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# Specificity of Spleen Acid DNAase

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The kinetics of liberation of 3′-phosphate terminal and of 5′-hydroxy terminal and penultimate nucleotides (termini) by spleen acid DNAase was investigated using as substrates calf thymus DNA, three bacterial DNAs of different base composition, and poly-[d(A-T) · d(A-T)].

The relative amounts of the nucleotides deoxyguanosine, deoxyadenosine, thymidine and deoxycytidine in the termini released by the enzyme from all DNAs were found to be constant in the terminal phase of digestion. In contrast, some variations were seen during the middle phase, particularly in the amount of 3′-phosphate terminal deoxyguanosine, which decreased with digestion time, specially in the calf thymus DNA digest. This phenomenon appears to be due to an initial accumulation of breaks in some nucleotide sequences which are preferentially split at the beginning of digestion, possibly by a diplotomic mechanism, with predominant release of 3′-phosphate deoxyguanosine. In contrast, no change in the composition of 3′-phosphate terminals released from poly[d(A-T) · d(A-T)] took place in the 40-15 average size range; deoxyadenosine and thymidine formed 80% and 20%, respectively, of these terminals.

The composition of the 3′-phosphate terminal nucleotides was very little or not at all affected by changes in the ionic environment of the incubation mixture, whereas it was significantly changed in degradations of native DNA digested at neutrality and low ionic strength, or of heat-denatured DNA.

Linear relationships were found to hold, at all degradation stages investigated here, between the relative amounts of termini liberated from different bacterial DNAs and their (dG + dC) contents. In contrast, the termini obtained from calf thymus DNA were released in relative amounts which did not fit the relationships established for bacterial DNAs.

Recent investigations carried out in our laboratory have led to the establishment of methods for the determination of 3′-phosphate terminal [1,2], 5′-hydroxy terminal and penultimate [3] and 3′-phosphate penultimate nucleotides [4] in oligonucleotides released from calf thymus DNA by acid DNAase from hog spleen. These techniques rely upon: (a) the use of very highly purified enzymes which were obtained from hog spleen: acid DNAase B [5,6], acid exonuclease [7] and acid phosphomonoesterase B [8]; (b) the development of new analytical techniques for the determination of nucleosides [1,9,10]. The availability of methods for the quantitative determination of the termini[1] of oligonucleotides allowed us to identify specific patterns in the termini of the fragments obtained by degrading calf thymus DNA with spleen DNAase and to demonstrate that this enzyme can recognize sequences of at least four nucleotides in DNA.

In the present work, we have investigated the kinetics of liberation of the 3′-phosphate terminal and the 5′-hydroxy terminal and penultimate nucleotides liberated by spleen DNAase from calf thymus DNA, from bacterial DNAs having different (dG + dC) contents and from poly [d(A-T) · d(A-T)]. The main findings of these investigations are: (a) the demonstration that the specificity of spleen DNAase does not change during the course of DNA degradation; some changes in the relative

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

*Enzymes.* DNAase or deoxyribonuclease (EC 3.1.4.5); acid deoxyribonuclease (EC 3.1.4.6); snake venom and spleen acid exonuclease (EC 3.1.4.1); 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; isostichs are oligonucleotide size groups, regardless of their composition [28].

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

amounts of termini taking place early in the degradation and essentially concerning the 3'-phosphate terminals can be explained as due to the existence of two different mechanisms of degradation, involving different sequences; such changes are not seen in the degradation of poly [d(A-T) · d(A-T)] by spleen DNAase, nor in the degradation of calf thymus and bacterial DNAs by the other three enzymes investigated so far, the snail DNAase [11,12] pancreatic DNAase and *E. coli* DNAase [13,14]; (b) the demonstration that linear relationships exist, at all digestion stages investigated here, between the relative amounts of termini released from different bacterial DNAs and their (dG + dC) contents; in contrast the termini obtained from calf thymus DNA are released in relative amounts which do not fit the relationships established with bacterial DNAs; this deviation appears to be due to the presence of repetitive nucleotide sequences in calf thymus DNA (unpublished results). (c) The establishment of a new approach to the studies of nucleotide sequences in DNA, the quantitative analysis of termini; such an approach appears to be particularly interesting in connection with investigations on repetitive nucleotide sequences.

## MATERIALS AND METHODS

The calf thymus DNA digests were those already used for size determinations by Soave *et al.* [2].

Bacterial DNAs were obtained from *Haemophilus influenzae, Escherichia coli* and *Micrococcus luteus* (*M. lysodeikticus*), respectively, according to the detergent procedure of Kay *et al.* [15]. These DNA preparations were chromatographed on hydroxyapatite columns [16,17], in order to remove small amounts of ultraviolet-absorbing material (presumably formed by degraded RNA) eluting before DNA.

Poly[d(A-T) · d(A-T)] was a commercial sample obtained from Miles Laboratories (Kankakee, Ill.); this samples showed a single symmetrical peak when chromatographed on a hydroxyapatite column and had an $s_{20,w}$ value equal to 8.8 S.

The enzyme preparations used were described in the preceding paper [2] except for the snake venom exonuclease preparation, which was described elsewhere [3]. Pancreatic DNAase (code D) was obtained from Worthington (Freehold, N.J.).

