleen acid phosphomonoesterase B (0.1 unit/ml) at room temperature for 19 h.

DEAE-Cellulose-Urea Chromatography of Dephosphorylated Spleen-DNAase Digest

The dephosphorylated digest was slightly diluted (9.95 A_{260} units) and loaded on a DEAE-cellulose column (1.6×25.5 cm). A flow rate of 120 ml/h was used. The column was first washed with water to elute the nucleosides and then equilibrated with 7 M urea; oligonucleotides were then eluted with a 0—0.675 M ammonium acetate linear gradient in 7 M urea (total volume, 8 l). 19-ml fractions were collected and pooled together to provide the isostichs.

Desalting of Oligonucleotides. 250-ml aliquots were loaded on a BioGel P-2 column (6×48.5 cm,

minus 400 mesh; BioRad, Richmond, Calif.) equilibrated with 2 mM $(NH_4)_2CO_3$ pH 10.4. Elution was performed with the same buffer at a flow rate of 230 ml/h. Oligonucleotides were freeze-dried, dissolved in a small volume of water and purified by rechromatography on DEAE-cellulose columns (no urea was used).

Digestion of Spleen DNAase-Produced Tetranucleotides by Pancreatic DNAase

2.6 A_{260} units dephosphorylated tetranucleotides were digested with 0.6 mg/ml pancreatic DNAase (code D, Worthington, Freehold, N.J.) in 2.5 mM $MnCl_2$ for 2 h at 37 °C. The pH of the incubation mixture was maintained close to 7 by addition of 0.1 N NH_4OH.

The pancreatic DNAase digests were loaded on columns (0.5×10 cm) of QAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden: only particles less than 50 μm diameter, obtained by sieving, were used). Elution of dinucleoside monophosphates and dinucleotides, which were the only products formed, was done with a 0—1 M linear ammonium acetate gradient pH 6 (total volume: 80 ml, flow rate 6 ml/h). These products were freeze-dried, dephosphorylated (in the case of dinucleotides) and analyzed as described by Thiery *et al.* [3].

Digestion of Acid DNAase-Produced Tetranucleotides by Venom and Spleen Exonuclease

5′-OH terminal and penultimate nucleotides of dephosphorylated tetranucleotides were determined by venom exonuclease digestion as described by Thiery *et al.* [3] (method II).

3′-Phosphate terminal nucleotides were determined according to Thiery *et al.* [3]. 3′-Phosphate penultimate nucleotides were estimated in this case by the difference between mononucleotides produced by venom exonuclease digestion (see Fig. 6) and the 3′-phosphate terminal nucleotides determined by spleen exonuclease digestion.

RESULTS

Kinetics of Pancreatic DNAase Digestion

Fig. 4 shows the kinetics of pancreatic DNAase digestion of spleen DNAase hydrolysate ($\bar{P}_n = 14$), as followed by chromatography on DEAE-cellulose-urea columns of the dephosphorylated digests. It is evident that hydrolysis proceeds very rapidly at the beginning; the composition of the hydrolysate shows only small changes at later times of digestion. Di- and trinucleotides predominate among the hydrolysis products, the former accumulating to reach a level of about 65% and the latter

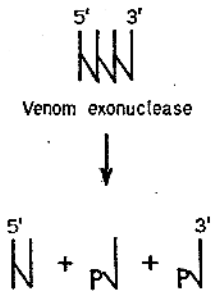

Fig. 6. *Products formed by hydrolysis of tetranucleotide triphosphates by venom exonuclease.* 3′-*P* penultimate nucleotides were estimated by difference between mononucleotides produced by venom exonuclease digestion and the 3′-*P* terminal nucleotides determined by spleen exonuclease digestion

decreasing to about 30%. The level of tetranucleotides after 8 h of digestion is negligible; nucleosides increase very slowly to attain a level of about 5%.

Terminal Doublets of the Spleen-DNAase Hydrolysates as Determined by Digestion with Pancreatic DNAase

A DEAE-cellulose-urea chromatogram obtained with a pancreatic DNAase digest of spleen DNAase hydrolysate ($\bar{P}_n = 14$) is shown in Fig. 5. Five main fractions were obtained. These were formed by: (a) dinucleoside monophosphates, (b) trinucleoside diphosphates, (c) dinucleotides, (d) trinucleotides and nucleoside diphosphates and (e) dinucleoside triphosphates and trinucleoside tetraphosphates. The identification of the components present in each fraction was done on the basis of their elution position, their relative amounts for trinucleoside diphosphates and dinucleotides, and by degradative and chromatographic procedures given in the preceding section for all other components.

