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Studies on Acid Deoxyribonuclease. IX. 5′-Hydroxy-Terminal and Penultimate Nucleotides of Oligonucleotides Obtained from Calf Thymus Deoxyribonucleic Acid

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ABSTRACT: A new procedure has been developed in order to determine the 5′-hydroxy-terminal and penultimate nucleotides of oligonucleotides derived from calf thymus DNA by digestion with hog spleen acid DNase. The essential point in this procedure is the isolation in high yield of the 5′-hydroxy-terminal dinucleoside monophosphates produced by the action of snake venom exonuclease. The dinucleoside monophosphates are then split with spleen exonuclease; the resulting nucleosides and nucleotides, derived from the penultimate and terminal positions of oligonucleotides, respectively, are separated and analyzed. The results obtained on oligonucleotides having an average chain length close to 10 show that purine nucleotides largely predominate at the 5′-hydroxy-penultimate positions (A forming 52% of the nucleotides, G 23%, T 16%, and C 9%), whereas the 5′-hydroxy-terminals show a predominance of G and C (G 34%, C 31%, A 22%, and T 13%). Since the 3′-phosphate-terminal nucleotides of the fragments are formed by G 39%, A 31%, T 21%, and C 9%, respectively, it appears that acid DNase can recognize sequences of at least three nucleotides in native DNA. A novel purification procedure for snake venom exonuclease is described.

It is widely believed that the specificity of DNases is too low to be of any use in the study of nucleotide sequences in DNAs. This opinion is rather due to the difficulty of demonstrating a specificity in DNases than to a well-demonstrated lack of specificity. As a matter of fact, DNases having the evident specificity of pancreatic RNase or T1 RNase, for instance, have not been found yet. Since all four nucleotides are present in the terminals formed by DNases, precise quantitative determinations of the terminal nucleotides are required in order to obtain information on the enzyme specificity. This means that one needs very accurate methods for separation and analysis, besides extremely pure DNases and contaminant-free ancillary enzymes (exonucleases and phosphatases). An effort has been made in recent years in our laboratory to set up an improved technology in this area. This has been used, so far, to investigate the specificity of acid DNase from hog spleen.

Some results concerning the 3′-phosphate-terminal nucleotides of the oligonucleotides obtained from calf thymus DNA have already been published (Carrara and Bernardi, 1968). We wish to present here a new procedure developed in order to determine the 5′-hydroxy-terminal and penultimate nucleotides of oligonucleotides derived from calf thymus DNA by acid DNase digestion. Initially, we tried the classical approach of degrading the dephosphorylated fragments with snake venom exonuclease in order to release the 5′-hydroxy-terminal nucleotides as nucleosides. We rapidly realized, however, that 5′-hydroxy-terminal dinucleoside monophosphates were accumulating in the digestion mixture as a result of enzyme action. The possibility of isolating them in a very high yield encouraged us to set up a procedure for the analysis of the 5′-hydroxy-terminal and penultimate nucleotides. Very briefly, the procedure, summarized in Figure 1, is the following. Oligonucleotide 3′-phosphates, released from calf thymus DNA by spleen acid DNase digestion, are dephosphorylated (step 1), treated with pancreatic DNase in order to decrease their average size (step 2), and digested with venom exonuclease (step 3). The 5′-hydroxy-terminal dinucleoside monophosphates thus liberated are isolated and split with spleen exonuclease (step 4) to release the 5′-hydroxy-terminal nucleotide as a nucleoside 3′-phosphate and the 5′-hydroxy-penultimate nucleotide as a nucleoside. These are subsequently separated and analyzed. This procedure, involving several new techniques to be described below, has led to the quantitative determination of 5′-hydroxy-terminal and penultimate nucleotides of oligonucleotides released by acid DNase.

The results obtained on oligonucleotides having an average chain length close to 10 show that purine nucleotides largely predominate at the 5′-hydroxy-penultimate positions (A forming 52% of the nucleotides, G 23%, T 16%, and C 9%), whereas the 5′-hydroxy-terminals show a predominance of G and C (G 34%, C 31%, A 22%, and T 13%). On the other hand, the 3′-phosphate-terminal nucleotides of the

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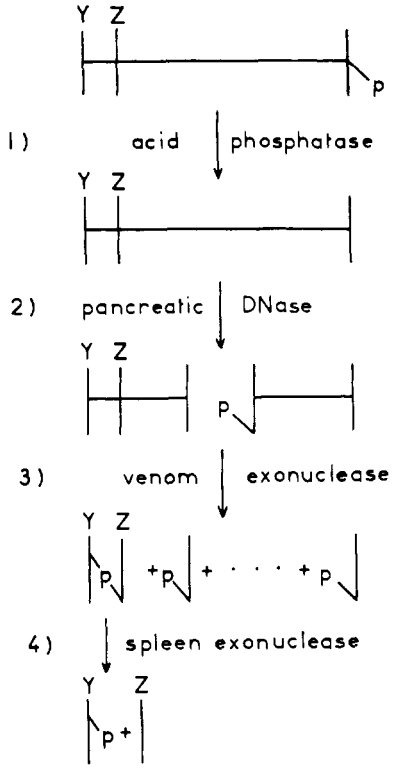

FIGURE 1: Scheme of the procedure used to determine the 5′-hydroxy-terminal and penultimate nucleotides in oligonucleotides obtained by degrading calf thymus DNA with hog spleen acid DNase.

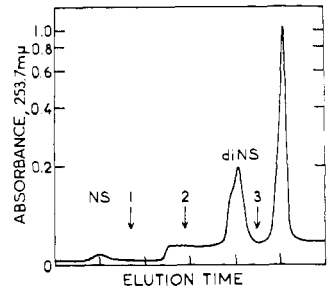

FIGURE 2: DEAE-cellulose chromatogram obtained with a venom exonuclease digest. A 2.0-ml aliquot from the incubation mixture (digestion time 90 min; A_{260} 10.8) was adjusted to pH 7 with 1 M acetic acid, then diluted with 6 ml of water and loaded on a 1.1 × 12 cm DEAE-cellulose column (acetate form) equilibrated with water. Nucleosides (NS) were washed through with water; the column was then washed with 7 M urea (arrow 1); the base line shift is due to the breakthrough of the urea solution. As soon as the column was equilibrated with urea, a linear molarity gradient (0–0.25 M) of ammonium acetate, containing 7 M urea (100-ml total) was started (arrow 2) with flow rate of 50 ml/hr. Dinucleoside monophosphates (diNS) were eluted at the beginning of the gradient, and then a step of 1 M ammonium acetate containing 7 M urea was applied (arrow 3) in order to elute the rest of the material from the column. The figure shows the absorbancy at 253.7 mμ of the column effluent as recorded by a LKB-Uvicord equipped with a 0.3-cm cell. One division on the abscissa corresponds to about 20 min.

fragments are formed by G 39%, A 31%, T 21%, and C 9%, respectively. These results are very different from those expected if the enzyme was breaking phosphodiester bonds at random and suggest that acid DNase can recognize sequences of at least three nucleotides in native DNA.

Material and Methods

Three independent acid DNase hydrolysates (I, II, and III) were investigated in the present work. The experimental conditions and the results reported below refer to hydrolysate II, except where otherwise stated.

