Studies on Acid Deoxyribonuclease. IX. 5'-Hydroxy-Terminal and Penultimate Nucleotides of Oligonucleotides Obtained from Calf Thymus Deoxyribonuclease 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 high spool acid DNAse. The essential point in this procedure is the release, in high yield of the 5'-hydroxy-terminal dinucleotide monophosphates produced by the action of snake venom exonuclease. The dinucleotide monophosphates are then split with snake venom exonuclease; the resulting nucleoside 5'-monophosphates, 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-terminus 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 DNA. This opinion is rather due to the difficulty of demonstrating a specificity in DNases than to a well-documented lack of specificity. As a matter of fact, DNases having the evident specificity of pancreatic RNAse or T4 RNAse, for instance, have not been found yet. Since all four nucleosides are present in the termini formed by DNase, 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 extensively 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 technique in this area. This has been used, so far, to investigate the specificity of acid DNase from hog spool.

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 dinucleotide 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 new 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 spool acid DNase digestion, are dephosphorylated (step 1); treated with pancreatic RNAse and digested with venom exonuclease (step 2) to release the 5'-hydroxy-terminal mononucleotides and 3'-phosphate-terminal mononucleotides as nucleosides. 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-terminus 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.
Acid DNase Digestion. A DNA solution (268.5 μl; preparation CTIR II, obtained from calf thymus using the detergent procedure; Bernardi and Sodiro, 1964, method 20, 4 ml, 0.05 M ammonium acetate-0.008 μM EDTA (pH 5.6) were digested at room temperature (22-23 °C) with 0.12 μl 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 μm reached 35%, the enzyme was inactivated by shaking the digestion mixture with one-tenth volume of chloroform-isomyl alcohol (24:1, v/v, CA).1

1 Abbreviations that are not listed in Biochemistry: 3, 144° (1966), 20% CA, chloroform-isomyl alcohol mixture (24:1, v/v).

Diagnosis of Acid DNase Digest. Viscosity % in unw. tubes, bold first in 105 °C sodium carbonate-0.03 μM EDTA, then in water, were used. Dialysis was done at room temperature by letting water flow through a glass tube having a diameter slightly larger than the dialysis tubing. Acid DNase digest (25 ml) was dialyzed against 175 l. of distilled water, using a flow rate of 7.41 l/hr. The removal of salt was complete as judged by refractometric measurement.

Pepsin digestion. Acid DNase (5.1 ml) (Chesi, A., Bernardi, A., and Bernardi, G., 1971, submitted for publication; 0.07 unit/ml of digestion mixture; units as defined by Chesi et al., 1966). 0.25 ml of 1 M Tris (final concentration 1.2 M) and enough 0.1 M acetic acid to reach pH 5.6 were added to 204 ml of dialyzed acid DNase digest (Aoa 10 M). Digestion was done at 25° for 19 h. The enzyme was inactivated with CA. Control experiments showed that the average chain length of the oligonucleotides was constant between 14- and 18-mer digestion, thus indicating that dephosphorylation at pH 19 hr was complete.

Pepsin Diastase Digestion. McCh (1 ml) was added to the dephosphorylated oligonucleotides to reach a final concentration of 5 mg/ml and enough 1 M Tris solution to reach pH 7.5 (final concentration 3.25 M). Crystalline pancreatic DNase (13 μg) (Worthington, Freehold, N. J., code D) was then added to 190 ml of oligonucleotides (Aoa 9.5). After 3.5 hr digestion at 37° the enzyme was inactivated with CA. No nucleic acids could be detected. The supernatant was dialyzed against 1M phosphate buffer, pH 7.5. When an aliquot of 7 ml was loaded on a 1-10 × 10 cm DEAE-cellulose column (acetate form) indicating that no 5'-hydroxyl terminal nucleoside had been released by pancreatic DNase.