Table 1 shows the results of the analysis of terminal doublets released by spleen DNAase from calf thymus DNA. The composition of the terminal doublets was determined by analyzing dinucleoside monophosphates for the 5′-end and dinucleoside triphosphates for the 3′-end. The contributions of trinucleoside diphosphates to the 5′-OH terminal and penultimate nucleotides (which is equal to 33% of these termini) and of trinucleoside tetraphosphates to the 3′-phosphate penultimates (which is equal to 8.5% of the these termini) were neglected in this analysis.

Nucleotide Sequences of Tetranucleotides

Table 1 shows the results obtained in the analysis of the nucleotides as present in the four different positions of tetranucleotides. The data obtained by

Table 1. *Analysis of terminal doublets released by acid DNAase*
The methods of determination are as follows: (I) by digestion with pancreatic DNAase; (II) by digestion with venom exonuclease for the 5′-OH terminal doublets and with spleen exonuclease for the 3′-*P* terminal nucleotides; (III) by adding the contributions of nucleoside diphosphates (which were formed by 27% thymidine, 39% deoxyguanosine, 23% deoxyadenosine and 12% deoxycytidine), dinucleoside triphosphates and trinucleoside tetraphosphates (whose 3′-*P* terminals were formed by 14% thymidine, 45% deoxyguanosine, 30% deoxyadenosine and 11% deoxycytidine) which represented 43%, 48% and 8.5% of the 3′-*P* ends, respectively

	Terminal doublet	Nucleotide	Method of determination	Nucleoside composition			
				dT	dG	dA	dC
				%	%	%	%
Whole digest	3′-*P*	Penultimate	I	22	16	46	16
		Terminal	III	20	42	27	11
			II	20	43	29	8
	5′-OH	Terminal	I	9	42	20	29
			II	11	43	18	28
		Penultimate	I	14	17	62	7
			II	14	26	52	8
Tetranucleotides	3′-*P*	Penultimate	I	27	19	44	10
			II	26	17	44	12
		Terminal	I	21	42	28	10
			II	21	41	26	11
	5′-OH	Terminal	I	15	25	47	14
			II	13	28	52	7
		Penultimate	I	13	35	24	29
			II	14	34	27	25

analyzing the dinucleoside monophosphates (corresponding to the 5′-ends of tetranucleotides) and the dinucleotides (corresponding to the 3′-ends of tetranucleotides) as liberated by the quantitative splitting of the tetranucleotides into their component doublets with pancreatic DNAase, are in good agreement with the results obtained by degrading the tetranucleotides with venom and spleen exonuclease. In this latter case, the 3′-phosphate penultimate nucleotide was estimated by difference as indicated in Fig. 6 and its legend.

DISCUSSION

The procedure developed for the determination of the terminal doublets released by spleen DNAase by further degradation of the oligonucleotides with pancreatic DNAase in the presence of Mn^{2+} deserves several comments.

(a) 5′-OH terminal doublets: pancreatic DNAase releases these doublets partly as dinucleoside monophosphates and partly as trinucleoside diphosphates. The analysis of the former presents no special problems; that of the latter requires the purification from contaminating mononucleotides (these formed about 10% of the trinucleoside diphosphates and were originated by pancreatic DNAase from the inner sequences of the fragments; see legend of Fig. 2) and the degradation to dinucleoside monophosphates plus nucleotides by venom exonuclease [2] or *E. coli* exonuclease I [9]. In the present work, trinucleoside diphosphates were not analyzed, since previous work had already provided information on the 5′-OH terminal doublets [2]; it should be pointed out, however, that this analysis is quite feasible. The analysis of dinucleoside monophosphates showed that the composition found for the 5′-OH terminal was in excellent agreement with results obtained using the venom exonuclease digestion [2]. In contrast, the 5′-OH penultimate was lower in deoxyguanosine and higher in deoxyadenosine than that determined using the venom exonuclease procedure, the other two nucleotides being equal in the two cases. This finding may be explained by the fact that pancreatic DNAase preferentially liberates deoxyadenosine over deoxyguanosine as 3′-OH terminal [10].