Acid DNase Digestion. A DNA solution (268.5 ml; preparation CTR II, obtained from calf thymus using the detergent procedure: Bernardi and Sadron, 1964, method B), A_{260} 8.0, in 0.05 M ammonium acetate–0.001 M EDTA (pH 5.6) were digested at room temperature (22–23°) with 0.12 ml of spleen acid DNase (Bernardi *et al.*, 1966; preparation HS 24; 225 units/ml; units as defined by Bernardi *et al.*, 1966). When the absorption increase at 260 mμ reached 31%, the enzyme was inactivated by shaking the digestion mixture with one-tenth volume of chloroform–isoamyl alcohol (24:1, v/v, CA).[1]

Dialysis of Acid DNase Digest. Visking $^{8}/_{32}$-in. tubings, boiled first in 10% sodium carbonate–0.1 M EDTA, then in water, were used. Dialysis was done at room temperature against water flowing through a glass tube having a diameter only slightly larger than the dialysis tubings. Acid DNase digest (235 ml) was dialyzed against 75 l. of distilled water, using a flow rate of 7.5 l./hr. The removal of salt was complete as judged by refractive index measurement.

Dephosphorylation of Acid DNase Digest. Spleen acid phosphomonoesterase B (5.1 ml) (Chersi, A., Bernardi, A., and Bernardi, G., 1971, submitted for publication; 0.07 unit/ml of digestion mixture; units as defined by Chersi *et al.*, 1966), 0.225 ml of 1 M Tris (final concentration 1.2 mM) and enough 0.1 M acetic acid to reach pH 5.6 were added to 204 ml of dialyzed acid DNase digest (A_{260} 10.1). Digestion was done at 23° for 19 hr. The enzyme was inactivated with CA. Control experiments showed that the average chain length of the oligonucleotides was constant between 14- and 38-hr digestion, thus indicating that dephosphorylation at 19 hr was complete.

Pancreatic DNase Digestion. $MgCl_2$ (1 M) was added to the dephosphorylated oligonucleotides to reach a final concentration of 5 mM, and enough 1 M Tris solution to reach pH 7.5 (final concentration 3.25 mM). Crystalline pancreatic DNase (13 mg) (Worthington, Freehold, N. J., code D) was then added to 190 ml of oligonucleotides (A_{260} 9.5). After 3.5-hr digestion at 37° the enzyme was inactivated with CA. No nucleosides could be detected in the digest when an aliquot of 7 ml was loaded on a 1.1 × 10 cm DEAE-cellulose column (acetate form) indicating that no 5′-hydroxy-terminal nucleoside had been released by pancreatic DNase.

Venom Exonuclease Digestion. Oligonucleotide solution (158 ml; A_{260} 9.9) in 0.018 M Tris-acetate–0.005 M $MgCl_2$ (pH 8.9) (a solvent obtained by adding 1 M Tris to the pancreatic DNase digest) was digested at 37° with 4 ml of snake venom exonuclease, having an activity of 1.8 units/ml (see Appendix).

DEAE-cellulose–urea chromatography of venom exonuclease digests was done on 2-ml aliquots taken at different digestion times (see legend of Figure 2 for experimental details) in order to obtain quantitative estimations of the amounts of nucleosides and dinucleoside monophosphates. It should be pointed out that in the absence of urea dinucleoside mono-

[1] Abbreviations that are not listed in *Biochemistry 5*, 1445 (1966), are: CA, chloroform–isoamyl alcohol mixture (24:1, v/v).

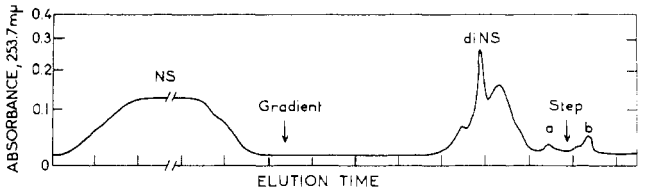

FIGURE 3: DEAE-cellulose chromatogram of a dephosphorylated venom exonuclease digest. An 8-ml sample (digestion time 300 min; OD_{260} 11) was diluted to 160 ml with CO_2-free water and loaded on a 1.1 × 24 cm DEAE-cellulose column equilibrated with water, using a flow rate of 11 ml/hr. The column was then washed with CO_2-free water, using the same flow rate, until all the nucleosides (NS) were eluted. Elution was then performed, at 50 ml/hr, with an ammonium acetate gradient. The total volume was 150 ml; three Varigrad (Technicon, Chauncey, N. J.) chambers were used, two of them filled with water, the third with 0.25 M ammonium acetate. Dinucleoside monophosphates were eluted from the column as a partially resolved fraction (diNS). Pyrimidine nucleotides, present in the mixture because of incomplete dephosphorylation, were eluted at the end of the gradient (peak a); then a step of 1 M ammonium acetate was applied to the column to elute a "high-eluting fraction" (peak b), which consisted of nondephosphorylated purine nucleotides and of oligonucleotides of higher size then dinucleoside monophosphates (if present). The figure shows the absorbancy at 253.7 mμ, of the column effluent as recorded by a LKB-Uvicord, equipped with a 0.3-cm silica cell. One division on the abscissa corresponds to about 30 min. The time interval between the breaks on the abscissa is equal to 12 hr. Recovery of material absorbing at 271 mμ was quantitative.

phosphates are poorly separated from the large amounts of nucleotides present in the digests.

Isolation of Dinucleoside Monophosphates. Since we needed urea-free dinucleoside monophosphates for the further steps described below and since we had, at the time these experiments were done, no fully satisfactory method for freeing dinucleoside monophosphates from urea (a method was developed later and used for hydrolysate III, see below), we used the following approach.

Aliquots (8–20 ml) of venom exonuclease digests were dephosphorylated using 0.07 unit/ml of acid phosphatase

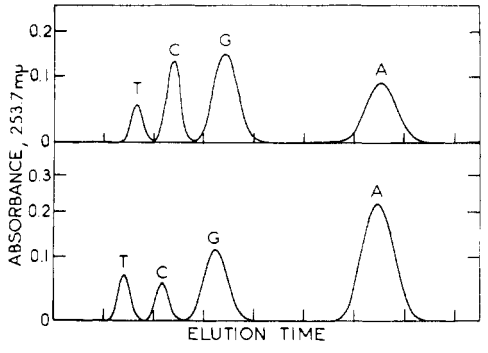

FIGURE 4: Chromatography of nucleosides on Sephadex G-10 columns. Nucleosides (2–3 A_{260} units) in 0.1–0.15 ml of 0.025 M ammonium carbonate (pH 10.4) were loaded on a 0.5 × 50 cm columns of Sephadex G-10, fractionated by decantation (particle size 20–40 μ), equilibrated with the same buffer; elution was done with the same buffer using flow rates equal to 5–7 ml/hr. The chromatography was recorded by an LKB-Uvicord, using a silica cell with an optical path equal to 0.3 cm. Each division on the abscissa is equal to about 22 min. The fractions were collected in volumetric cylinders; solutions were acidified to pH 1–2 and the quantity of each nucleoside was determined by reading its absorption at λ_{max}. Chromatography of 5′-terminal nucleosides (digestion time with venom exonuclease 130 min) and 5′-penultimate nucleosides (digestion time 215 min) are represented by the upper and lower diagram, respectively.

