Hydrolysis of Dinucleoside Monophosphates Containing Arabinose in Various Internucleotide Linkages by Exonuclease from the Venom of *Crotalus adamanteus**

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ABSTRACT: A method of purification of exonuclease from the venom of *Crotalus adamanteus* is described. It involves fractionation with acetone between 42 and 50% at −17° and pH 4.0, fractionation with ethanol between 50 and 67% at −17° and pH 8.9, gel filtration on Sephadex G-75, and chromatography on CM-Sephadex. The enzyme thus obtained is 160-fold purified, and is of similar quality as the preparations obtained previously in this laboratory from the venom of *Bothrops atrox*. The originally low value of the nonspecific monophosphatase per unit of exonuclease has been further reduced to $^1/_{30}$ of the original value during the purification. The corresponding value for 5′-nucleotidase has been reduced (2×10^6)-fold. Although the ratio of endonuclease to exonuclease is 1000-fold lower than in venom, endonuclease activity is still measurable by a modified viscosimetric procedure. The values of V_m and K_m have been measured for several synthetic dinucleoside monophosphates in two different ionic media, and only small differences have been found. All of the three linkages investigated (2′–5′, 3′–5′, and 5′–5′) are susceptible to the action of venom exonuclease, with V_m decreasing in the order 3′–5′ > 5′–5′ > 2′–5′. K_m decreases in the opposite direction. The enzyme is capable of hydrolyzing derivatives of all three sugars tested. Derivatives containing arabinose are equally good or slightly poorer substrates than the corresponding derivatives containing ribose or deoxyribose. The hydrolysis is slowest with the compounds which lead to the formation of 5′-arabinose mononucleotides. Compounds which lead to the formation of arabinose nucleosides are hydrolyzed relatively faster.

Several years ago it was realized (Laskowski, 1959, 1961) that some nucleases are capable of hydrolyzing both RNA and DNA. Enzymes with this property are found among both endo- and exonucleases. When this criterion was introduced to define the new subgroups of nucleases, the author did not mean to imply that the rates of hydrolysis of both substrates must be identical. Indeed, even a superficial analysis indicated that gradations occur in the ability to hydrolyze both types of nucleic acids. Some enzymes of the group, *e.g.*, mung bean nuclease (Sung and Laskowski, 1962), appear to hydrolyze RNA faster than DNA; whereas others, *e.g.*, venom endonuclease, attack DNA faster (Georgatsos and Laskowski, 1962). In neither case is it possible to attach much quantitative significance to such observations since it is obvious that only some of the existing internucleotide bonds are susceptible under the conditions of the experiment.

Dinucleoside monophosphates have only one internucleotide bond and therefore are convenient substrates to investigate the effect of component nucleosides. Recently, several dinucleoside monophosphates containing arabinose were synthesized by Wechter (1966). They were found to be susceptible (Wechter, 1966, 1967) to the action of commercial (Worthington) venom exonuclease (phosphodiesterase).[1] Paper chromatography was used to detect hydrolysis, but no rate measurements were made.

It was decided to investigate the quantitative aspects of hydrolysis using a pH-Stat. Since it was imperative to ascertain that the observed rate of reaction was attributable solely to venom exonuclease and was not influenced by a contaminant, a highly purified exonuclease from the venom of *Crotalus adamanteus* was used. For several years we were unable to obtain from Brazil the venom of *Bothrops atrox* which was previously our source of highly purified exonuclease. The venom of *C. adamanteus* was chosen because the species is native to North America and because it resembles *B. atrox* with respect to the content of major nucleolytic enzymes (Richards *et al.*, 1965).

This paper presents the values of V_m and K_m for several dinucleoside monophosphates hydrolyzed by a purified exonuclease from the venom of *C. adamanteus*.

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[1] Throughout the remainder of this paper, the term exonuclease will be used.

The modifications of the method of preparation of venom exonuclease imposed by the change of the species are also described.

