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Studies on the Specificity of an Acid Deoxyribonuclease from *Helix aspersa* (Müll.)

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The kinetics of degradation of calf thymus DNA by the DNAase from *Helix aspersa* has been investigated in its middle and terminal phase by determining both hyperchromic shift and average chain length of the digest. The results obtained are practically identical with those previously reported for acid DNAase from hog spleen. In contrast, the distribution of the isostichs in snail DNAase digests is significantly different from those found for spleen acid DNAase digests of similar sizes.

The 3′-phosphate and 5′-hydroxy terminal nucleotides were found not to vary in their compositions in the average size range 34 to 10 and 70 to 12, respectively. In the 3′-phosphate terminal position, deoxyadenosine is by far predominant (78%) and deoxycytidine almost absent (less than 1%); deoxyguanosine and thymidine form 6% and 16%, respectively, of the terminals. In the 5′-hydroxy terminal position deoxyguanosine is predominant (45%), followed by deoxycytidine (31%), thymidine (14%) and deoxyadenosine (10%); in the 5′-hydroxy penultimate position thymidine forms 38% of the nucleotides, deoxyguanosine 24%, deoxyadenosine 21% and deoxycytidine 17%.

The availability of an acid DNAase from the hepatopancreas of *Helix aspersa* (Müll.), free of contaminating exonuclease and phosphatase [1,2], suggested to us that an investigation on the termini[1] released by this enzyme might be of interest. In fact, the results obtained on the specificity of acid DNAase from hog spleen [3–7] indicate that our understanding of the general problem of DNAase specificity could be much improved by a detailed knowledge of the specificity of other DNAases. A screening program on this subject might, therefore, be extremely rewarding in providing new enzymatic tools with different specificity spectra for the study of nucleotide sequences in DNAs. The choice of the snail DNAase as the first enzyme to be investigated in such a screening is justified by the fact that both the spleen and the snail enzyme, though different proteins, are 3′-phosphate formers and have very close pH optima. The procedures developed for the study of the specificity of spleen DNAase can therefore be applied without any modification to the snail enzyme.

The most interesting results obtained in this work were the findings that the snail DNAase releases from calf thymus DNA 3′-phosphate terminal nucleotides in which deoxyadenosine is by far predominant (78%) and deoxycytidine almost absent (less than 1%), and that the termini liberated by the enzyme are invariant over a wide range of degradation levels.

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

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

Enzymes. DNAase from bovine pancreas (EC 3.1.4.5); acid DNAase from snail hepatopancreas (EC 3.1.4.–); acid DNAase from porcine spleen (EC 3.1.4.6); snake venom exonuclease from *Crotalus admanteus* and acid exonuclease from porcine spleen (EC 3.1.4.1); acid phosphomonoesterase from porcine spleen (EC 3.1.3.2); polynucleotide kinase (EC 2.7.1.–).

Definition. Isostichs are oligonucleotide size groups, regardless of their composition [13].

MATERIALS

Calf-Thymus DNA

Preparation CTR2 [5] was used, except where otherwise stated.

Enzymes

The *Helix aspersa* DNAase preparation was fraction VIII, described elsewhere [1]. Acid exo-

nuclease and acid phosphomonoesterase B were obtained from hog spleen [8,9]. Polynucleotide kinase was prepared according to Richardson [10]. Pancreatic DNAase was purchased from Worthington (Freehold, N. J., Code DPFF). Venom exonuclease from *Crotalus adamanteus* was prepared as previously described [4]. A commercial preparation was also used in some experiments (see below).

METHODS

3′-Phosphate terminal nucleotides were determined as previously described [5]; acid phosphomonoesterase B digestion was done at 37 °C for 2 h using 0.5 U/ml substrate solution; acid exonuclease was used at 27 °C, at a concentration of 0.7 U/ml, for 2 h.

The determination of the average size and of the distribution of the oligonucleotides was done as previously described [5].

5′-Hydroxy terminal and penultimate nucleotides were determined as described [4,6].