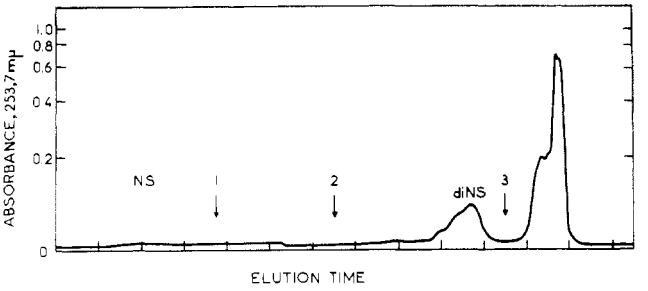

FIGURE 5: DEAE-cellulose chromatogram obtained with a venom exonuclease digest from hydrolysate III. All the experimental conditions were the same as for the Figure 3, except that the 7 M urea was replaced by 1 M urea.

for 17 hr at 22° on digests adjusted to pH 5.4–5.5 by adding 1 M acetic acid.

Dinucleoside monophosphates were then isolated by running the dephosphorylated samples on DEAE-cellulose columns (see legend of Figure 3 for the experimental conditions). In this case dinucleoside monophosphates were obtained completely free of the residual mononucleotides; in addition this step permitted a good estimation of the amount of material eluting higher than dinucleoside monophosphates.

Spleen Exonuclease Digestion of Dinucleoside Monophosphates. These were lyophilized after chromatography on DEAE-cellulose, dissolved in water, and adjusted with 1 M ammonium acetate (pH 5.5) to 0.02–0.03 M. Spleen exonuclease (Bernardi and Bernardi, 1968) was then added to a concentration of 0.01–0.1 unit/ml (units as defined by Bernardi and Bernardi, 1968). Digestion was done at 22° for 1–4 hr.

DEAE-cellulose Chromatography of Spleen Exonuclease Digests. In a typical experiment, a 3-ml aliquot of dinucleoside monophosphates was digested with spleen exonuclease and loaded on a 0.8 × 7 cm DEAE-cellulose column (carbonate form). The nucleosides were eluted with water using a flow rate of 40 ml/hr; nucleotides were subsequently eluted with a step of 0.7 M $(NH_4)_2CO_3$.

Dephosphorylation of Mononucleotides. Nucleotides derived from the previous step were lyophilized, in order to get rid of $(NH_4)_2CO_3$, dissolved in water, and adjusted with

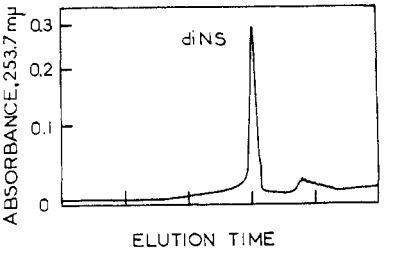

FIGURE 6: A Bio-Gel P-2 chromatogram of the dinucleoside monophosphates obtained from hydrolysate III. The solution of dinucleoside monophosphates in 1 M urea (see Figure 5) was concentrated by rotary evaporation under reduced pressure to a volume of 8 ml. A total of 4 ml was loaded on a 1.1 × 50 cm Bio-Gel P-2 column, equilibrated with 2 mM ammonium carbonate, (pH 10.4). The elution was done with the same buffer using a flow rate of 20 ml/hr. The figure shows absorbancy at 253.7 mμ of the column effluent as recorded by a LKB-Uvicord equipped with a 0.3-cm cell. One division on the abscissa corresponds to about 20 min. The small peak with indentations corresponds to the breakthrough of urea.

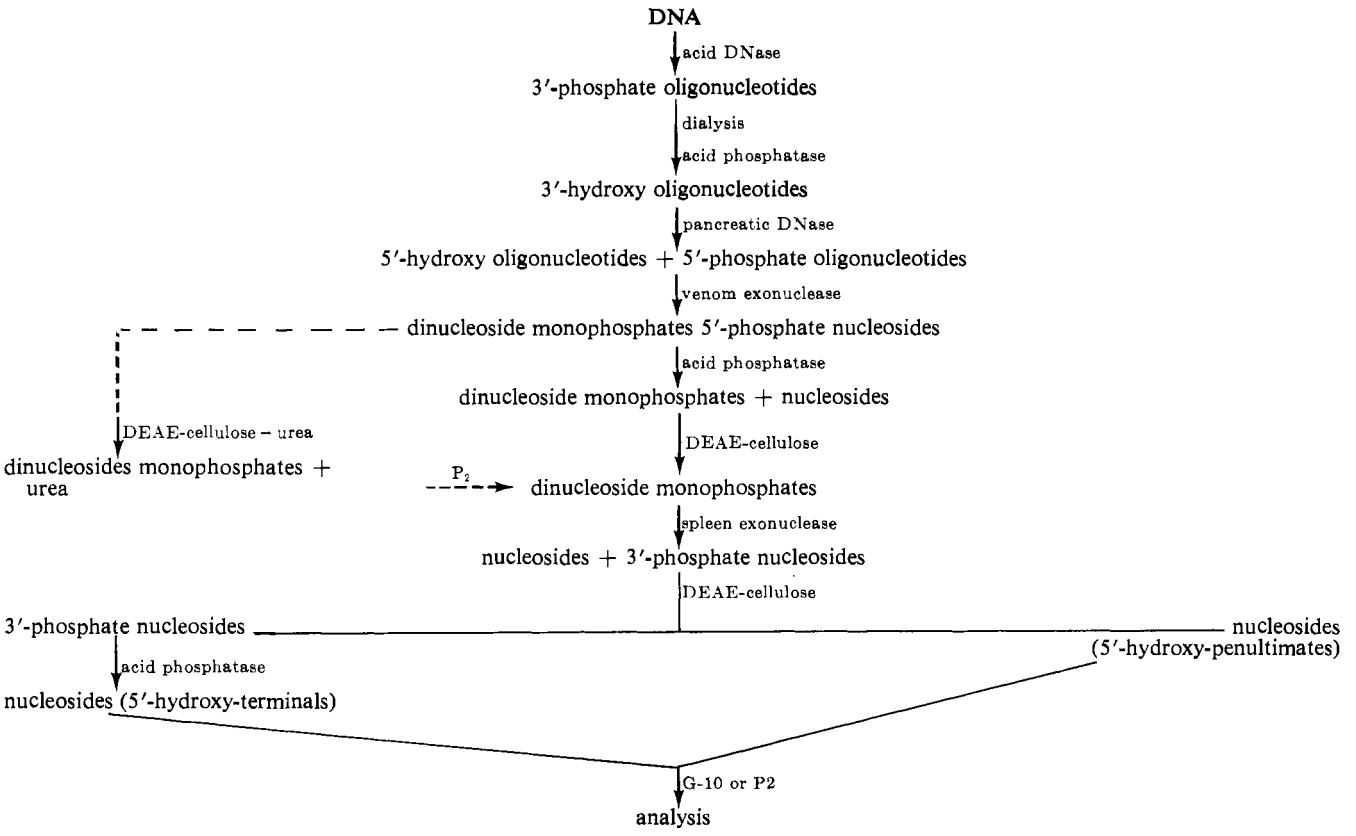

FIGURE 7: Scheme of the procedure used for the analysis of 5′-hydroxy-terminal and penultimate nucleotides. The broken line indicated the procedure followed for preparation III.