Experimental Procedure

Materials. The majority of the dinucleoside monophosphate substrates[2] were synthesized as described previously (Wechter, 1967). The synthesis of two additional dinucleoside monophosphates, rC5′p5′dC and rC5′p5′rC, is described below. To prepare rC5′p5′dC, N^4,3′-*O*-diacetyldeoxycytidine 5′-phosphate [derived from 1.0 g of deoxycytidine 5′-phosphate (Khorana and Vizsolyi, 1961)] and 2.0 g of N^4,2′,3′-*O*-tribenzoylcytidine (Rammler and Khorana, 1962) were shaken for 1 week at room temperature with 10 g of dicyclohexylcarbodiimide in 50 ml of anhydrous pyridine. After addition of 25 ml of water, dicyclohexylurea was removed by filtration, and the filtrate was taken to dryness under vacuum. The protective acetyl and benzoyl groups were removed by hydrolysis in methanol saturated with anhydrous NH_3. A small amount of insoluble material was removed by filtration and the filtrate was dried under vacuum. The dry residue was extracted with water, and the water-soluble portion was extracted with ether. After removal of ether, the aqueous extract was further purified by continuous-flow electrophoresis in 0.5 N acetic acid (Ko *et al.*, 1967) and chromatography on Dowex 1 formate. The lyophilized final product was homogeneous by thin layer chromatography on cellulose and high-voltage electrophoresis in pH 3.6 formate. The structure of the product was confirmed by its nuclear magnetic resonance spectrum in D_2O (Varian A60A spectrometer, SDSS[3] external standard. Chemical shifts are given in cycles per second; coupling constants in parentheses): H-6, 469 (7.5) and 467 (8.0); H-5, 360; H-1′ of dC, 378 (5); H-1′ of C, <355; H-4′ and H-5′, 247 (br); and H-2′ of dC, 148 (m).

The compound rC5′p5′rC was prepared and evaluated in a similar manner, starting with N^4,2′,3′-*O*-triacetylcytidine 5′-phosphate and N^4,2′3′-*O*-tribenzoylcytidine. The continuous-flow electrophoresis step was replaced with a preliminary chromatography on Dowex 1 formate. Nuclear magnetic resonance data were as follows: H-6, 468 (7.5); H-5, 358 (7.5); H-1′, 355 (~2.5); and H-4′ and H-5′, 251 (br s).

Lyophilized *C. adamanteus* venom was purchased from Miami Serpentarium. DNA from calf thymus was prepared by the method of Kay *et al.* (1952).[4] Sephadex G-75, CM-Sephadex C-50, and Ficoll were purchased from Pharmacia, Inc. Carbowax 20 M was obtained from Union Carbide Corp. Sodium *p*-nitrophenyl phosphate and calcium [bis(*p*-nitrophenyl) phosphate]$_2$ were purchased from Sigma Chemical Co., AMP from P-L Biochemicals, Inc., bovine serum albumin from Pentex Inc., and rC3′p5′rC from Zellstoffsfabrik Waldhof.

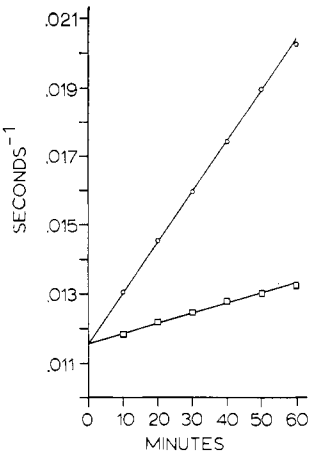

FIGURE 1: Determination of endonuclease activity by viscometry. Assays were performed as described in the Experimental Section. The vertical axis shows the reciprocal of flow time in the viscometer, and the horizontal axis shows time from the start of the reaction. The lower curve was obtained with 0.01 ml of a solution of partly purified venom endonuclease containing approximately 7×10^{-3} Kuntz unit/ml, while the upper curve was obtained with five times as much enzyme.

Assay of Venom Exonuclease and Its Contaminants. Exonuclease, 5′-nucleotidase, and the nonspecific phosphatase of venom were determined with the respective substrates calcium [bis(*p*-nitrophenyl) phosphate]$_2$, 5′-AMP, and sodium *p*-nitrophenyl phosphate, using procedures previously described (Sulkowski *et al.*, 1963; Richards *et al.*, 1965). Endonuclease activity at pH 5 was estimated by the viscometric method described below. Reagents at room temperature were mixed in the following order: enzyme plus water to make 0.2 ml; 0.1 ml of 1.2 M NaAc (pH 5.0) 0.1 ml of 0.12 M EDTA (pH 5.0); and 0.4 ml of DNA, 2.2 mg/ml in 0.01 M NaCl. With the addition of DNA, a stopwatch was started. The mixture was gently agitated for 1 min at room temperature and 0.4 ml was introduced into an Ostwald microviscometer held at 37°. Readings of flow time were taken every 5 or 10 min for 1 hr. Designating f = flow time, the quantity f^{-1} was plotted *vs.* the time of reaction t, recorded at the start of each flow-time measurement. The slope of the resulting straight line was proportional to the amount of