5′-Hydroxy terminal nucleotides were also determined by ^{32}P labelling using [γ-^{32}P]ATP and polynucleotide kinase. Samples (155 µl) containing 82.5 nmol hydrolyzed DNA-*P*, 20 µmol Tris buffer pH 8.0, 3 µmol $MgCl_2$, 5 µmol 2-mercaptoethanol, 16 nmol [γ-^{32}P]ATP (53 counts × $min^{-1}pmol^{-1}$) and 0.3 µg polynucleotide kinase were incubated at 37 °C for 30 min, a second identical aliquot of enzyme being added after 15 min. Samples were then dialyzed against two changes of 0.01 M Tris buffer, 0.01 M $MgCl_2$, pH 7.5, for 16 h and against distilled water for 2 h. The phosphorylated oligonucleotides were dried under a nitrogen stream, dissolved in 45 µl 0.05 M Tris buffer pH 7.5, 4 mM $MgSO_4$, 0.5 mM $CaCl_2$ and incubated for 75 min at 37 °C with 5 µg pancreatic DNAase. At this point, the pH was raised by addition of 1.5 µmol NaOH and the mixture was incubated for 40 min at 37 °C with 25 µg venom exonuclease (VPH, Worthington). Digestion was stopped by addition of 1 µl 16% $HClO_4$ and carrier nucleotides. Electrophoretic separation of the nucleotides was carried out according to Richardson [10].

RESULTS

Kinetics of DNA Degradation

Fig. 1 shows the time course of the hyperchromic shift and the reciprocal average degree of polymerization, $\bar{P}_n^{-1}$, of calf thymus DNA during digestion by the *Helix* DNAase.

The hyperchromic shift curve is biphasic, the initial steep slope being followed by an increasingly flatter one; the initial time lag is evident in the particular experiment shown. The $\bar{P}_n^{-1}$ curve shows an initial linear increase with time, whereas in the later stage of the reaction it appears to increase at a slower rate compared to the relative absorbance. A plot of

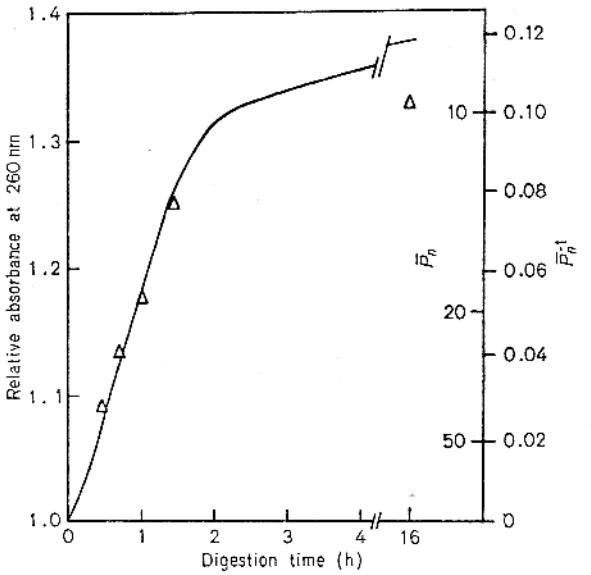

Fig. 1. *Hyperchromic shift (——) and reciprocal average size (△) of calf-thymus DNA during* Helix *DNAase digestion.* The incubation mixture (15 ml) contained DNA ($A_{260} = 8.0$) in 0.05 M ammonium acetate, 0.001 M EDTA, pH 5.5 and 334 U *Helix* enzyme (fraction VIII [2]). Incubation temperature was 26 °C. Samples were withdrawn from the incubation mixture and used for size determinations (△)