1 M ammonium acetate (pH 5.5) to 0.015–0.03 M. If necessary, the pH was corrected by addition of 1 M acetic acid. Acid phosphatase was added to a concentration of 0.1 unit/ml. Digestion was done at 22° for 12–20 hr. Nucleosides were isolated from the digestion mixture by chromatography on DEAE-cellulose columns (acetate form). Typically, a sample of dephosphorylated nucleotides (4 ml; A_{260} 0.976) was loaded on a 0.8 × 7 cm column. Nucleosides were eluted with water, using a flow rate of 40 ml/hr. The recovery of ultraviolet-absorbing material eluted with water was 87–90%. The residual ultraviolet-absorbing material was eluted with a step of 1 M ammonium acetate, and was shown to consist of nondephosphorylated nucleotides, residual dinucleoside monophosphates, and ultraviolet-absorbing material present in the ammonium carbonate solution used in the previous step to elute nucleotides.

Analysis of Nucleosides. This was done on Sephadex G-10 columns (S. D. Ehrlich, J.-P. Thiery, and G. Bernardi, 1971, submitted for publication; see legend of Figure 4).

Hydrolysate III was not dephosphorylated after the venom exonuclease digestion. Further processing of this sample involved some steps different from those used for hydrolysates I and II: a) dinucleoside monophosphates were isolated from 2-ml aliquots of venom exonuclease digest, on a DEAE-cellulose column in the presence of 1 M urea (see legend of Figure 5), freed from urea by passing them on a Bio-Gel P-2 column (see legend of Figure 6), lyophilized to get rid of ammonium acetate and digested with spleen exonuclease under conditions similar to those described above. The digests (0.5 A_{260} unit) were loaded on 0.5 × 4 cm DEAE-cellulose columns (acetate form) to separate nucleosides which were eluted with water, from nucleotides which were subsequently eluted with 0.4 M ammonium acetate. Nucleotides were dephosphorylated essentially as already described. In this case, analyses of nucleosides were carried out on Bio-Gel P-2 columns on 0.02-A_{260} unit samples (Piperno and Bernardi, 1971). Figure 7 summarizes all the steps described above.

Preparation of Dinucleoside Monophosphates with Micrococcal Nuclease. This was done to provide material to use in investigations of the inhibition of venom exonuclease digestion of dinucleoside monophosphates by nucleotides. In addition, this material was used for several preliminary tests. Calf thymus DNA was digested with micrococcal nuclease (Worthington, code NFCP). Dinucleotides were isolated from the digest in 43% yield by chromatography on a DEAE-cellulose–urea column, readsorbed on a DEAE-cellulose column, and eluted with a solution of $(NH_4)_2CO_3$. This was then eliminated by submitting the dinucleotide solution to rotary evaporation and chromatography on a Sephadex G-10 column. Dinucleotides were dephosphorylated with *Escherichia coli* alkaline phosphatase (Worthington, code BAPF). Dinucleoside monophosphates were freed from residual dinucleotides and enzyme by chromatography on a Sephadex G-10 column. The purity of dinucleoside monophosphate preparation was checked by chromatography on a DEAE-cellulose–urea column: less than 0.1% dinucleotides were present.

Results

Acid DNase hydrolysate II had an average chain length of 9.3, as determined by estimating the amount of 3′-phosphate-terminal nucleotides (Carrara and Bernardi, 1968).

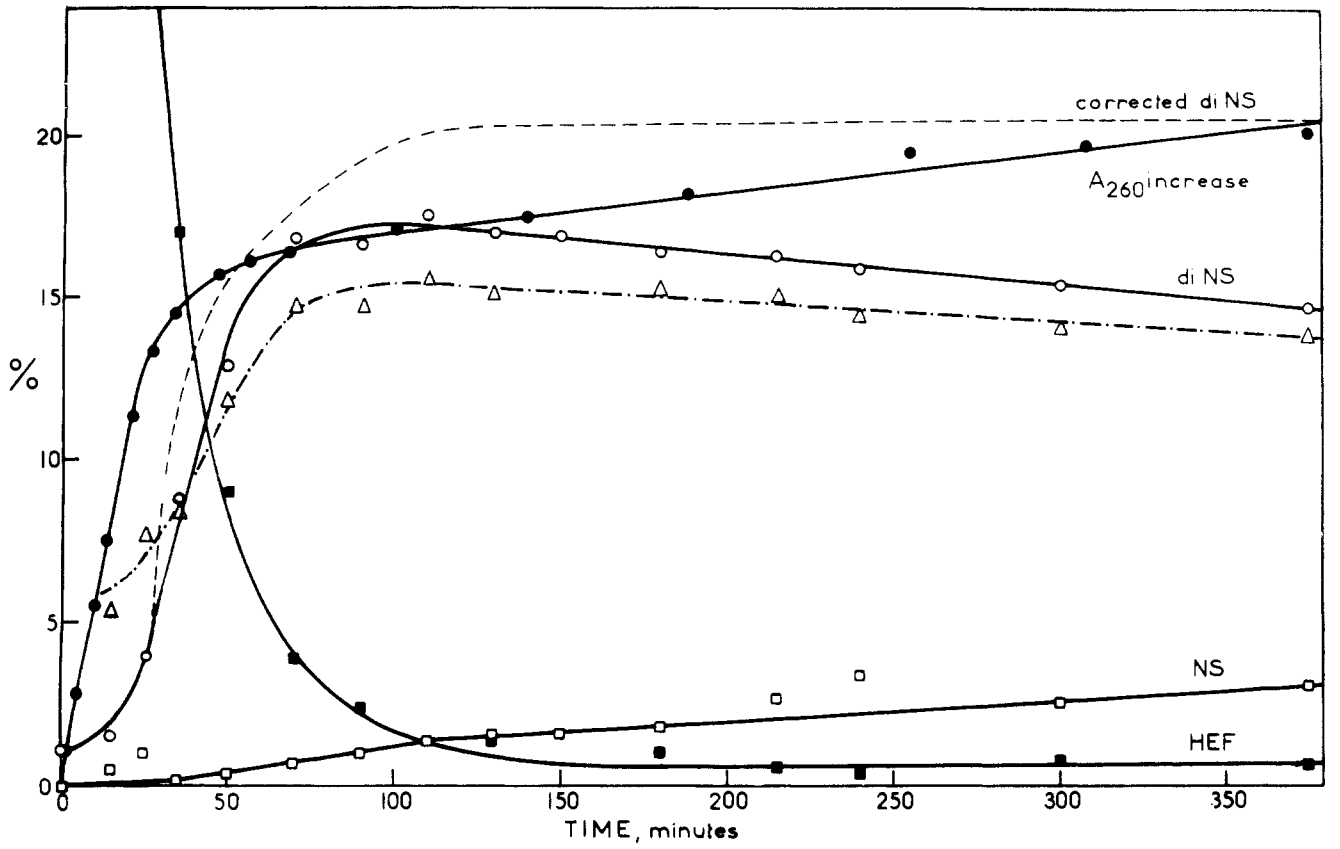

FIGURE 8: Snake venom exonuclease digestion of the acid DNase digest II treated with pancreatic DNase. Experimental conditions are given in Materials and Methods. The relative A_{260} increase (filled circles) was measured in 0.1-cm silica cells. Dinucleoside monophosphates (open circles) and nucleosides (open squares) were determined by chromatography on DEAE-cellulose–urea columns. The broken line shows the level of dinucleoside monophosphates after correction for their slow degradation; this was done by using the nucleoside determinations. Triangles indicate the dinucleoside monophosphates, as determined in independent experiments on the dephosphorylated exonuclease digests, using DEAE-cellulose columns. The high-eluting fraction (filled squares) was also determined in the latter series of experiments. Percentages of various classes of digestion products are given relative to the total material recovered from the columns.