[2] Since the compounds used contained different sugars and different internucleotide linkages the following system of abbreviations is used, to serve the needs of this paper only. Nucleotides are abbreviated by two letters, a small letter denoting the sugar, and a capital letter denoting the base. Thus, aC is arabinosylcytosine, rC is cytidine, and dC is deoxyribocytidine. Three types of internucleotide linkages are abbreviated by placing appropriate numbers designating the carbons involved, before and after p, as: 2′p5′, 3′p5′, and 5′p5′. The above system of abbreviations evolved from discussion with Dr. Waldo E. Cohn, Director, NAS-NRC Office of Biochemical Nomenclature (Cohn, 1967), to whom we express our gratitude.

[3] Abbreviations used: SDSS, sodium dimethylsilapentanesulfonic acid; AMP, adenosine monophosphate.

[4] The authors are indebted to Mr. George Kuzmycz, Jr., for the preparation of this material.

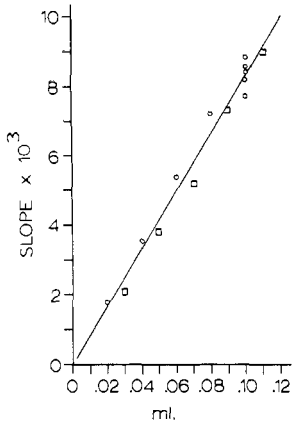

FIGURE 2: Linear range of the viscometric method. Assays were performed as described in the Experimental Section. The horizontal axis indicates the volume of stock endonuclease solution diluted to 1.0 ml. Of this dilution, 0.01 ml was used in the assay. The vertical axis gives the slope derived from viscometric measurements as in Figure 1. (□—□) Assay performed with a 6-month-old substrate solution having a DNA concentration of 33.6 A_{260} units/ml (determined in 0.15 M sodium acetate, pH 5.0). (○—○) Assay performed with a freshly prepared substrate solution having a DNA concentration of 32.4 A_{260} units/ml. The several points at 0.10 on the horizontal axis were obtained on different days. Over the course of all measurements, the activity of the stock endonuclease solution as determined by the method of Kunitz (1950) varied between 0.330 and 0.378 unit/ml in an irregular manner.

enzyme added over the fivefold range investigated. Figure 1 shows typical plots of f^{-1} *vs.* t at the extremes of this concentration range.[5] Figure 2 illustrates the proportionality between amount of enzyme added and the slope of f^{-1} *vs.* t. The endonuclease used in obtaining these curves was prepared by carrying *C. adamanteus* venom through step 3 of the method of Georgatsos and Laskowski (1962). Relatively little improvement in over-all potency of endonuclease was gained in these steps; in contrast to venom which contains interfering substances, the resulting material could be assayed by the method of Kunitz (1950), the latter performed using 0.2 M sodium acetate at pH 5.0, without added Mg^{2+}. It was determined that the amount of enzyme giving a slope of 0.01 sec^{-1}/hr in the viscometric assay (the upper end of the enzyme range) corresponds to 4×10^{-4} Kunitz unit. Using this factor, endonuclease levels reported below have been expressed in Kunitz units.

Rates of Hydrolysis of Dinucleoside Monophosphate Substrates. Substrates were dissolved in water to give 0.01 M solutions, which were adjusted to pH 6 with NaOH. Hydrolysis was followed at pH 9 in a water-jacketed cell at 37°, using a slightly modified version of the pH-Stat described by Peanasky and Szucs (1964). The following steps were taken to overcome difficulties encountered in the use of low concentrations of NaOH (0.002–0.01 N). A reaction chamber made of polyallomer was used (Beckman 326820 tube, the bottom flattened with heat); NaOH was delivered from a closed system consisting of polyethylene storage bottle, CO_2 trap, glass syringe, three-way nylon stopcock, and narrow diameter polyethylene tubing; and the reaction mixture was protected with a slow stream of nitrogen. The use of a 0.5-in. diameter reaction chamber, close-fitting magnetic stirrer, and a semimicro combination electrode (Sargent S-30070-10) allowed the reaction volume to be held to 1.5 ml.