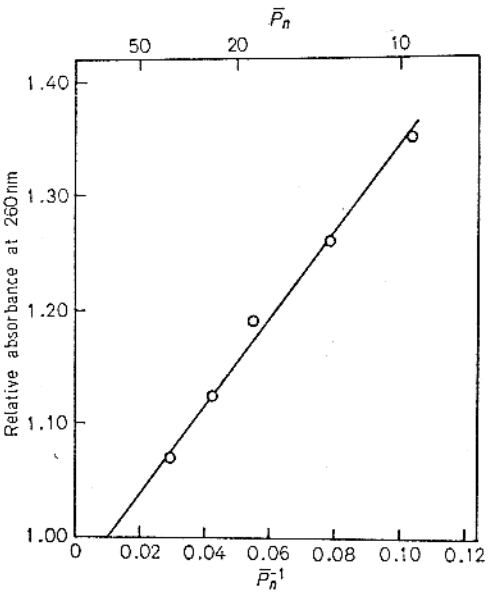

Fig. 2. *Plot of hyperchromic shift* versus *reciprocal average size of calf-thymus DNA during* Helix *DNAase digestion.* Data of Fig. 1

the relative absorbance of the snail DNAase hydrolysate against its reciprocal average size shows a linear relationship (Fig. 2), at least up to a $\bar{P}_n^{-1}$ value of 0.10. The abscissa axis is cut at a $\bar{P}_n^{-1}$ value of about 0.01. All these results are identical with those reported for the spleen enzyme [5].

Table 1. *Chromatography of snail and spleen DNAase digests of calf-thymus DNA on DEAE-cellulose-urea columns*
Fractions I to VI refer to mono to hexanucleotides. R is formed by the material eluted after the hexanucleotide peak

Digest	Reference for details	Digestion temperature	$\bar{P}_n$	Fractions						
				I	II	III	IV	V	VI	R 8
		°C		%						
Snail DNAase	Fig.3	26	9.7	0.1	0.5	1.6	2.3	5.3	4.1	85.2
Spleen DNAase	[5]	22	10.4	0.37	0.7	4.1	7.2	5.2	82.5[b]	
Snail DNAase	[2]	37	6.4		≈1[a]		5.5	16.5	23.7	53.3
Spleen DNAase	[7]	22	6.5	0.7	0.8	6.3	11.4	8.8	8.4	63.6

[a] Fractions I, II and III combined.
[b] Fractions VI and R combined.

Isostich Distribution of the DNA Digest

The distribution of the isostichs in the snail DNAase digest (average size 9.7; last point of Fig. 1) was investigated after dephosphorylation. Table 1 shows a comparison with that obtained on a spleen DNAase digest having a similar size level (average size 10.4 from [5]). As shown in Table 1, the distribution of the isostichs shows some significant differences, the relative amount of mono to tetranucleotides being smaller than in the spleen DNAase digest; in addition, the tetranucleotide peak is no more predominant among the resolved peaks.

At a lower size level (average size 6.5) the differences in the isostich distribution of the two hydrolysates are even more striking, the snail DNAase digest showing less mono to tetranucleotides, but more penta and hexanucleotides than the spleen DNAase digest. Another difference concerns the oligonucleotides having a size larger than 6; they are present in smaller amount and are completely resolved in the snail DNAase digest, whereas they are more abundant and larger in their average size in the spleen DNAase digest; this difference would have been even larger if the spleen DNAase digest had been obtained at 37 °C like the snail DNAase digest.

Termini Released by the Snail DNAase

Fig. 3 shows the relative amounts of 3'-phosphate terminal nucleotides released by the snail enzyme from calf thymus DNA. The levels of different terminals are practically invariant, at least at size levels between 34 and 9.7; however, a very slight decrease in the amount of terminal deoxyadenosine at the lowest size levels is noticeable. Among the terminals, deoxyadenosine is by far predominant (78%) and deoxycytidine almost absent (less than 1%); deoxyguanosine and thymidine form 6 and 16%, respectively of the terminals.

The 5'-hydroxy terminal nucleotides released by the snail DNAase, as determined with polynucleotide kinase, are shown in Fig. 3. The levels of different terminals appear to be practically constant at average size levels between 70 and 12. Deoxyguanosine is predominant among the 5'-hydroxy terminals.