### *Determination of the 3'-Phosphate Terminal Nucleotides*

This was done as already described [1,2], except in the case of poly[d(A-T) · d(A-T)], where this was done using BioGel P-2 (BioRad, Richmond, Calif.) columns [9].

### *Determination of the 5'-Hydroxy Terminal and Penultimate Nucleotides*

This was done according to one of the following methods.

Method I was essentially the same as already described by Ehrlich *et al.* for hydrolysate III [3] except that only 10 $A_{260}$ units oligonucleotides were digested with venom exonuclease; reaction was stopped at the beginning of the hyperchromicity plateau.

Method II is similar to method I, except that only 2 $A_{260}$ units of oligonucleotides were treated, and that excellent separation of digestion products (dinucleosides monophosphates from mononucleotides) was obtained on a DEAE-cellulose column without 7 M urea by using very fine particles of DEAE-cellulose, eliminating thus an additional chromatographic step. The dinucleoside monophosphates thus isolated were further treated as already described ([3] hydrolysate II), except that all steps were done on a lower scale.

Oligonucleotides derived from the calf thymus DNA were processed according to method I; very similar results were obtained when method II was applied, except that values for terminal deoxycytidine were slightly lower. Oligonucleotides from bacterial DNA were all processed according to method II, which was preferred because of its greater simplicity.

### *Nucleoside Analyses*

Nucleoside analyses were done on BioGel P-2 columns [9], on Sephadex G-10 (Pharmacia, Uppsala, Sweden) columns [10], or by a modification of the method of Uziel *et al.* [18]. In the latter case, 0.04 $A_{260}$ unit nucleosides in 5 µl 0.4 M ammonium formiate pH 4.65, were loaded on a column (0.2 × 5 cm) of Aminex A-6 (BioRad, Richmond, Calif.), equilibrated with the same solvent. Elution was done at room temperature using a flow rate of 0.75 ml/h; a typical separation required about 90 min; the detection and evaluation of the nucleoside peaks were done as already described [9,10].

## RESULTS

### *3'-Phosphate Terminal Nucleotides Liberated from Calf-Thymus and Bacterial DNAs*

The results concerning the composition of 3'-phosphate terminals have been plotted against the reciprocal average degree of polymerization $\bar{P}_n^{-1}$ rather than against digestion time. This permits comparison of results obtained at different initial reaction rates and eliminates the very marked decrease in reaction rate following the melting of DNA fragments which takes place once they become short enough.

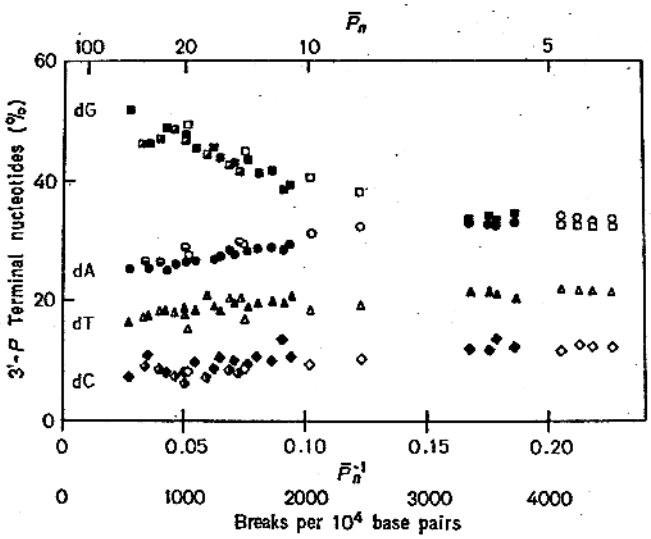


Fig. 1. *3'-Phosphate terminal nucleotides obtained at different degradation levels of calf-thymus DNA by acid DNAase.* Squares, circles, triangles and lozenges correspond to terminal the nucleotides of deoxyguanosine, deoxyadenosine, thymidine and deoxycytidine, respectively. Filled and half-filled symbols show the percentages of terminal nucleotides of undialyzed and dialyzed oligonucleotides, respectively, as obtained by digestion of DNA preparation CTR 2 at 22 °C. Data corresponding to $\bar{P}_n^{-1}$ lower than 0.1 values were obtained on the digests presented in Fig. 2 of the preceding paper. Data corresponding to $\bar{P}_n^{-1}$ values comprised between 0.15 and 0.20 were obtained on the digests described in Table 3 of the preceding paper. Open symbols correspond to the percentage terminal nucleotides of undialyzed oligonucleotides obtained by digestion at 0 °C of DNA preparations CTR 2 and V 391. The CTR 2 data concern the digests described in Table 3 of the preceding paper and correspond to $\bar{P}_n^{-1}$ values higher than 0.15; the V 391 data concern the digests presented in Fig. 3 of the preceding paper and correspond to $\bar{P}_n^{-1}$ values lower than 0.15