Two series of reactions were run. The first of these was carried out in 6.7×10^{-4} M $MgCl_2$, at substrate concentrations of between 0.33 and 5.5 mM, using 0.0132 N NaOH as titrant. However, under these conditions the initial reaction slopes often were curved unless the reaction was carried out at a very low rate, restricting the precision with which V_m and K_m could be determined. Using readily available rC3'p5'rC as a test substrate, more satisfactory conditions were developed for a second series of measurements. These were carried out in a mixture of 1.0 M sodium acetate, 0.01 M $CaAc_2$, and 0.1 mg/ml of bovine serum albumin, at substrate concentrations of between 0.11 and 1.3 mM, using 0.002 N NaOH as titrant. Typical initial reaction rates varied between 5 and 50×10^{-9} mole of NaOH/min per 1.5 ml of reaction mixture.

To run a reaction, substrate, water, and ionic medium totaling 1.5 ml were placed in the pH-Stat and brought to pH 9.0 with NaOH. After pH stability was attained, enzyme was added, contained in from 0.01 to 0.1 ml of reaction ionic medium. Activity of the enzyme solutions was checked before and after each day's series of pH-Stat runs, using calcium [bis(*p*-nitrophenyl) phosphate]$_2$. Concentration of the 0.002 N NaOH was checked in a similar manner by titration of a standard potassium hydrogen phthalate solution in the pH-Stat. The concentration of NaOH remained constant throughout the series of measurements. Final values for the enzyme activities were taken from a plot of all the enzyme assays.

Products were identified by carrying the reactions in low salt to completion. Samples of the reaction mixtures were chromatographed on Whatman No. 1 filter paper, using a mixture of 2.5 volumes of 95% EtOH and 1.0 volume of 1 M NH_4Ac (pH 7.5) for development. The nucleoside and nucleotide products of hydrolysis were eluted from the chromatogram with water and examined spectrophotometrically at 259 and 271 mμ. In the case of aC5'p5'dC and aC5'p5'rC the eluted spots of nucleotide and nucleoside were digested with 12 N HCl at 100° for 8 hr and neutralized with NaOH. The neutralized solutions were then reacted with 10^{-4} M $NaIO_4$ and the reaction was followed spectrophotometri-

[5] The upper end of this range corresponds approximately to the amount of enzyme which, under the conditions described, reduces the flow time of the reaction mixture from the initial six times that of water to less than four times that of water, within 1 hr.

cally at 220 mμ. Deoxyribose was identified by the lack of reactivity with this reagent; ribose and arabinose by comparing their rates of the reaction to those of authentic ribose and arabinose. It was found that ribose reacted with $NaIO_4$ twice as fast as arabinose.

Results

Purification of Venom Exonuclease. The enzyme was prepared from the venom of *C. adamanteus* by a four-step method based partly on procedures developed previously in this laboratory for the venom of *B. atrox* (Felix *et al.*, 1960; Williams *et al.*, 1961; Björk, 1963).

STEP 1. Fractionation with acetone. Venom (10 g) was suspended in 600 ml of water at room temperature and stirred for 1 hr before centrifuging briefly at low speed. The supernatant liquid was placed in a −17° bath and 400 ml of 0.5 M sodium acetate (pH 4.0) was added with stirring. When the temperature reached 0°, 725 ml of acetone, precooled to −17°, was slowly added. Stirring was continued until the temperature returned to 0°, after which the suspension was transferred to an ice bath and stirred for 30 min. The suspension was then centrifuged at 7500*g*, 0°, for 15 min. Without stopping the centrifuge, the temperature was lowered to −17° and centrifugation was continued for 1 hr.[6] The supernatant liquid was returned to the −17° bath and stirred while 275 ml of precooled acetone was added. Stirring was continued for 30 min, after which the suspension was centrifuged at 7500*g*, −17°, for 15 min. The supernatant solution was discarded, and the precipitate was taken up in cold water to make 100 ml. This solution was stored at 0 to 4°.