The composition of the latter is given in Table I. Digests I and III had average chain lengths of 10.6 and 13.7, respectively.

As a first step, digests were dialyzed against water in order to reduce the ionic strength. If this is omitted, the pH changes needed in further steps lead to an ionic strength too high to allow the complete adsorption of dinucleoside monophosphates on DEAE-cellulose. The recovery of dialyzed fragments, as judged from A_{260} measurements, was 98%; this value is, however, somewhat overestimated because of the hyperchromic shift undergone by oligonucleotides when dialyzed against water. Expectedly, the average chain length of the dialyzed oligonucleotides was found to be slightly higher than before dialysis, a finding suggesting some loss of the smallest fragments. No change in terminal nucleotide composition was detected, however (Table I). Dialysis was done before and not after dephosphorylation since in the latter case losses were severe.

TABLE I: 3′-Phosphate-Terminal Nucleotides of Acid DNase Oligonucleotides.[a]

	Before Dialysis		After Dialysis	
T	20.2	21.0	20.5	20.3
C	9.4	9.4	9.4	8.3
G	38.5	39.1	39.0	40.7
A	31.8	30.5	31.2	30.7
Chain length	9.3		9.9	

[a] Analytical data from duplicate experiments on hydrolysate II are reported.

The 3′-phosphate oligonucleotides were then dephosphorylated since otherwise the 3′-phosphate ends would later inhibit venom exonuclease. Acid phosphatase was preferred to *E. coli* phosphatase, since a phosphatase-free incubation mixture is required for the next step, and the spleen enzyme can be easily inactivated.

The dephosphorylated acid DNase digest was degraded with pancreatic DNase to an average chain length close to 5. This step is very useful as it causes the breakdown of the larger oligonucleotides, which are rather resistant to venom exonuclease, presumably because of their secondary structure; besides, it leads to the formation of 5′-phosphate-ended oligonucleotides, which are better substrates for the enzyme than dephosphorylated oligonucleotides, as observed by both Razzell and Khorana (1959) and ourselves.

The digest was subsequently hydrolyzed with snake venom exonuclease. An investigation on the kinetics of hydrolysis showed the following features (Figure 8). (1) The ultraviolet absorption, which is the only readily measurable parameter while digestion is proceeding, shows an increase which is

TABLE II: 5′-Hydroxy-Terminal and Penultimate Nucleotides of Acid DNase Oligonucleotides.[a]

		Digests				
		I	II (90 min)[b]	II (110 min)[b]	II (Calcd)[c]	III
Terminal nucleotides	T	13.5	12.8	13.6	12.5	11.4
	C	29.6	31.1	30.8	30.9	30.4
	G	35.1	34.7	33.9	34.2	37.8
	A	21.8	21.4	21.7	22.3	20.2
Penultimate nucleotides	T	14.7	15.3	15.5	15.9	14.7
	C	9.7	9.4	8.8	9.2	8.7
	G	21.9	22.9	22.5	22.6	23.5
	A	53.6	52.4	53.2	52.3	53.1

[a] Results obtained at digestion times of maximal yield. [b] See Figures 7 and 10. [c] By extrapolation (see Figure 10 and text).

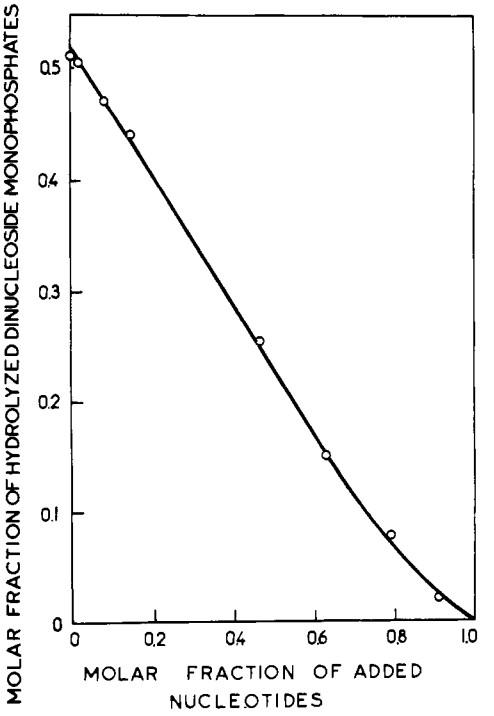

FIGURE 9: Inhibition of digestion of dinucleoside monophosphates by 5′-mononucleotides. Dinucleoside monophosphates obtained by micrococcal nuclease digestion (A_{260} 10.6 in 0.05 M ammonium acetate–0.025 M $MgCl_2$, pH 8.9) were incubated at 45° with venom exonuclease (0.05 unit/ml) for 30 min. The molar fraction of dinucleoside monophosphates hydrolyzed in the presence of different concentrations of 5′-mononucleotides was determined by isolating liberated nucleosides on DEAE-cellulose columns. The two points obtained at the highest levels of added nucleotides are affected by a relatively large experimental error for technical reasons.

rapid at the beginning of digestion and then becomes very slow. (2) Fragments higher than dinucleoside monophosphates (see also Figure 3) disappear in an exponential way during the fast increase of ultraviolet absorption. (3) The dinucleoside monophosphate level shows the following changes: (a) the initial low level due to the release of some 5′-hydroxy-terminal dinucleoside monophosphates by the action of pancreatic DNase is increased at the beginning of the digestion only very slowly, presumably because snake venom exonuclease is mainly active on the larger fragments; (b) a rapid linear increase leads then to a maximum, where about 90% of all expected terminals are present as dinucleoside monophosphates; this coincides with the transition between the fast and slow phases of ultraviolet hyperchromic shift; (c) a very slow decrease follows; this is accompanied by a slow liberation of nucleosides; if the level of dinucleoside monophosphates is corrected for their slow splitting, by using the nucleoside determinations, a plateau value equal to the theoretical one is found. (4) The pattern found for the dinucleoside monophosphates as determined in the dephosphorylated venom exonuclease digests (Figure 8; triangles) is similar to that found in undephosphorylated digests with two differences, however: (a) in the early phase of digestion the values found in the dephosphorylated digests are higher because of the contribution of the dinucleotides produced by pancreatic DNase; these, however, disappear rapidly because they are digested venom exonuclease; (b) in the middle and late phases of digestion the values are systematically lower; this is, in all likeliness, due to the hypochromism of dinucleoside monophosphates in the absence of urea.

Digestion with venom exonuclease was not pushed to the end with complete liberation of 5′-hydroxy-terminal nucleosides for the reason that the reaction is very strongly inhibited by 5′-mononucleotides as shown by the experiment of Figure 9. In an attempt to reach a complete digestion, the enzyme was used at an activity level 16 times higher than indicated. This required concentrating the enzyme by ultrafiltration (using an Amicon, Oosterhout, Holland, ultrafiltration system) a step which caused a 50% loss on specific activity of the enzyme. Hydrolysis under these conditions still left 10% dinucleoside monophosphates undigested and led, in addition, to a slight dephosphorylation of the mononucleotides by traces of phosphatase present in the enzyme. Terminal nucleotides liberated in this experiment were T 14.3%, C 27.6%, G 37.0%, and A 21.1%, a result not too far from those found by the analysis of dinucleoside monophosphates (see below and Table II).