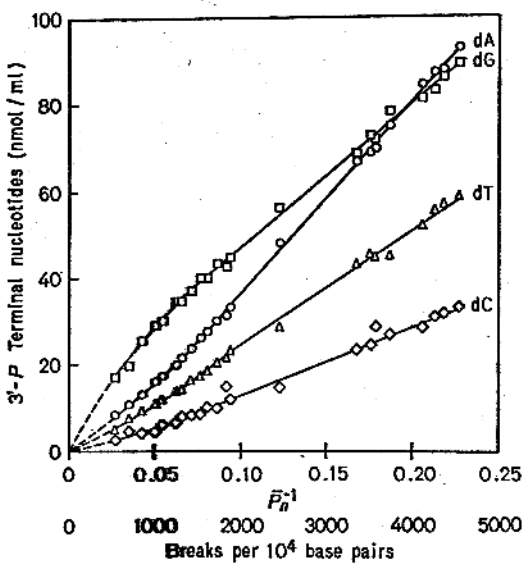


Fig. 2. *Plot of the concentration (nmol/ml) of the 3'-P terminal nucleotides released from calf-thymus DNA by acid DNAase against* $\bar{P}_n^{-1}$. See Fig. 1 for all other indications

The composition of termini has been presented in terms of either percentages or concentrations. The first type of plot gives the relative amounts of termini which are present in the digest at any given degradation level; all the termini which have been accumulated up to that level are seen. The second one shows, in addition, by its slope at any $\bar{P}_n^{-1}$ value, the absolute amount (strictly speaking, a quantity proportional to it, *i.e.* the concentration) of termini liberated per break; we will call this amount the "production" of termini.

Fig. 1 shows the percentage of 3'-phosphate terminal nucleotides from calf thymus DNA as determined over a wide range of $\bar{P}_n^{-1}$ values, during spleen acid DNAase digestion at 0 °C at 22 °C. In this latter case terminal nucleotides were also determined after dialysis of the digests against running distilled water [3]. The use of the different experimental conditions just mentioned affected the results only very little. In the early digestion times (corresponding to the middle phase and to the early terminal phase) the percentages of the terminals are not constant, a rather strong decrease of terminal deoxyguanosine nucleotide being accompanied by a slow increase of the other three terminal nucleotides. Below an average size of about 7 ($\bar{P}_n^{-1} > 0.15$), the percentages of the terminals reach plateau values. The variation in the percentage of 3'-phosphate terminals released by spleen DNAase from calf thymus DNA had already been observed by Vanecko and Laskowski [19] on three hydrolysates having average sizes of 95, 54 and 17, respectively; these authors did not see, however, the terminal plateau region.

Fig. 2 shows a plot of the concentration of 3'-phosphate terminal nucleotides from calf thymus DNA (data of Fig. 1 for undialyzed digest) *versus* $\bar{P}_n^{-1}$ values. In the middle phase, therefore at average size levels higher than 15 ($\bar{P}_n^{-1} > 0.07$), all lines of Fig. 2 show curvatures, indicating that the production of terminals (defined as the amount of terminals released per break: see above) is not constant in this phase. The production of the nucleotide deoxyguanosine, which is much larger than that of other terminals, decreases in the middle phase, whereas that of the other terminals increases. In the terminal phase ($\bar{P}_n^{-1} > 0.07$) the production of terminals, though different for each one of them, is constant: the ratios of the concentrations of different terminals to each other is, therefore, constant. The different shapes of the concentration curves of different terminals in the middle phase cause the extrapolations from the linear parts of the plots

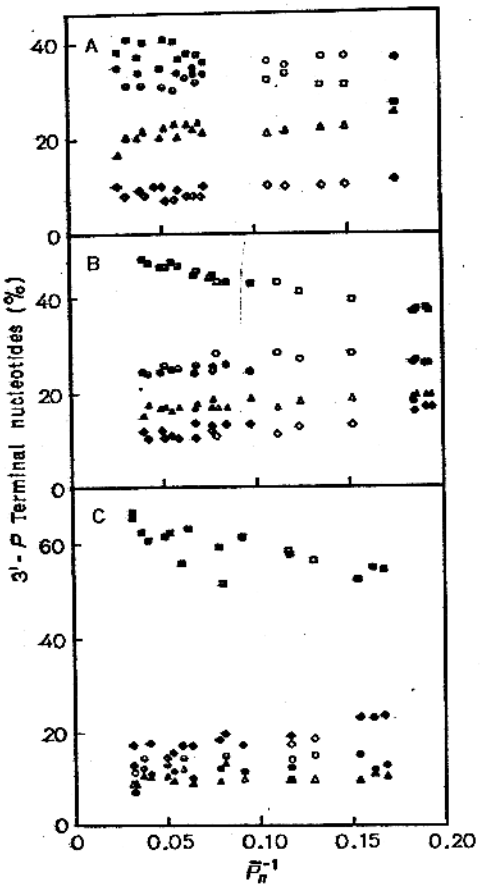


Fig. 3. *3'-Phosphate terminal nucleotides obtained from acid DNAase digests at different degradation levels of* H. influenzae *(A),* E. coli *(B) and* M. luteus *(C) DNAs.* Symbols of Fig. 1 were used

to zero $\bar{P}_n^{-1}$ value to show a high positive intercept for deoxyguanosine and slight negative intercepts for the other terminals.