(b) 3′-*P* terminal doublets: pancreatic DNAase releases nucleoside diphosphates, dinucleoside triphosphates and trinucleoside tetraphosphates from the 3′-ends of the acid DNAase fragments. The 3′-*P* terminals obtained from all these fragments showed a composition which is in excellent agreement with results obtained by dephosphorylation

Table 2. *Average composition of sequences split by acid DNAase in calf-thymus DNA at* $\bar{P}_n = 15$
The values in parentheses indicate the composition of each terminus calculated from its nearest neighbor(a). For the terminal positions, calculated values could be obtained from both neighboring positions

	3′-*P* penultimate		3′-*P* terminal			5′-OH terminal			5′-OH penultimate	
	exp.	calc.	calc.	exp.	calc.	calc.	exp.	calc.	calc.	exp.
T	22	(31)	(29)	20	(32)	(29)	11	(29)	(29)	14
G	16	(21)	(22)	43	(21)	(23)	43	(23)	(19)	26
A	46	(30)	(29)	29	(30)	(29)	18	(30)	(31)	52
C	16	(19)	(20)	8	(17)	(18)	28	(18)	(20)	8

and spleen exonuclease degradation of spleen DNAase oligonucleotides [3]. The presence of nucleoside diphosphates in the pancreatic DNAase digest implies that, in any case, the 3′-*P* penultimate nucleotides originally linked to the ends and released as nucleoside diphosphates are lost. Since the latter form 43% of the 3′-*P* ends (Table 2), a large percentage of the 3′-*P* penultimates cannot be accounted for. The trinucleoside tetraphosphates, forming 8.5% of the 3′-*P* ends, were not analyzed in the present work, although this could be done. Therefore the analysis of the 3′-*P* penultimate nucleotides was limited to the remaining 48% which were present in the dinucleoside triphosphates. Since pancreatic DNAase preferentially releases thymidine at 5′-*P* end [10, 11], it might be expected that, if anything, our results might be overestimated in this nucleotide.

(c) In order to check the validity of the analytical data concerning the 3′-*P* penultimate nucleotides, these were also isolated from tetranucleotides formed by spleen DNAase, and analyzed. It should be noted: (a) that tetranucleotides appear to be the main product of spleen DNAase in the terminal phase of digestion, since they can represent as much as 25% of the isostichs in calf thymus DNA [4], and (b) that the analysis of nucleotides present in the other three positions of tetranucleotides showed values in agreement with those obtained on total digests having an average chain length of about 15 [3]. These findings support the idea that the 3′-*P* penultimate nucleotides of tetranucleotides are representative of those of total digests as obtained in the middle phase. Indeed, 3′-*P* penultimate nucleotides, as obtained from tetranucleotides, showed a composition which was in very good agreement with that just reported when total digests were analyzed.

(d) The nucleosides observed in the dephosphorylated pancreatic DNAase digest originate to a large extent (as judged from their amount in the digest, Fig. 4 and 5) from the nucleoside diphosphates released from the 3′-*P* end of the acid DNAase oligonucleotides (Fig. 2); the kinetic data of Fig. 4 indicate that in the range explored they are formed at the expense of the trinucleotides. This conclusion is in agreement with previous results of Vanecko and Laskowski [12].

Conclusion

The main conclusion of the present work, is that spleen acid DNAase recognizes sequences at least four-nucleotides long. This conclusion can be arrived at as follows. The composition of each terminus strongly differs from that expected from random splitting (thymidine 28%; deoxyadenosine 28%; deoxyguanosine 22% and deoxycytidine 22%). In order to rule out that some of these differences merely reflect the composition of the nearest neighbors of the termini actually recognized by the enzyme, it was checked that the composition of each terminus strongly differs from that calculated from its nearest neighbor(s), using the data of Josse *et al.* [13]. This type of analysis is shown in Table 2 for calf thymus DNA. An extension of this approach to the results obtained on three bacterial DNAs having different base composition confirm our conclusion [3] and rules out the unlikely possibility that the calf thymus DNA results are due to some special tetranucleotide pattern.

The present work concludes a series of investigations in which methods for the quantitative analysis of termini released by DNAases were set up. At the same time, it presents the first clear indication that at least one DNAase, spleen acid DNAase, is sequence specific in that it recognizes nucleotide sequences at least four nucleotides long. One further point which requires clarification before the enzyme can be put to any practical use is the kinetics of liberation of termini by spleen DNAase. This is the subject of investigations reported elsewhere [4]. It may be added that subsequent work on three other DNAases (snail acid DNAase, pancreatic DNAase and *E. coli* endonuclease I) has shown that they also recognize short nucleotide sequences, suggesting that the present finding may have a general significance.

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