The dinucleoside monophosphates were isolated from the venom exonuclease digests (Figure 3) and subsequently split with spleen exonuclease into terminal nucleotides and penultimate nucleosides; these were separated on DEAE-cellulose and analyzed on Sephadex G-10 (Figure 5). When the analytical results obtained with terminal and penultimate nucleotides are plotted against digestion time (Figure 10), it appears that some changes in the composition of both terminals and penultimates occur in the early phase of dinucleoside monophosphate liberation. These changes can be explained as follows. (a) Dinucleoside monophosphates formed by pancreatic DNase action by splitting bonds next to the 5′-hydroxy-terminal end, and reflecting therefore in their composition the specificity of this enzyme, are already present in the digest before the addition of venom exonuclease; these dinucleoside monophosphates, forming about 7% of the maximal amount found later in the digest are barely split by venom exonuclease as shown by the extremely slow initial release of nucleosides (Figure 7); the reason for this is that they are a very poor substrate compared to the much more abundant 5′-phosphate oligonucleotides present in the incubation mixture (see Discussion). (b) Dephosphorylation

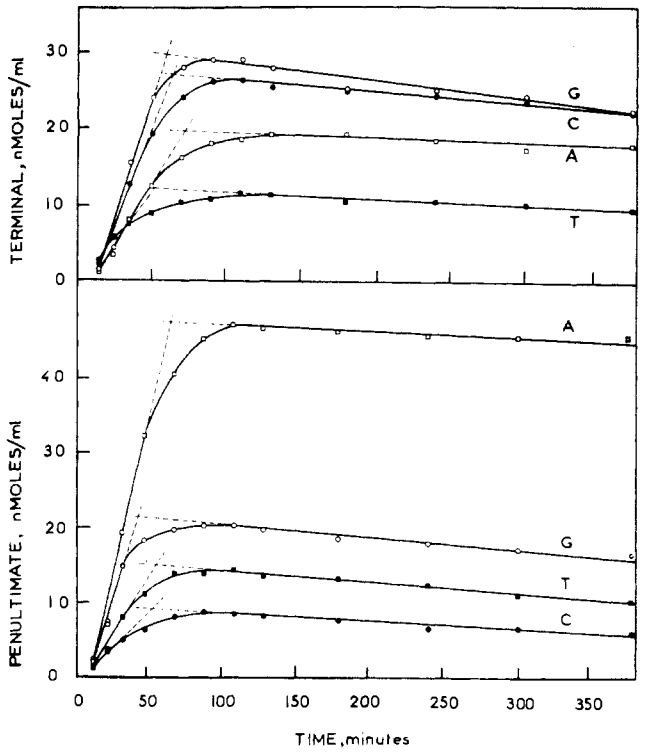

FIGURE 10: Kinetics of liberation of 5′-terminal (upper frame) and penultimate (lower frame) nucleotides released by digestion of acid DNase hydrolysate II with venom exonuclease. Values are given as nanomoles of nucleotides per milliliter of the incubation mixture.

of the venom exonuclease digest may lead to the liberation of dinucleoside monophosphates from dinucleotides released by pancreatic DNase. The formation of dinucleoside monophosphates by this mechanism is, however, offset by the fact that dinucleotides are very rapidly split by venom exonuclease, as shown by the comparison of the level of dinucleoside monophosphates in undephosphorylated and dephosphorylated samples (Figure 8) and by the results obtained on hydrolysate III, which was not dephosphorylated after the venom exonuclease digestion (see Table II). (c) An additional factor contributing to the variation of the composition of monophosphates may also be their nonrandom liberation from longer oligonucleotides by venom exonuclease, since the first ones will be liberated from the oligonucleotides which are digested most rapidly.

When the initial phase is over, a much smaller variation in the composition of terminal and penultimate nucleotides takes place as shown by the slightly different final slopes of Figure 10. This reflects the preferential splitting of some classes of dinucleoside monophosphates by venom exonuclease. In order to obtain correct results, we determined the composition of terminal and penultimate nucleotides at the intersection of the initial and final slopes present in the release curve of each nucleotide (Figure 10). It appears from the data of Table II that these calculated values agree very well with the two sets of experimental values obtained at digestion times corresponding to the maximal yields of dinucleoside monophosphates. It is, therefore, possible to obtain correct results simply by analyzing samples taken at digestion times giving maximal yields of dinucleoside monophosphates. The choice of these digestion times can be easily made by following the hyperchromic shift, thus avoiding any need for a detailed investigation in the kinetics of dinucleoside monophosphate liberation.

Discussion

Several points concerning the steps used in the procedure developed in the present work have been discussed in the previous section for the sake of clarity. We will therefore restrict the discussion to the following problems: (a) the accumulation of dinucleoside monophosphates in the venom exonuclease digest, and (b) the reproducibility of the results.

The successful determination of the 5′-hydroxy-terminal and penultimate nucleotides is essentially due to the recognition that 5′-hydroxy-terminal dinucleoside monophosphates accumulate during the venom exonuclease degradation to the point that almost theoretical yields can be obtained. It is interesting to understand why this accumulation takes place. Razzell and Khorana (1959) showed that the affinity of venom exonuclease for oligothymidilates increases exponentially with increasing chain length: for example, the affinity for $pT(pT)_6$ is about three orders of magnitude higher than that for pTpT. Dephosphorylated oligomers show the same phenomenon, except that the affinity of the enzyme for this homologous series is systematically lower than that for the phosphorylated ones. Dinucleoside monophosphates are therefore the oligonucleotides for which the enzyme has the least affinity. In addition, TpT is degraded by venom exonuclease at a V_{max} about 25 times lower than pTpT (Razzell and Khorana, 1959). The very low K_m and V_{max} of the enzyme for dinucleoside monophosphates certainly would lead by themselves to an accumulation of these products in the incubation mixture. In fact, as shown here, an additional, very important factor is the exponential formation of 5′-mononucleotides which are competitive inhibitors of the enzyme. As already mentioned, the accumulation of 5′-mononucleotides is such that it prevents the splitting of terminal dinucleoside monophosphates to go to completion even in the presence of large amounts of enzyme.

Concerning the reproducibility of the results obtained, it should be stressed that careful control experiments were done on each single step of the rather complex procedure used. Some of these experiments were already mentioned in the two previous sections. Other controls and further comments are given here. (a) Table II shows that the terminal and penultimate results obtained on three independent acid DNase digests are in satisfactory agreement with each other. The slight differences found in the results from hydrolysate III compared with the other two might be due to the fact that all steps were done on much smaller amounts of materials; particularly, the analysis was done on a scale 100 times lower than in the other two series. (b) The reproducibility of the analytical results obtained by running duplicate samples taken from venom exonuclease digests (hydrolysate I) through the different steps of our procedure: analyses of 5′-hydroxy-terminal and penultimate nucleotides were found to be within 1% from each other. (c) The procedure used for hydrolysate III was checked by running previously analyzed dinucleoside monophosphates, derived from hydrolysate II, through all the steps. The final analysis agreed within 2% with the original one. (d) Finally, it should be stressed that the results reported here were obtained under a single set of experimental conditions (solvent and temperature) and at, or near, a single average chain length value.