Vesicle Exonuclease Digestion. Oligonucleotide solution (155 ml; Aoa 9.9) in 0.018 M Tris-acetate-0.005 M McCh (pH 8.9) (a solut obtained by adding 1 M Tris to the pepsin-diastase 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 chromophotometry of venom exonuclease digest was done on 2-mm discs 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. 8 should be pointed out that in the absence of urea dinucleoside mono-

FIGURE 2: DEAE-cellulose chromatogram obtained with a venom exonuclease digest. 1.296 ml aliquots from the equilibration mixture (digest time 90 min.; Aoa 10.3) was adjusted to pH 7 with 1 M acetic acid, then dialysed with 6 ml of water and loaded on a 1.1 × 12 cm DEAE-cellulose column (initial form) equilibrated with water. Nucleotides (N) were washed through with water, the column was then washed with 7 ml of water (arrow 3); the base line shift is due to the breakthrough of the urea solutions. As soon as the columns was equilibrated with urea, a linear molarity gradient (0-0.25 M) of ammonium acetate containing 7 M urea (10 M Tris) (arrow 2) with flow rate 50 ml/hr. Dinucleoside monophosphates (NMP) were eluted at the beginning of the gradient and the 0.1 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 absorbance at 263.7 μm of the column effluent as recorded by a 1-WA/B-3A equipped with a 0.3 cm cell. One division on the abscissa corresponds to about 20 min.
phosphates are poorly separated from the large amounts of nucleotides present in the digest.

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

The 80 mg of unextracted urine digests were dephosphorylated using 0.07 unit/ml of acid phosphatase.

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**Figure 3**: DEAE-cellulose chromatogram of a dephosphorylated venous exonucleolus digest. An 8-ml sample (dissolution time, 30 min; (Celite, 10 g) was diluted to 160 ml with CO₂-free water and loaded on a 1.1 X 24 cm DEAE-cellulose column equilibrated with water, using a flow rate of 11 ml/hr. The column was then washed with CO₂-free water, using the same flow rate, until all the nucleotides (NS) were eluted. Elution was then performed, at 90 ml/hr, with an ammonium acetate gradient. The total volume was 150 ml. Three Varel glass (Technicon, Chvaletz, 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 (AN). Pyrimidine nucleotides, present in the mixture because of incomplete dephosphorylation, were eluted at the end of the gradient peak as, then a step of 1.4 m ammonium acetate was applied to the column to elute a "high-affinity fraction" (peak B), which contained mononucleotide and purine nucleotides and di- and oligo-nucleotides of higher size than dinucleoside monophosphates (figure). The figure shows the absorbancy at 257.5 m of the column effluent as recorded by a LKB-UV/Vis, equipped with a 0.9-

**Figure 4**: Chromatography of nucleosides on Sephadex G-10 cell umns. Nucleosides (27-40 mg final) in 0.1-0.15 ml of 0.025 m ammonium carbonate (pH 10.5) were loaded on a 0.5 X 30 cm columns of Sephadex G-10. Fractionation by deacylation (parlaidic acid 2.40) and equilibrated with the same buffer, elution was done with the same buffer for a time equal to 5.2 ml/hr. The chromatography was recorded by an LKB-UV/Vis, using a silica cell with an optical path equal to 0.1 cm. Each fraction on the absorbance was equal to about 22 min. The fractions were collected and counted (spectrometer, centrifuge). The solution was adjusted to pH 3.0 and the content of each nucleoside was determined by its absorption at 249 m. The chromatography of 5'-terminal nucleosides (elution time with respect to "high--NS 13 min") and 5' phosphonate nucleosides (elution time 215 min) are expressed by the upper and lower diagonal, respectively.

**Figure 5**: Chromatography of nucleosides on Agarose G-10 cell umns. Nucleosides (27-40 mg final) in 0.1-0.15 ml of 0.025 m ammonium carbonate (pH 10.5) were loaded on a 0.5 X 30 cm columns of Sephadex G-10. Fractionation of nucleosides was performed on an LKB-UV/Vis, using a silica cell with an optical path equal to 0.1 cm. Each fraction on the absorbance was equal to about 22 min. The fractions were collected and counted (spectrometer, centrifuge). The solution was adjusted to pH 3.0 and the content of each nucleoside was determined by its absorption at 249 m. The chromatography of 5'-terminal nucleosides (elution time with respect to "high--NS 13 min") and 5' phosphonate nucleosides (elution time 215 min) are expressed by the upper and lower diagonal, respectively.