The 5'-hydroxy terminal (and penultimate) nucleotides were also determined using the method

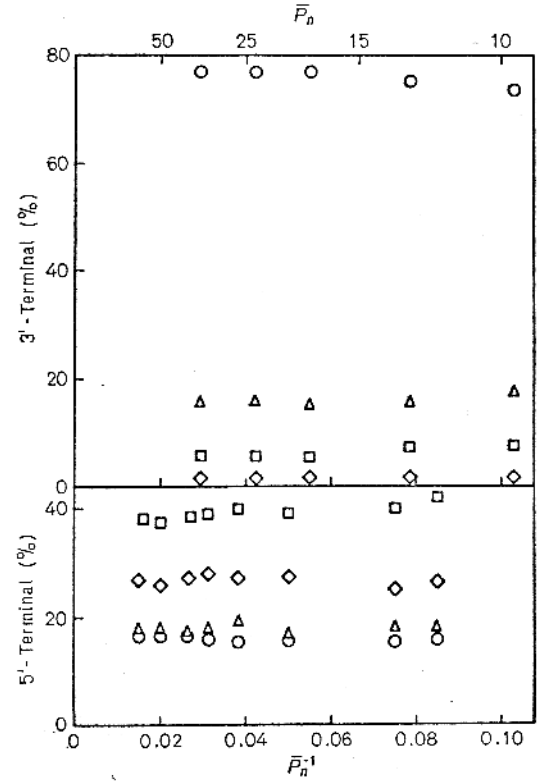

Fig. 3. *Percentages of 3'-phosphate and 5'-hydroxy terminal nucleotides obtained from* Helix *DNAase digest at different degradation levels of calf-thymus DNA.* (○) Deoxyadenosine; (△) thymidine; (□) deoxyguanosine; (◇) deoxycytidine

Table 2. *Termini released by snail and spleen DNAase from calf-thymus DNA*
Data for snail DNAase was obtained from: (A) CTR2 DNA in ammonium acetate buffer, average chain length of the digest (sample c, Fig. 1) was 18.1; (B) polynucleotide kinase experiments, calf thymus DNA (preparation CTS) in sodium acetate buffer was used (Fig. 4). Preliminary experiments were presented at the 6th Meeting of the Federation of European Biochemical Societies [12]. In the printed abstract, the deoxyodenosine and deoxycytidine values were exchanged by mistake. Data for spleen DNAase was from Thiery *et al.* [6]; average chain length of the digest was 18

Enzyme	Nucleotide	3′-P terminal	5′-OH terminal		5′-OH penultimate
		(A)	(A)	(B)	(A)
		%			
Snail DNAase	Thymidine	16	14	18	38
	Deoxycytidine	1	31	27	17
	Deoxyguanosine	6	45	39	24
	Deoxyadenosine	78	10	16	21
Spleen DNAase	Thymidine	21	11		14
	Deoxycytidine	7	28		7
	Deoxyguanosine	45	43		26
	Deoxyadenosine	28	18		53

of Ehrlich *et al.* [4]. Since no variation of the terminals had been seen using the polynucleotide kinase, this experiment was performed only on a hydrolysate having an average size of 18. The results obtained are shown in Table 2. The agreement between the two methods, as far as the 5′-hydroxy terminals are concerned, is fairly satisfactory, though the amounts of terminal deoxyguanosine and deoxycytidine appear to be underestimated by the kinase procedure. In this latter case, however, snail DNAase digestion was carried out at a different temperature (37 °C instead of 26 °C).