STEP 2. Fractionation with ethanol. The aqueous solutions of exonuclease (200 ml) from two 10-g batches of venom were combined and diluted with 400 ml of 0.15 M Tris-HCl (pH 8.9). The mixture was placed in a −17° bath and 300 ml of 95% ethanol, precooled to −17°, was added immediately with stirring. Stirring was continued until the temperature returned to 0°, after which the suspension was transferred to an ice bath and stirred 30 min longer. The suspension was centrifuged at 7500*g*, 0°, for 15 min; then the supernatant liquid was returned to the −17° bath and 900 ml of precooled ethanol was added with stirring. After 30 min of stirring, the suspension was centrifuged at 7500*g*, −17°, for 1 hr. The precipitates were drained thoroughly at −17° and taken up in the minimum volume of cold water giving a clear solution, generally less than 100 ml. The material was kept between 0 and 4° in all subsequent steps.

STEP 3. Chromatography on Sephadex G-75. The aqueous solution of exonuclease was reduced essentially to dryness by dialysis overnight against dry Carbowax 20-M. The nearly dry bag was washed free of Carbowax with distilled water and stirred in water for 2–4 hr, after which the contents of the bag were removed and the bag was rinsed with sufficient water to make a total of about 20 ml. Insoluble material, if present, was removed by centrifugation. The supernatant solution was diluted with 0.11 volume of 0.1 M Tris-HCl–0.5 M in NaCl (pH 8.9) and was applied to a column of Sephadex G-75 (2.5 × 90 cm) which had been washed previously with the above Tris-NaCl buffer, tenfold diluted (0.01 M Tris-HCl–0.05 M NaCl). Elution was carried out with the same dilute buffer at 0.5 ml/min. Exonuclease appeared in the first peak of A_{280}-absorbing material emerging from the column, at the void volume, as described by Björk (1963). Fractions falling under this peak were combined, reduced in volume with Carbowax, and recovered as an aqueous solution as described previously, except that the sample volume was kept to a minimum.

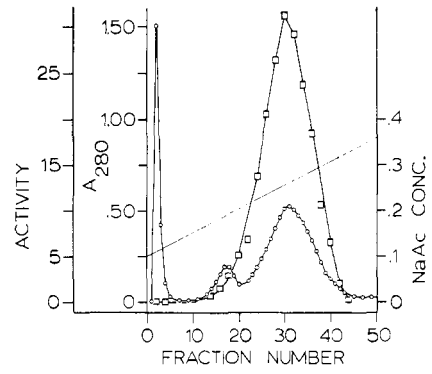

FIGURE 3: Step 4 of the purification procedure. Elution profile from the chromatography of venom exonuclease on CM-Sephadex. Column 1.5 × 25 cm, flow rate 16 ml/hr, collecting 11.5-ml fractions. Elution with a gradient of sodium acetate (pH 6.0) as indicated. (— - - —) Eluent concentration; (O——O) absorbancy at 280 mμ; (□——□) activity of exonuclease. The final product consisted of pooled fractions 21–42.

STEP 4. Chromatography on CM-Sephadex. Despite the fact that the preceeding step had been carried out at pH 8.9, the pH of the final concentrated solution of exonuclease was 5.9. The addition of one-third volume of 0.4 M sodium acetate (pH 6.0) brought the sample to 0.1 M in sodium acetate at pH 6. The sample was then applied to a column of CM-Sephadex C-50 (1.5 × 25 cm) previously equilibrated with 0.1 M sodium acetate (pH 6.0). Elution was carried out at 16 ml/hr, using a linear gradient developed with 335 ml of 0.1 M sodium acetate and 335 ml of 0.4 M sodium acetate (pH 6.0). The profile of the eluate (Figure 3) shows three ultraviolet-absorbing peaks, the third and largest of which appeared at a sodium acetate concentration of 0.25 M and contained the bulk of the exonuclease. Fractions falling under this peak were combined and reduced essentially to dryness by dialysis against 1.0 g of Ficoll/2.0 ml of sample. The contents of the bag were recovered as an aqueous solution of minimum volume, as described previously, and were frozen.

The final concentrated preparation has been stored

[6] The precipitate from this centrifugation routinely was saved as a source of 5′-nucleotidase and endonuclease.

TABLE I: Yield and Extent of Purification of Venom Exonuclease.