**Figure 6**: A Bio-Gel P-2 chromatogram of the dinucleoside monophosphates obtained from hydrolysate III. The solution of dinucleoside monophosphates in 1 ml ura (see Figure 3) 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 X 90 cm Bio-Gel P-2 column, equilibrated with 2.5 m ammonium carbonate, (pH 10.4). The figure shows absorbance at 257.5 m of the column effluent as recorded by a LKB-UV/Vis equipped with a 0.9-cm cell. One-dim us of the absorbance corresponds to about 1 cm. The small peak at 30 min indicates the breakthrough of ura.

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DNA

3'-phosphate oligonucleotides

analyzed

3'-hydroxy oligonucleotides

3'-hydroxy oligonucleotides

5'-hydroxy oligonucleotides

5'-phosphate nucleotides

nucleotides monophosphates 5'-phosphate nucleotides

monophosphates + nucleotides

nucleotides + 3'-phosphate nucleotides

DEAE-cellulose

nucleotides (5'-hydroxy-terminals)

(5'-hydroxy-terminals)


diagram 7: Scheme of the procedure used for the analysis of 5'-hydroxy-terminal and penultimate nucleotides. The broken line indicates the procedure followed for preparation III.

1 M ammonium acetate (pH 5.5) to 0.015-0.05 M. If necessary, the pH was corrected by addition of 1 N acetic acid. Ammonium phosphate was added to a concentration of 0.1 unit/ml. Digestion was done at 22 °C 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} 0.075) was loaded on a 0.8 x 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 95-99%. The residual ultraviolet-absorbing material was eluted with a step of 1 M ammonium acetate, and was shown to consist of dephosphorylated nucleotides, residual dinucleoside monophosphates, and ultraviolet-absorbing material present in the ammonium carbonate solution used at the previous step to elute nucleosides.

Analysis of Nucleotides. This was done on Sephadex G-20 columns (2.6 x 20 cm) (D. Ehrlich, J. P. Thierry, and G. Bernardi, HPL, submitted for publication; see legend of Figure 4). Hydrolyzate III was not dephosphorylated after the venom nucleosome digestion. Further processing of this sample involved some steps different from those used for hydrolysates I and II: a dinucleoside monophosphates were isolated from the aliquot of venom exonucleolytic 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 trichloroacetic acid under conditions similar to those described above. The digests (0.3 A_{260} unit) were loaded on 0.5 x 4 cm DEAE-cellulose columns (acetate form) to separate nucleotides 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 nucleotides were carried out on a Sephadex G-20 column on 0.05-0.4 M 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 NHCP). Dinucleotides were isolated from the digest in 43% yield by chromatography on a DEAE-cellulose-urea column, redissolved in a DEAE-cellulose column, and eluted with a solution of (NH₄)₂CO₃. 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 RAPP). 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 hydrolyzate H had an average chain length of 9.3, as determined by estimating the amount of 3'-phosphate-terminal nucleotides (Carrara and Bernardi, 1968).
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.

A first step, digestes 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 dinucleotide 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. Fortunately, the average chain length of the dialyzed oligonucleotides was found to be slightly higher than before dialysis, thus suggesting some loss of the longer fragments. No change in the terminal nucleotide composition was detected, however (Table I). Dialysis was done before and not after dephosphorylation, since in the latter case losses were severe.

The 5'-phosphate oligonucleotides were then dephosphorylated since, otherwise the 3'-phosphate end, which inhibits venom exonuclease, Acid phosphatase was prefered to E. coli phosphatase, since a phosphate-free inclusion 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 3. 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 exopeptidase than dephosphorylated oligonucleotides, as observed by both Buzzell and Khoury (1959) and ourselves.

The digest was subsequently hydrolyzed with snake venom exonuclease. An investigation in 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 I: 5'-Phosphate-Terminal Nucleotides of Acid DNase Oligonucleotides

<table>
<thead>
<tr>
<th>Before Dialysis</th>
<th>After Dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>20.2, 21.0</td>
</tr>
<tr>
<td>C</td>
<td>9.4, 9.6</td>
</tr>
<tr>
<td>G</td>
<td>38.5, 38.5</td>
</tr>
<tr>
<td>S</td>
<td>31.5, 30.5</td>
</tr>
</tbody>
</table>

*Analytical data from duplicate experiments on hydrolysate II are reported.*

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TABLE II: 5'-Hydroxy-Terminal and Penultimate Nucleotides of Acid DNase Digests of 5'-Mononucleotides.