The percentages of the 3'-phosphate terminal nucleotides, as determined on dialyzed and undialyzed digests obtained at 22 °C, and on undialyzed digests obtained at 0 °C from the bacterial DNAs also are not constant with $\bar{P}_n^{-1}$ in the early phase of digestion, deoxyguanosine showing a decrease (Fig. 3). This decrease, which is concomitant with a slight increase of the other three terminal nucleotides, is less pronounced than in the case of calf thymus DNA. It is important to notice that the relative amounts of different terminals released by the enzyme from different bacterial DNAs vary considerably (Table 1).

Fig. 4 shows the concentrations of 3'-terminal nucleotides plotted as a function of $\bar{P}_n^{-1}$, as obtained on undialyzed oligonucleotides liberated from the three bacterial DNAs. Very similar results, not shown here, were obtained on dialyzed hydrolysates. The general features of these plots

Table 1. *Termini release by acid DNAase from four different DNAs*

| DNA source | Nucleotide position | $\bar{P}_n$ | Composition | | | |
|---|---|---|---|---|---|---|
| | | | dG | dT | dC | dA |
| | | | % | % | % | % |
| Calf-thymus | 3'-Terminal | 20 | 47 | 18 | 6 | 29 |
| | | 15 | 43 | 20 | 8 | 29 |
| | | 5.5 | 35 | 20 | 12 | 33 |
| | 5'-Terminal | 28 | 41 | 10 | 29 | 20 |
| | | 23 | 45 | 8 | 30 | 17 |
| | | 14 | 43 | 11 | 28 | 18 |
| | 5'-Penultimate | 28 | 27 | 14 | 6 | 53 |
| | | 23 | 28 | 13 | 7 | 53 |
| | | 14 | 26 | 14 | 8 | 52 |
| *H. influenzae* | 3'-Terminal | 20 | 40 | 22 | 7 | 31 |
| | | 15 | 37 | 22 | 8 | 33 |
| | | 5.5 | 27 | 25 | 12 | 36 |
| | 5'-Terminal | 14 | 30 | 15 | 34 | 22 |
| | 5'-Penultimate | 14 | 22 | 18 | 11 | 49 |
| *E. coli* | 3'-Terminal | 20 | 46 | 17 | 11 | 26 |
| | | 15 | 46 | 17 | 11 | 26 |
| | | 5.5 | 38 | 19 | 17 | 26 |
| | 5'-Terminal | 23 | 35 | 10 | 37 | 10 |
| | | 14 | 32 | 12 | 39 | 17 |
| | 5'-Penultimate | 23 | 28 | 12 | 14 | 45 |
| | | 14 | 26 | 14 | 15 | 45 |
| *M. luteus* | 3'-Terminal | 20 | 62 | 10 | 14 | 14 |
| | | 15 | 56 | 12 | 17 | 15 |
| | | 5.5 | 55 | 11 | 23 | 11 |
| | 5'-Terminal | 31 | 50 | 3 | 41 | 5 |
| | | 20 | 46 | 4 | 45 | 5 |
| | | 13 | 44 | 5 | 44 | 7 |
| | 5'-Penultimate | 31 | 43 | 11 | 15 | 32 |
| | | 20 | 42 | 9 | 18 | 30 |
| | | 13 | 42 | 8 | 18 | 32 |

resemble those exhibited by calf thymus DNA hydrolysates, in that the same curvatures are seen in the middle phase. These curvatures are, however, less pronounced than those exhibited by calf thymus DNA hydrolysates.

### *Degradation of Poly[d(A-T) · d(A-T)]*

Two very important results were found when studying the kinetics of liberation of 3'-phosphate terminals from poly[d(A-T) · d(A-T)]: (a) no change in the composition of terminals took place in the 40-15 $\bar{P}_n$ range; (b) 3'-phosphate terminals were formed by 80% deoxyadenosine and 20% thymidine.

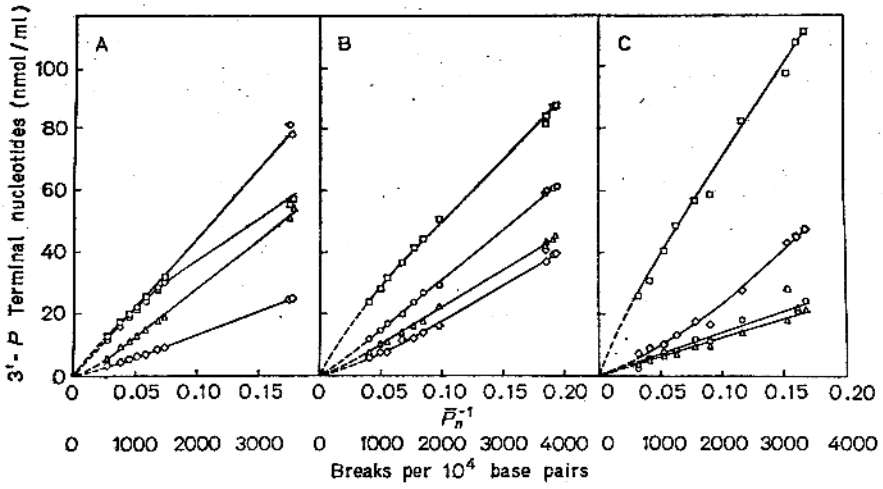


**Fig. 4.** *Plot of the concentration (in nmol/ml) of the 3'-phosphate terminal nucleotides appearing during hydrolysis of* H. influenzae *(A),* E. coli *(B) and* M. luteus *(C) DNAs by spleen acid DNAase against* $\bar{P}_n^{-1}$. Symbols of Fig. 1 were used