In conclusion the two main results of the present work are (a) the first determination of the 5′-hydroxy- and penulti-

TABLE III: Average Composition of Sequences Split by Acid DNase.[a]

	3′-Phosphate Terminal	5′-Hydroxy Terminal	5′-Hydroxy Penultimate
T	21	13 (26.8)[b]	16 (27.9)[b]
C	9	31 (22.0)	9 (22.9)
G	39	34 (23.6)	23 (19.7)
A	31	22 (27.6)	52 (29.5)

[a] Average chain length of the digest (hydrolysate II) was 9.3. [b] Nearest neighbors of 3′-phosphate and 5′-hydroxy terminals, respectively, as calculated on the basis of the data of Swartz *et al.* (1962).

mate nucleotides of DNA oligonucleotides obtained by an enzymatic degradation; (b) the demonstration that acid DNase can recognize a sequence of at least three nucleotides on native DNA as shown by the fact that the enzyme produces 3′-phosphate-terminal nucleotides which are far from those expected from the random degradation of a DNA having the base composition of calf thymus DNA, and that the 5′-hydroxy-terminal and penultimate nucleotides are far from those expected as the nearest neighbors of the 3′-phosphate terminals and 5′-terminals, respectively. Table III summarizes the average composition of the sequences split by acid DNase. We will refrain here from commenting on the specificity of acid DNase, since this will be more appropriately done after current investigations on the nucleotide sequences in 5′-hydroxy-terminal dinucleoside monophosphates and on the composition of terminal nucleotides as a function of average chain length will be completed. We will also not comment on results obtained by previous authors (Laurila and Laskowski, 1957; Koerner and Sinsheimer, 1957; Doskocil and Sorm, 1961a,b, 1962; Vanecko and Laskowski, 1962) since the prerequisites for valid end group determinations (particularly enzyme purity) were only partially met in those pioneering investigations. We should mention, however, that the 5′-hydroxy-terminal nucleotides of oligonucleotides recently obtained from ox brain acid DNase (Rosenbluth and Sung, 1969) are not very far from those found in the present work.

Acknowledgment

The spleen exonuclease preparation used in the present as well as in other recent investigations from this laboratory had been obtained by Dr. Alberto Bernardi, to whom we wish to express our gratitude.

Appendix: Chromatographic Purification of Snake Venom Exonuclease

The following is a new procedure for obtaining snake venom exonuclease in a phosphatase-free form. The essential step in the procedure is a chromatography on hydroxylapatite. Since all the steps were done in phosphate buffers, a new assay had to be set up for detecting contaminating phosphatase.

Materials. Crotalus adamanteus venom (lot 125 B-0560) was obtained from Sigma (St. Louis, Mo.). Adenosine 5′-phosphate and the *p*-nitrophenyl ester of thymidine 5′-phosphate were purchased from Calbiochem (Los Angeles, Calif.).

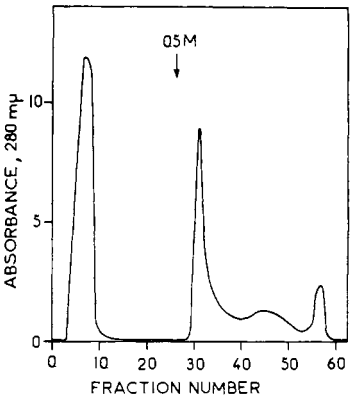

FIGURE 11: Chromatography of snake venom of DEAE-Sephadex A-25. Snake venom (1 g) dissolved in 49 ml of 0.001 M KH_2PO_4 was loaded on a 2.5 × 17 cm column equilibrated with the same solvent. This was then used to wash through the exonuclease and phosphatase activities. The endonuclease activity was eluted by a 0.5 M K_2HPO_4 step. Fractions (12 ml) were collected. Flow rate was 60 ml/hr. The continuous line indicates the absorption at 280 mμ (left-hand scale).

Assay of Enzymatic Activities. Exonuclease activity was assayed by measuring the liberation of *p*-nitrophenol from the *p*-nitrophenyl ester of thymidine 5′-phosphate under conditions similar to those described by Razzell and Khorana (1959). The reaction mixture (total volume 1 ml) contained: (a) 0.5 μmole of substrate; 100 μmoles of Tris adjusted to pH 8.9 with HCl; (b) enzyme; this was diluted, if necessary, with 0.1 M Tris-HCl buffer (pH 8.9) containing 0.05% bovine serum albumin (crystallized and lyophilized, Sigma). After 10-min incubation at 37°, the reaction was stopped by adding

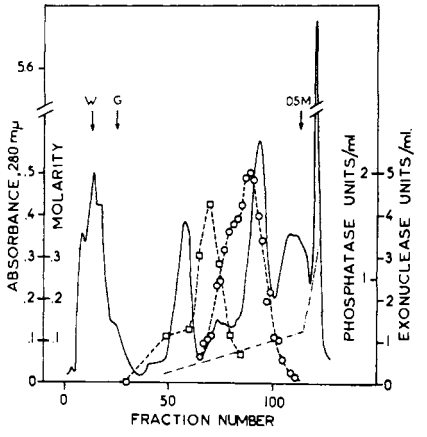

FIGURE 12: Fractions 3–9 (84 ml) from the chromatogram shown in Figure 1 were adjusted to pH 6.8 with 1 M KH_2PO_4 (0.84 ml) and to 0.005 M phosphate with 1 M phosphate buffer (pH 6.8, 0.25 ml). They were then loaded on a 2 × 55 cm CM-Sephadex C-50 column equilibrated with 0.005 M potassium phosphate. The column was then washed with 120 ml of this buffer. A phosphate molarity gradient (0.005–0.15 M; total volume 1200 ml) was used to elute phosphatase and exonuclease activities. A 0.5 M phosphate step was used to elute other proteins retained by the column. Flow rate was 40 ml/hr. Circles and squares indicate the exonuclease activity (right-hand outer scale) and phosphatase (right-hand inner scale), respectively.

TABLE IV: Chromatographic Purification of Snake Venom Exonuclease.

	Vol (ml)	Total A_{280}	Exonuclease Total Units	Exonuclease Sp Act.	Exonuclease Recov (%)	Phosphatase Total Units	Phosphatase Sp Act.	Exonuclease/ Phosphatase
Snake venom	48	1296	1977	1.5	100	2429	1.8	0.8
Step I (DEAE-Sephadex)	84	511	1974	3.6	100	1865	3.4	1.0
Step II (CM-Sephadex)	480	112	1358	12.1	69	457	4.1	2.9
Step III (hydroxylapatite)								
Fractions 75–87	149	21	460	21.2	23	0.08	0.0038	5750
Fractions 88–106	218	35	689	19.5	35	0.46	0.013	1500

0.2 ml of 2 N NaOH. The absorbance at 400 mμ was measured within 10 min (the yellow color being stable for at least 1 hr) and a suitable blank was subtracted. One activity unit is defined as the amount of enzyme that liberates 1 μmole of *p*-nitrophenol/min under the conditions specified above. The specific activity was calculated by dividing the activity by the absorbance at 280 mμ of the enzyme solution. Assays were performed using enzyme concentrations such as to obtain A_{400} readings, corrected for the blank, not higher than 1. Under these conditions, a linear relationship was obtained between enzyme concentration and *p*-nitrophenol liberation.