<table>
<thead>
<tr>
<th>Digests</th>
<th>II (90)</th>
<th>II (110)</th>
<th>II (Calc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>13.5</td>
<td>12.8</td>
<td>13.6</td>
</tr>
<tr>
<td>nucleotides</td>
<td></td>
<td></td>
<td>12.5</td>
</tr>
<tr>
<td>C</td>
<td>29.6</td>
<td>31.1</td>
<td>30.8</td>
</tr>
<tr>
<td>G</td>
<td>35.1</td>
<td>34.7</td>
<td>33.9</td>
</tr>
<tr>
<td>A</td>
<td>21.7</td>
<td>22.1</td>
<td>22.2</td>
</tr>
</tbody>
</table>

Penultimate nucleotides

| C       | 9.7     | 9.4      | 8.8       | 8.2       |
| G       | 21.9    | 22.9     | 22.5      | 22.6      |
| A       | 53.6    | 52.4     | 53.2      | 52.3      | 33.1      |

- Results obtained at digestion times of maximal yield.

† See Figures 7 and 10. By extrapolation (see Figure 10 and text).

![Graph](image)

**Figure 9:** Inhibition of digestion of dinucleoside monophosphates by 5'-mononucleotides. Dinucleoside monophosphates obtained by micrococcal nuclease digestion (4.0 l, 10.0 in 0.05 M ammonium acetate; 0.025 M MgCl₂, pH 8.9) were incubated at 25° 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 noting the liberation of 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.

The dinucleoside phosphates were isolated from the venom exonuclease digests (Figure 3) and subsequently split with spleen exonuclease into terminal nucleotides and penultimate nucleotides; these were separated on DEAE-cellulose and analyzed on Sephadex G-10 (Figure 5). When the analytical results obtained with terminal and penultimate nucleo-
tides are plotted against digestion time (Figure 10), it appears that some changes in the composition of both terminals and penultimate nucleotides occur in the early phase of dinucleoside monophosphate liberation. These changes can be ex-
plained as follows. (a) Dinucleoside monophosphates formed by pancreatic DNase action by splitting bonds next to the 5'-hydroxyl-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 nucleotides (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) Diphosphorylation...
of the venom exocinase 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 undenatured and denatured and samples (Figure 8) and by the results obtained with hydrolytic III, which was not denatured after the venom exonuclease digestion (see Table I). (c) An additional factor contributing to the variation of the composition of mononucleotides may also be their nonrandom liberation from longer oligonucleotides by venom exonuclease, since the free ones will be liberated from the oligonucleotides which are digested most readily.

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 effect 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 hyperbolic shift, thus avoiding

any need for a detailed investigation in the kinetics of dinucleoside monophosphate liberation.

Discussion

Several points concerning the steps used is the procedure developed in the present section for the sake of clarity. We will therefore retract the discussion to the following problems: (a) the accumulation of dinucleoside monophosphates in the venom exonuclease digest, and (b) the reproductibility of the results. The successful determination of the 5'-hydroxy-terminal and penultimate nucleotide was an indication that 5'-hydroxy-terminal dinucleoside monophosphates accumulate during the venom exonuclease degradation is the point that allows here. It is interesting to understand why this accumulation takes place. Razzell and Khorana (1959) showed that the affinity of the venom exonuclease for oligonucleotides increases exponentially with increasing chain length; for example, the affinity for pTTP, is about three orders of magnitude higher than that for pTTP. Denatured oligonucleotides show the same phenomenon, except that the affinity of the enzyme for the homologous series is systematically lower than for the phosphoribosylated ones. Dinucleoside monophosphates are therefore the oligonucleotides for which the enzyme has the least affinity. In addition, pTTP is degraded by venom exonuclease at a rate about 25 times lower than pTTP (Razzell and Khorana, 1959). The very low R, and F,, 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.