DISCUSSION

The kinetics of degradation of calf thymus DNA by the snail DNAase as followed by both hyperchromic shift and chain length determination, is essentially identical to that already found with the spleen enzyme and many comments already made in connection with this enzyme [5] apply here too. It is very likely that the biphasic character of the hyperchromic shift and $\overline{P}_n^{-1}$ curves is due to the same reasons as for the spleen DNAase, namely to the exhaustion of double-stranded substrate (which is preferred over single-stranded DNA [2]). In this case, too, it has been ruled out that the slowing down of the reaction rate is due to enzyme inactivation. It can be noticed that the relative absorbance *versus* reciprocal average size plot of Fig. 2 is very similar to that obtained with the spleen enzyme to the point that its slope and its intercept on the abscissa axis are identical. The empirical equation concerning the hyperchromic shift and $\overline{P}_n^{-1}$ in the case of spleen acid DNAase digestion of calf thymus DNA [6] is therefore also valid for snail DNAase digests. This finding underlines the fact that a precise relationship exists between number of bonds broken and hyperchromic shift, and that this is not affected by differences in sequence specificity.

While a distinction between spleen acid DNAase and the snail enzyme would be impossible on the basis of the kinetic data, differences clearly appear when the isostich distribution is considered and, much more so, when one considers the kinetics of liberation and the composition of the termini released.

The isostich distribution of the snail DNAase digest at a size level of about 10, appears to be different from that obtained by degradation with the spleen enzyme, particularly in that di- to tetranucleotides are less abundant and tetranucleotides do not constitute anymore the predominant class among the resolved peaks. These differences are even more striking at a lower size level (6.5). In this case, the oligonucleotides of the snail DNAase digest are characterized by a narrower and more symmetrical isostich distribution compared to those released by the spleen DNAase.

The largest differences between the two enzymes appear, however, when the released termini are considered. The first difference is that essentially no change takes place in the relative amounts of the terminal nucleotides released from calf thymus DNA over a very wide range of size levels. Therefore, the snail enzyme does not seem to cause the accumulation of terminals having a particular composition in the early digestion period, as the spleen enzymes does. As in the case of the spleen enzyme, no change in the composition of released termini is caused by the melting of DNA fragments.

The second difference concerns the composition of the termini released. The 5′-hydroxy terminals are not very far in composition from those released by the spleen enzyme; the 5′-hydroxy penultimate nucleotides do not show any high degree of specificity (see following paper). The 3′-phosphate terminals, on the other hand, are not only very different from those released by the spleen enzyme, but they are also extremely characteristic because of the great abundance of deoxyadenosine the almost complete lack of deoxycytidine. The following paper [11] presents data concerning the termini released by the snail enzyme from bacterial DNAs of different (dG + dC) contents.

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REFERENCES

1. Laval, J. & Paoletti, C. (1972) *Biochemistry, 11*, 3596—3603.
2. Laval, J. & Paoletti, C. (1972) *Biochemistry, 11*, 3604—3610.
3. Carrara, M. & Bernardi, G. (1968) *Biochemistry, 7*, 1121—1132.
4. Ehrlich, S. D., Torti, G. & Bernardi, G. (1971) *Biochemistry, 10*, 2000—2009.
5. Soave, C., Thiery, J. P., Ehrlich, S. D. & Bernardi, G. (1973) *Eur. J. Biochem. 38*, 422—433.
6. Thiery, J. P., Ehrlich, S. D., Devilliers-Thiery A. & Bernardi, G. (1973) *Eur. J. Biochem. 38*, 434—442.
7. Devillers-Thiery, A., Ehrlich, S. D. & Bernardi, G. (1973) *Eur. J. Biochem. 38*, 416—422.
8. Bernardi, A. & Bernardi, G. (1968) *Biochim. Biophys. Acta, 155*, 360—370.
9. Chersi, A., Bernardi, A. & Bernardi, G. (1971) *Biochim. Biophys. Acta, 246*, 51—60.
10. Richardson, C. C. (1965) *Proc. Natl. Acad. Sci. U. S. A. 54*, 158—165.
11. Ehrlich, S. D., Devillers-Thiery, A. & Bernardi, G. (1973) *Eur. J. Biochem. 40*, 139—141.
12. Laval, J. (1969) *Proc. 6th Meet. Fed. Eur. Biochem. Soc. Abstract*, 782.
13. Shapiro, H. S. & Chargaff, E. (1964) *Biochim. Biophys. Acta, 91*, 262—270.

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