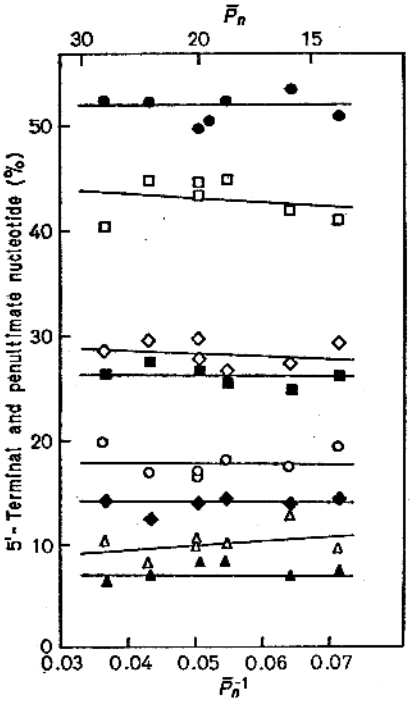


**Fig. 5.** *Percentages of 5'-hydroxy terminal (open symbols) and penultimate (filled symbols) nucleotides obtained from acid DNAase digests at different degradation levels of calf-thymus DNA.* Symbols of Fig. 1 are used

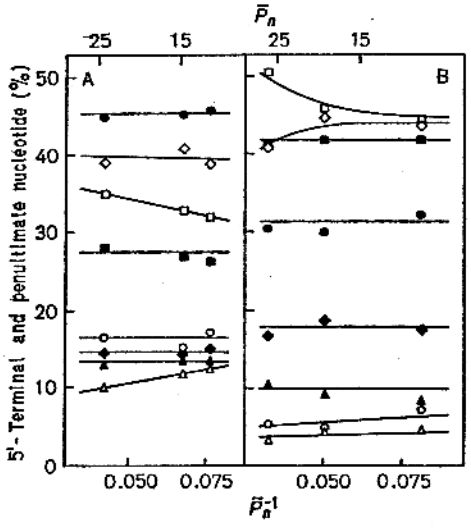


**Fig. 6.** *Percentages of 5'-hydroxy terminal and penultimate nucleotides obtained from acid DNAase digests at different degradation levels of* E. coli *(A) and* M. luteus *(B) DNAs.* Symbols of Fig. 5 were used

### *5'-Hydroxy Terminal and Penultimate Nucleotides from Calf-Thymus and Bacterial DNAs*

For results see Table 1 and Fig. 5 and 6. Penultimate nucleotides never appeared to vary on the $\bar{P}_n$ range 30-14. In contrast, some variation in the percentages of terminal nucleotides was observed. In the case of calf thymus and *E. coli* DNAs, these variations are close to the limit of precision of the analytical method used; it might be significant, however, that in both cases the percentage of terminal deoxyguanosine is decreasing and that of terminal thymidine is increasing with digestion time. For the *M. luteus* DNA the variation of the percentage of terminal deoxyguanosine is higher, and follows the same trend as in the case of calf thymus and *E. coli* DNA.

Table 1 summarizes the results obtained on the termini released by spleen DNAase.

Table 2. *3'-Phosphate terminals released by acid DNAase under different experimental conditions*
Buffers used were 0.05 M ammonium acetate, pH 5.5 (Ac) or 0.15 M sodium acetate (NaAc); concentrations of EDTA, $Mg^{2+}$, $Ca^{2+}$, $Mn^{2+}$, $Co^{2+}$ were 1 mM

| DNA | Conditions: DNA conen ($A_{260}$) | Buffer | Temp. | $\bar{P}_n$ | Composition: dT | dG | dA | dC |
|---|---|---|---|---|---|---|---|---|
| | | | °C | | % | % | % | % |
| Native | 1.35 | Ac + EDTA | 23 | 19.1 | 17.0 | 48.5 | 26.0 | 7.6 |
| | | Ac + $Mg^{2+}$ | | 19.1 | 17.1 | 49.5 | 26.0 | 7.4 |
| | | Ac + EDTA | | 16.9 | 19.6 | 48.8 | 25.6 | 6.1 |
| | | Ac + $Ca^{2+}$ | | 15.6 | 19.9 | 43.7 | 29.7 | 6.7 |
| | | Ac + $Mn^{2+}$ | | 15.9 | 18.6 | 45.2 | 30.1 | 6.1 |
| | | Ac + $Co^{2+}$ | | 14.5 | 18.3 | 43.9 | 31.1 | 6.7 |
| | | Ac + EDTA | | 16.9 | 19.6 | 48.8 | 25.6 | 6.1 |
| | | NaAc | | 16.4 | 16.2 | 46.8 | 30.0 | 7.1 |
| Native | 8.0 | Ac + EDTA | 0 | 15 | 16.5 | 45 | 29.6 | 8.6 |
| | | | 23 | 15 | 20.6 | 44.5 | 27.5 | 7.3 |
| Native | 1.35 | Ac[a] | 23 | 15 | 22.7 | 35.8 | 30.7 | 10.8 |
| Heat-denatured | 1.35[b] | Ac + EDTA | 23 | 20.9 | 19.6 | 37.9 | 32.0 | 10.5 |

[a] 1 mM acetate pH 7.5 was used.
[b] As measured in the native state.