Conversely, the reproducibility of the results obtained, it should be stressed that careful control experiments were made on each step of the rather complex procedure used. Some of these experiments were already mentioned in the previous sections. Other controls and further details on the procedure used can be obtained on request. The differences found in the preliminary and penultimate results obtained on three independent independent dinucleoside digestes are in satisfactory agreement with each other. The slight differences found in the 5'-hydroxy III compared with the other two might be due to the fact that all steps were done on much smaller amounts of material. Particularly, the analysis was done on a scale 100 times lower than the other two series. (b) The reproducibility of the analytic results obtained by running duplicate samples taken from the venom exonuclease digestes (hydrolytic IV) through all the steps. The final analysis was made within 27 with the original one. (b) Finally, it should be stressed that the results reported here were obtained under a single set of experimental conditions (cuvettes 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 penultimate
S T U D I E S O N A C I D D N A S E

Table II: Average Composition of Sequences Split by Acid D N A s e.

<table>
<thead>
<tr>
<th>Phosphate Terminal</th>
<th>5′-Hydroxy Terminal</th>
<th>5′-Hydroxy Penultimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>21</td>
<td>13 (26.8%)</td>
</tr>
<tr>
<td>G</td>
<td>39</td>
<td>34 (23.6%)</td>
</tr>
<tr>
<td>A</td>
<td>31</td>
<td>22 (27.6%)</td>
</tr>
</tbody>
</table>

* Average chain length of the digest (hydrolysis II) was 53. Nearest neighbors of 3′-phosphate and 5′-hydroxy terminal, respectively, as calculated on the basis of the data of Swart et al. (1962).

was obtained from Sigma (St. Louis, Mo.). Adenosine 5′-phosphate and the 5′-nucleotidase of thymidine 5′-phosphate were purchased from Calbiochem (Los Angeles, Calif.).

Assay of Enzymatic Activity. Enzyme activity was assayed by measuring the liberation of phosphoric acid from the 5′-nucleotidase of thymidine 5′-phosphate in water at pH 8.5 containing 0.5% bovine serum albumin (crystallized and lyophilized, Sigma). After 10-min incubation at 20°C, the reaction was stopped by adding...
TABLE IV: Chromatographic Purification of Snake Venom Exonuclease.

<table>
<thead>
<tr>
<th>Exonuclease</th>
<th>Total</th>
<th>Recov</th>
<th>Phosphatase</th>
<th>Exonuclease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snake venom</td>
<td>48</td>
<td>1266</td>
<td>1977</td>
<td>1.5</td>
</tr>
<tr>
<td>Step 1 (DEAE-Sephadex)</td>
<td>844</td>
<td>511</td>
<td>1974</td>
<td>3.6</td>
</tr>
<tr>
<td>Step II (CM-Sephadex)</td>
<td>480</td>
<td>112</td>
<td>1358</td>
<td>12.1</td>
</tr>
<tr>
<td>Step III (hydrolysate)</td>
<td>Fractions 7-8</td>
<td>149</td>
<td>21</td>
<td>460</td>
</tr>
<tr>
<td>Fractions 9-10</td>
<td>218</td>
<td>35</td>
<td>689</td>
<td>19.5</td>
</tr>
</tbody>
</table>

0.2 ml of 2% NaOH. The absorbance at 400 nm 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 µ mole of p-nitrophenol/min under the conditions specified above. The specific activity was calculated by dividing the activity by the absorbance at 280 µ of the enzyme solution. Assays were performed using enzyme concentrations such as to obtain A_{280} 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 Kuenzer and Sinnhuber (1957). The reaction mixture (total volume 1 ml) contained: (a) 3 µ moles of 5'-AMP; 10 µ moles of MgCl₂; 500 µ moles of glycine adjusted to pH 9.0 with NaOH; 0.6 enzyme units; this was diluted, if necessary, with 0.1 M glycine buffer. 0.1 M glycine (pH 9.0) containing 0.05 M sodium acetate buffer was incubated at 37° for 60 min. The reaction was stopped by adding 0.1 M glycine buffer and 0.5 M NaCl and measured in a SF 10 cm column of DEAE-cellulose (Sorva, Hesdler, Germany), 0.37 cm diameter; washed with 1 N NaCl, water, 1 N NaOH, water, 0.9 M glycine (pH 9.0), and water.

FIGURE 13: Fractions 61-105 (280 ml) from the chromatogram shown in Figure 2 were diluted to 600 ml with water and loaded on a 2 x 35.8 cm hydrolysate column equilibrated with 0.08 M phosphate buffer (pH 6.8). Elution was carried out with a relatively constant gradient of phosphate buffer (pH 6.8). Flow rate was 100 ml/hr. Other indications as in Figure 2.

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