Step	Exonuclease			Ratio of Contaminants:Exonuclease		
	Units/10 g of Venom	% Recov	Potency	Nonspecific Phosphatase	5′-Nucleotidase	Endonuclease
Venom	435	(100)	0.033	0.052[a]	56[a]	0.25
Acetone	315	72	0.11	0.0014	4.8×10^{-4}	
Ethanol	248	79	0.41	0.0015	3.0×10^{-5}	
Sephadex G-75[b]	152	61	3.7	0.0010	2.0×10^{-5}	0.00041
CM-Sephadex[c]	146	96	5.4	0.0017	3.0×10^{-6}	0.00025
Start to finish		34				

[a] Data on monophosphatases in venom from Richards *et al.* (1965). [b] Including concentration dialysis of sample before and after gel filtration. [c] Including concentration dialysis of sample after chromatography.

TABLE II: Comparison of Rates of Hydrolysis of 2′-5′, 3′-5′, and 5′-5′ Linkages in Dinucleoside Monophosphates of Similar Composition.

Expt	Substrate	V_m (moles/min unit)		K_m (moles/l.)		Products
		High Salt	Low Salt	High Salt	Low Salt	
1	aC2′p5′rA	2.0×10^{-6}	3×10^{-6}	1.0×10^{-3}	4×10^{-3}	aC + prA
2	aC3′p5′rA	2.7×10^{-5}	3×10^{-5}	2.6×10^{-4}	4×10^{-4}	aC + prA
3	aC5′p5′rA	6.0×10^{-6}	5×10^{-6}	3.9×10^{-4}	4×10^{-4}	aC + prA
4	rAe′p5′aC		2×10^{-6}		2×10^{-3}	rA + paC
5	rA3′p5′aC		2×10^{-6}		1×10^{-3}	rA + paC
6	rC3′p5′rC	8.1×10^{-6}		2.0×10^{-4}		rC + prC
7	rC5′p5′rC	4.4×10^{-6}		7.6×10^{-4}		rC + prC
8	aC3′p5′aC	1.5×10^{-6}	1×10^{-6}	7.3×10^{-4}	8×10^{-4}	aC + paC
9	aC5′p5′aC	1.9×10^{-6}	2×10^{-6}	3.3×10^{-3}	2×10^{-3}	aC + paC

frozen both at the original pH 6 and after adjustment to pH 9 with NaOH, for many months without appreciable loss of activity. Solutions diluted with water or sodium acetate, containing 0.1–1.0 unit of enzyme/ml, lose 50% of their activity in approximately 2 months when stored at 0°. Occasionally, a freshly thawed sample of enzyme appears to have lost up to 50% of its activity. Full activity reappears after the sample has been allowed to stand for several hours at 0°, or has been held for 5 or 10 min at 37°.

Table I summarizes the yield and extent of purification of exonuclease in the processing of 10 g of lyophilized venom. In practice, it is convenient to combine two 10-g batches after the acetone precipitation step. Over-all recovery is 34%, with a 1000-fold decrease in the ratios of endonuclease to exonuclease and the nonspecific phosphatase to exonuclease. Specific 5′-nucleotidase is reduced to the limit of detectability.

Rates of Hydrolysis of Dinucleoside Monophosphates. Results have been arranged into three Tables (II–IV). Table II shows the effect of different types of internucleotide linkage in substrates of the same composition, whereas Table III shows the effect of composition in substances of the same linkage type. Because of this arrangement many experiments appear in both tables. To indicate these repetitions the experiments are identified by number in the first column of each table. Table IV shows the results obtained with two phosphodiesters in which only one partner is a nucleoside.

The principal conclusion from these tables is that all of the substrates are hydrolyzed with quite similar rates. For the dinucleoside monophosphates, variations in V_m and K_m do not exceed a 20-fold range. Table II shows that exonuclease has a slight but definite preference for the natural 3′-5′ linkage. V_m for this linkage is four times greater than for the 5′-5′ linkage and 13 times greater than for the 2′-5′ linkage. Values for K_m decrease in opposite order from those of V_m (expt 1–3). Razzell and Khorana (1959) observed that dT3′p5′dT was hydrolyzed a little more slowly than the corre-

TABLE III: Comparison of the Rates of Hydrolysis of Dinucleoside Monophosphates of Different Composition, but Similar Linkage.