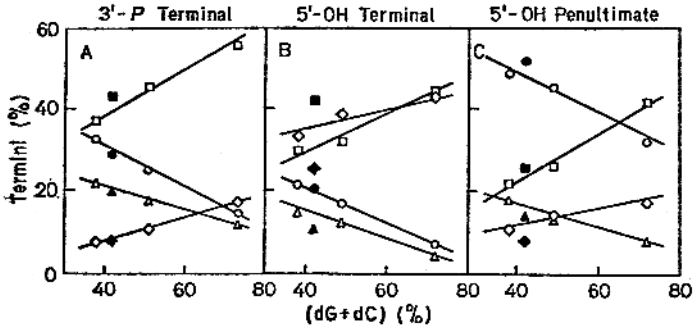


Fig. 7. *Plot of the percentage of the 3'-P terminal (A), 5'-hydroxy terminal (B) and 5'-hydroxy penultimate (C) nucleotides as function of the (dG + dC) content.* Values obtained at $\bar{P}_n = 15$ were used in all cases (Table 1). Symbols of Fig. 1 are used; open symbols for bacterial DNAs, filled symbols for calf thymus DNA

### *3'-Phosphate Terminal Nucleotides Released from Calf-Thymus DNA Degraded under Different Experimental Conditions*

See Table 2. No differences were seen whether digestions were made in the presence of 1 mM EDTA or 1 mM $Mg^{2+}$; in the presence of 1 mM $Ca^{2+}$, $Co^{2+}$, $Mn^{2+}$, a very slight increase was seen in 3'-phosphate terminal deoxyadenosine compared to deoxyguanosine; a similar trend was found in 0.15 M sodium acetate—0.01 M EDTA pH 5.0. Only very slight differences were found when digestions were performed at 0 or at 22 °C, or when using different DNA concentrations (1—8 $A_{260}$ units). In contrast, large differences in the terminals released at an average size level of 15 were seen when degrading either heat-denatured DNA in the usual buffer or native DNA in 1 mM acetate pH 7.5; in both cases, the composition of the terminals liberated was very similar to that found in the digestion of native DNA in the usual buffer at a $\bar{P}_n$ of about 9.

### *Relationships between the DNA Base Composition and the Termini Released by Acid DNAase*

Fig. 7 shows the plots of the percentages of 3'-phosphate terminal nucleotides ,5'-hydroxy terminal and penultimate nucleotides, respectively, as obtained at a $\bar{P}_n$ value equal to 15 for the three bacterial DNAs *versus* their (dG + dC) contents. Linear relationships were found in all cases. They also held at different average size levels, the slopes of the lines being different, as expected (Table 1 and Fig. 8). In the experiment of Fig. 8 mixtures of bacterial DNAs were also used, in addition to the bacterial DNAs; the results obtained with the former fit the straight-line relationships of the latter.

In contrast, the results found for calf thymus DNA, deviate from the relationships found for bacterial DNAs, the deviation of 5'-terminals deoxyguanosine and deoxycytidine being the most pronounced. These deviations increased with increasing size of the digest.

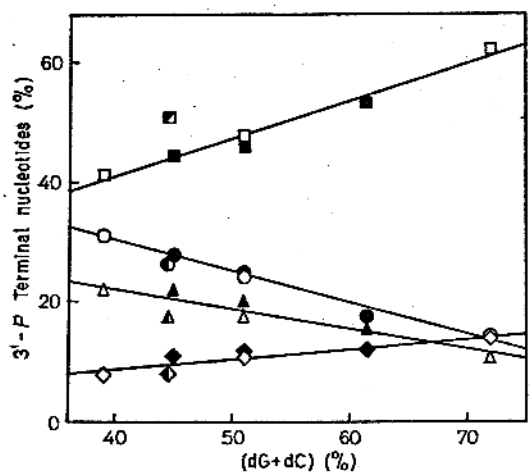


Fig. 8. *Plot of the percentage of the 3'P-terminal nucleotides at a* $\bar{P}_n = 30$ *as function of DNA (dG + dC) contents.* Open, filled and half-filled symbols correspond to results obtained with bacterial DNAs, mixtures of bacterial DNAs [*H. influenzae* + *E. coli* DNA, 44% (dG + dC); *H. influenzae* + *M. luteus* DNA, 51% (dG + dC); *E. coli* + *M. luteus* DNA, 61% (dG + dC)] and calf thymus DNA, respectively. Symbols of Fig. 1 are used