The phosphatase activity was assayed by measuring the dephosphorylation of 5′-AMP under conditions similar to those of Koerner and Sinsheimer (1957). The reaction mixture (total volume 1 ml) contained: (a) 3 μmoles of 5′-AMP; 10 μmoles of $MgCl_2$; 100 μmoles of glycine adjusted to pH 9.0 with NaOH; (b) enzyme; this was diluted, if necessary, with 0.1 M glycine buffer–0.01 M $MgCl_2$ (pH 9.0) containing 0.05% serum albumin. After 10-min incubation at 37°, the reaction was stopped by emulsifying the mixture with 0.2 ml of chloroform–isoamyl alcohol (20:1, v/v). 500 μl of the clear aqueous phase were loaded on a 1 × 8 cm column of DEAE-cellulose (Serva, Heidelberg, Germany; 0.57 mequiv/g; washed, in succession, with 1 N HCl, water, 1 N NaOH, water, 0.5 M glycine (pH 9.0), and water). The liberated adenosine was washed through with water, under ultraviolet monitoring, and collected in a 10-ml volumetric flask; 1 ml of N HCl was added; the solution was then brought to the mark and its A_{257} was measured. More than 12 samples could be loaded in succession on the same column. This could be regenerated by washing with 0.5 M glycine (pH 9.0) and water. One activity unit is defined as the amount of enzyme that liberates 1 μmole of adenosine in 1 min, under the conditions given above. The specific activity was calculated by dividing the activity by the absorbance at 280 mμ of the enzyme solution. Assays were performed using enzyme concentrations such as to obtain A_{257} readings not higher than 0.8. A linear relationship was obtained between enzyme concentration and *p*-nitrophenol liberation at least up to this value. The endonuclease activity was assayed as for spleen acid DNase (Bernardi *et al.*, 1966) except that the DNA solvent was 0.05 M ammonium acetate (pH 5.6).

The isolation of the enzyme involved three chromatographic steps (Table IV). All operations were performed at 4°. Snake venom (1 g) was dissolved in 0.001 M K_2HPO_4 (pH 9.1, 42 ml) and clarified by low-speed centrifugation. The sediment was dispersed in the same buffer (8 ml) and centrifuged. The pooled supernatants (49 ml) were loaded on a DEAE-Sephadex column (step I, Figure 11) equilibrated with the same solvent. Exonuclease and phosphatase activities were not retained, whereas endonuclease activity was, and could be eluted by a 0.5 M K_2HPO_4 step. The nonretained material was adjusted to 0.005 M potassium phosphate (pH 6.8) and chromatographed on CM-Sephadex column (step II, Figure 12). A partial separation of the phosphatase and exonuclease activities was obtained along with a relatively high increase in the specific activity of the latter enzyme.

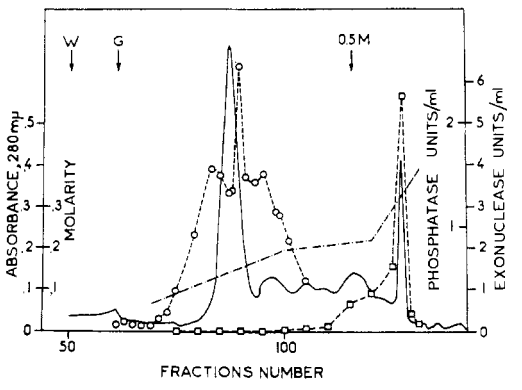

FIGURE 13: Fractions 65–105 (480 ml) from the chromatogram shown in Figure 2 were diluted to 600 ml with water and loaded on a 2 × 38.5 cm hydroxylapatite column equilibrated with 0.08 M phosphate buffer (pH 6.8). Elution was carried out with a molarity gradient of phosphate buffer (pH 6.8). Flow rate was 100 ml/hr. Other indications as in Figure 2.

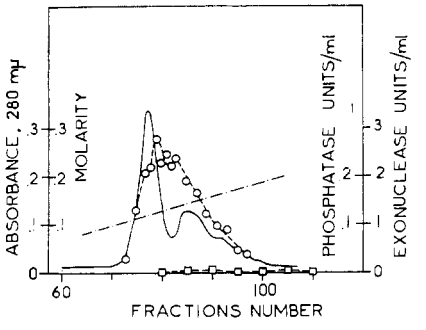

FIGURE 14: Fractions 88–106 (214 ml) from the chromatogram shown in Figure 3 were diluted to 508 ml with water, and loaded on the same column used in the previous step, after reequilibration with 0.08 M phosphate buffer. Elution was carried out as in the previous experiment.

The exonuclease-rich fractions from the previous step were chromatographed on hydroxylapatite (step III, Figure 13). An excellent separation of the phosphatase and exonuclease activities was obtained. The high-eluting side of the exonuclease activity peak still contained, however, some phosphatase activity and was therefore rechromatographed on hydroxyapatite (Figure 14). Interestingly enough, the exonuclease activity was eluted in a series of peaks, suggesting a heterogeneity in the enzyme molecules. The enzyme, as obtained by this method, is extremely stable at 4° in alkaline buffers. After dialysis against 0.1 M Tris-HCl (pH 9.0) in order to eliminate inhibitory phosphate, the low-eluting part of the enzyme from the rechromatography on hydroxylapatite (these fractions were used in all the work described above) showed no phosphatase activity under conditions where 2×10^{-4} phosphatase unit/exonuclease unit could have been detected.

This novel procedure has two main advantages over the method most recently developed in Laskowski's laboratory (Richards *et al.*, 1967): (a) the purification only involves chromatographic steps, avoiding cumbersome precipitations with acetone and ethanol at −17°; (b) enzyme yield is extremely high: 23% of the exonuclease activity present in the venom is obtained in an essentially phosphatase-free form after step III; an additional 35% of the activity can be obtained at an even higher level of purity after one more hydroxylapatite step.

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Serologic Specificities of Methylated Base Immune Systems*

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ABSTRACT: Rabbits immunized with conjugates of 1-methylguanosine–human serum albumin, N^2-dimethylguanosine–albumin, 7-methylguanosine–albumin, N^2-methylguanosine–albumin, and 5-methylcytidine–albumin produce antibodies that recognize the position of the methyl group(s) on the base. These serologic specificities were determined by gel diffusion experiments in agar containing an excess of the carrier albumin, thus eliminating the albumin antigen–antibody reaction. A minor population of the antibodies in several of the serums recognizes the normal bases. When measured by complement fixation, these latter antibodies are eliminated by dilution.

Hapten inhibition of complement fixation confirms the specificities of these immune systems.

The production of antibodies with narrow specificity toward nucleic acids is observed only in the T-even bacteriophage DNA (Murakami *et al.*, 1962; Townsend *et al.*, 1965), irradiated DNA (Levine *et al.*, 1966; Van Vunakis *et al.*, 1966), and double- and triple-stranded polyribonucleotide immune systems (Stollar, 1970). With the exception of the "conformation" specificity of antibodies to the multistrand polyribonucleotides, narrow specificities in nucleic acid immune systems result from the immunodominance of "unique" bases that either occur in nature or are produced by a chemical modification of a normal base, Thus, specificity of anti-bacteriophage DNA and the anti-irradiated DNAs has been shown to be due to the glucosylated hydroxymethylcytosine in the T-even coliphage DNAs and to the guanine photoproducts resulting from photooxidation of DNA (Van Vunakis *et al.*, 1966; Levine *et al.*, 1968) or thymine dimers resulting

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