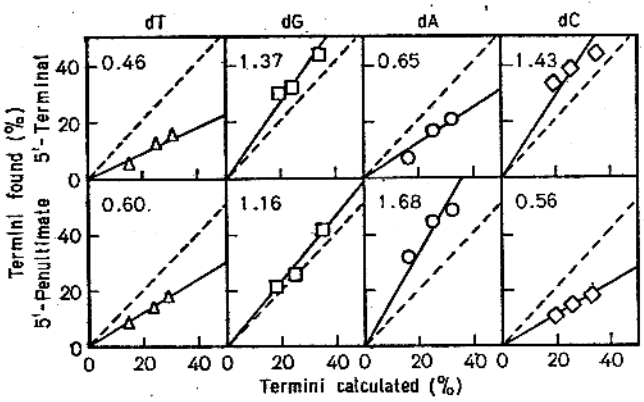


Fig. 9. *The experimentally determined compositions of the 5'-hydroxy terminal and penultimate nucleotides released from bacterial DNAs plotted against the compositions calculated for the nearest neighbors of the 3'-phosphate and 5'-hydroxy-terminal nucleotides, respectively.* The experimental values strongly deviate from those expected if the enzyme had not selected the nucleotides in the 5'-hydroxy terminal and penultimate positions: in this latter case data should align on the broken line of slope 1

### *Sequence Recognition by Spleen DNAase*

Previous work on calf thymus DNA [3,4] had shown that sequences at least four nucleotides long were recognized by spleen DNAase. The availability of data from bacterial DNAs permitted a further check on this point. In fact, it is possible to compare the composition of termini (the 5'-hydroxy terminal and penultimate positions were chosen) as determined experimentally with the composition expected for the nearest neighbors of the 3'-phosphate and 5'-hydroxy terminal nucleotides. As shown in Fig. 9, the experimental values strongly deviate from those expected if the enzyme had not selected the nucleotides in the 5'-hydroxy terminal and penultimate positions.

## DISCUSSION

### TERMINI RELEASED BY ACID DNAase

### *The 3'-Phosphate Terminal Nucleotides Released during the Middle Phase of Digestion*

These nucleotides from all four DNAs investigated in the present work exhibit a characteristic variation in their composition as digestion proceeds. A discussion of this phenomenon may be usefully done on the terminal which shows the largest variation, namely 3'-phosphate terminal deoxyguanosine, as released from calf thymus DNA. Fig. 2 shows that during the middle phase (down to $\bar{P}_n^{-1} = 0.07$), the production (defined as the amount liberated per break and corresponding therefore to the slope of the plot of Fig. 2 at an $\bar{P}_n^{-1}$ value; see Results) of this terminal is larger than that of the other terminals and at the same time is decreasing as digestion proceeds. This indicates that during the middle phase, sequences predominantly liberating 3'-phosphate deoxyguanosine are preferentially split and are being exhausted. Both phenomena are likely to start at the very beginning of the degradation, and to occur during the initial phase as well. The production of deoxyguanosine mimics therefore the overall kinetics of degradation as seen in the plot of $\bar{P}_n^{-1}$ *versus* digestion time (Fig. 2, see also Fig. 2 of ref. 2); expectedly, the extrapolation of the deoxyguanosine concentration plot to zero digestion time (not shown here) exhibits a positive intercept like the extrapolation of $\bar{P}_n^{-1}$ to zero digestion time. These results demonstrate that the sequences which are initially split at a faster rate, possibly by the diplotomic mechanism [2] predominantly liberate 3'-phosphate deoxyguanosine. These sequences appear to be completely exhausted by the end of the middle phase.

Two points worth stressing are the following: (a) the constant ratio of terminal deoxyadenosine to terminal thymidine as released from poly-[d(A-T) · d(A-T)] over a $\bar{P}_n$ range of 40 to 15 shows that the enzyme splits the two available sequences existing in this polymer with no exhaustion of either one; (b) the different magnitude of this phenomenon in different DNAs may be explained by the different concentration of the sequences preferentially split in these DNAs.

### *The 3'-Phosphate Terminals Released during the Terminal Phase of Digestion*

These nucleotides derive from the majority of the susceptible sequences, including those which are

split in melted oligonucleotides. It can be seen from plots like those of Fig. 2 and 3 that over 75% of all the sequences which can be split by the enzyme, under the experimental conditions used, are, indeed, split during the terminal phase. In this phase the production of terminals (as defined above) is constant, or, in other words, the ratio of the produced terminals to each other is constant (Fig. 2 and 3). It should be noticed that the production of termini is not affected by the melting of the fragments, which affects so dramatically the degradation rate, nor by the release of mononucleotides; this latter point being explained by their very low level in the digests.

The fact that the percentage terminals is constant at the end of the terminal phase, but changing during its early part, means that the terminals accumulated as a result of the splitting of the preferred sequences, are "diluted out" by those which are produced during the terminal phase and which have a less extreme composition. We tend to identify these "diluting sequences" as those which are split according to a haplotomic mechanism. Two observations may be quoted in favor of this interpretation: in the case of calf thymus DNA the compositions of the terminals as determined at a $\bar{P}_n$ value of 15 with either denatured DNA digested in the usual incubation solvent or with native DNA degraded at neutrality and low ionic strength is different from that found in native DNA of identical average size and similar to that observed at a lower size value ($\bar{P}_n = 9$). This may mean that in these cases the initial accumulation of preferred breaks in sequences releasing very large amounts of 3′-phosphate terminal deoxyguanosine is no longer possible, or reduced to a very large extent. In the case of denatured DNA the diplotomic mechanism of degradation may be very greatly reduced owing to the loss of the original pairing of complementary sequences and of their symmetry properties [20, 21]. In the case of the degradation of native DNA at a low ionic strength and neutrality, it is not yet clear whether this phenomenon is due to structural change in DNA ("breathing") or in the enzyme; some results obtained in the inactivation of transforming *H. influenzae* DNA by acid DNAase had already indicated that low ionic strength and neutrality favored the haplotomic over the diplotomic mechanism [22].

### *5′-Hydroxy Terminal and Penultimate Nucleotides Released by Acid DNAase*

The 5′-hydroxy terminal nucleotides show some slight variations in their composition during the middle phase. It is very likely that this variation is caused by the same reasons as that concerning the 3′-phosphate terminals. During the terminal phase the composition of the 5′-hydroxy terminal nucleotides is constant.

The 5′-hydroxy penultimate nucleotides never appeared to change in composition during enzymatic degradation. This finding is particularly interesting since these termini are, together with the 3′-phosphate penultimates [4], the most specific ones released by the enzyme.

### *Conclusions on the Kinetics of Release of Termini*

The kinetics of degradation of spleen DNAase may be considered as the sum of two different kinetics. On one hand, the enzyme preferentially splits a limited number of sequences characterized by a high level of deoxyguanosine in what will become the 3′-phosphate terminal position; this splitting takes place at a fast rate and leads to the exhaustion of the preferred sequences by the end of the middle phase of degradation; there are arguments favoring the identification of these sequences as those split by a diplotomic mechanism [2]. On the other hand, the enzyme splits the majority of the susceptible sequences with an initial slower rate; these sequences contain less deoxyguanosine than the former ones in what will become the 3′-phosphate terminal position; the production of termini from these sequences, and therefore their composition, are constant during the terminal phase and, in our view, during the whole degradation. In addition, the termini released are not affected by the melting of DNA fragments; we tend to identify the sequences split by these kinetics as those split by a haplotomic mechanism, mainly because it is known that the ratio of diplotomic to haplotomic splitting rates is decreasing from the beginning of the degradation [22, 23]. The findings that spleen DNAase release 3′-phosphate terminals of constant composition from poly-[d(A-T) · d(A-T)] and that snail DNAase [11], pancreatic DNAase [13] and *E. coli* DNAase I [14] release termini of constant composition from all DNAs investigated, very strongly support the idea that the specificity of at least the four DNAases investigated so far remains the same throughout the whole course of degradation. The variation in the 3′-phosphate terminals seen in the spleen acid DNAase hydrolysis appears to be only due to the existence of two different mechanisms of degradation involving different sequences. It appears, therefore, that the case of the spleen enzyme is just complicated by the presence of an additional mechanism of splitting. From a practical point of view, this greater complexity is compensated by the possibility of using the spleen enzyme at increasing levels of specificity simply by limiting the degradation of DNA.

The demonstration of the constant specificity of DNAases towards nucleotide sequences is one of the

major results of this series of investigations, since it gives a firm basis to their use in studies of nucleotide sequences in DNAs, putting an end to the long-held belief [24—26] that the specificity of DNAases changes during the course of degradation.

### APPARENT AND REAL FREQUENCIES OF TERMINI

It should be strongly pointed out that the sequences which are split are only seen through the termini they release and that much of the possible differences in $K_m$ and $V$ values associated with individual sequences are lost through the averaging of the termini released (see also next section). In the only case in which the split sequences are "seen", dA-dT-dA-dT and dT-dA-dT-dA in poly-[d(A-T) · d(A-T)], the fact that the first one is split in a ratio of 20:80 to the second one, whereas they are equally abundant in the starting product, shows that the enzyme has different $K_m$ and/or $V$ values for the two sequences. This finding points out that the frequencies of the termini as determined by our analysis are only apparent frequencies and should be clearly distinguished from the real frequencies of the termini recognized by the enzyme.

### RELATIONSHIPS BETWEEN TERMINI LIBERATED BY SPLEEN DNAASE AND BASE COMPOSITION OF DNAS

The finding of linear relationships between the relative amounts of termini released, at any given degradation level, from bacterial DNAs and the (dG + dC) contents of the latter is not surprising if one considers that: (a) the enzyme can recognize a very large number of sequences, since the average size of the final digest with concomitant dephosphorylation is about 4 [2] which means that 25% of all internucleotide bonds can be broken by the enzyme. (b) The termini deriving from the sequences which are split are averaged out.

The empirical relationships of Fig. 7 represent a new parameter which may be used to describe nucleotide sequences in DNAs; they resemble very much those previously found between physical properties (buoyant density, melting temperature, optical rotatory dispersion) and base composition of bacterial DNAs. Like the latter, they are not expected, as a rule, to apply to DNAs containing sequences strongly deviating from randomness. They do not hold, in fact, for DNAs containing repetitive nucleotide sequences like total nuclear mammalian DNAs (see, for instance, the results of calf thymus DNA) and satellite DNAs (unpublished results). In fact, plots of the deviations of the apparent frequencies of "repetitive" DNAs from the "base-line" of non-repetitive DNAs as given in Fig. 7, provide a new approach to the study of repetitive sequences in DNAs [27].

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