Expt	Substrate	V_m (moles/min unit)		K_m (moles/l.)		Products
		High Salt	Low Salt	High Salt	Low Salt	
7	rC5′p5′rC	4.4×10^{-6}		7.6×10^{-4}		rC + prC
10	rC5′p5′dC	4.2×10^{-6}		3.2×10^{-4}		rC + pdC dC + prC
11	aC5′p5′rC	1.9×10^{-5}	9×10^{-6}	1.7×10^{-3}	7×10^{-4}	aC + prC
12	aC5′p5′dC	3.0×10^{-5}	2×10^{-4}	2.5×10^{-3}	3×10^{-3}	aC + pdC
9	aC5′p5′aC	1.9×10^{-6}	2×10^{-6}	3.3×10^{-3}	2×10^{-3}	aC + paC
5	rA3′p5′aC		2×10^{-6}		1×10^{-3}	rA + paC
2	aC3′p5′rA	2.7×10^{-5}	3×10^{-5}	2.6×10^{-4}	4×10^{-4}	aC + prA
6	rC3′p5′rC	8.1×10^{-6}		2.0×10^{-4}		rC + prC
8	aC3′p5′aC	1.5×10^{-6}	1×10^{-6}	7.3×10^{-4}	8×10^{-4}	aC + paC

TABLE IV: Comparison of the Rates of Hydrolysis of Phosphodiesters Containing A Common 5′-Phosphoarabinosylcytosine.

Substrate	V_m (moles/min unit)	K_m (moles/l.)	Products
	High Salt	High Salt	
Cytosine arabinoside 5′-*O*-phenyl phosphate	8.5×10^{-5}	7.2×10^{-4}	C_6H_5 + paC
Cytosine arabinoside 5′-*O*-methyl phosphate	1.1×10^{-6}	5.1×10^{-3}	CH_3 + paC

sponding 5′-5′ compound, but the difference was not considered significant. In both their and our experiments K_m values are of a similar order of magnitude. In the remaining experiments in Table II (4,5 and 8,9) arabinose is present in the 5′ linked or second position (conventional notation), and in expt 8 and 9 it is present in both positions. In these experiments V_m is low in all three types of linkage. However, the value of K_m is still lowest for the natural 3′-5′ linkage and highest for the 2′-5′ linkage.

Table III shows that exonuclease does not discriminate between ribose and deoxyribose (expt 7 and 12). In contrast, Razzell and Khorana (1959) found that *p*-nitrophenyl 5′-thymidylate was hydrolyzed with V_m 30 times that of *p*-nitrophenyl 5′-uridylate. They ascribed this difference to the nature of the sugar. The remaining experiments in Table III show that exonuclease discriminates only slightly between arabinose, on one hand, and ribose and deoxyribose on the other. K_m is increased slightly by the presence of arabinose. When only one arabinose is present, occupying a position such that it can be released as a nucleoside, V_m is raised severalfold over the value for similar substrates lacking arabinose (expt 11, 12, and 2). However, when arabinose occupies a position from which it can be released only as a 5′-nucleotide, V_m is lowered severalfold from the value for similar substrates without arabinose. Such a situation occurs when both components of a 5′-5′ substrate contain arabinose (expt 9), or when arabinose occupies the 5′-linked position in a 3′-5′ substrate (expt 5 and 8). It is implicit in expt 9 and 8 that this latter effect (lowering of V_m) predominates.

Table IV gives data for the phenyl and methyl esters of arabinose 5′-phosphate. These are, respectively, the best and the poorest substrates measured. The high V_m of the phenyl ester is surprising, since upon hydrolysis arabinose 5′-phosphate is formed. However, K_m is still slightly higher than the value found by Razzell and Khorana (1959) for *p*-nitrophenyl 5′-thymidylate (7.2×10^{-4} *vs.* 5.0×10^{-4}). In addition, these authors found that *p*-nitrophenyl 5′-thymidylate was hydolyzed many times faster than the corresponding 3′-5′-dithymidylyl monophosphate.

In both tables it can be seen that values for V_m and K_m do not differ much in the two ionic media used. The single exception is V_m for aC5′p5′dC, expt 12. We are inclined to consider the result in low salt as spurious.

Discussion

The choice of *C. adamanteus* venom as a source of exonuclease was based on several factors. The species is

fairly common in North America, and the price is in the lower third for domestic venoms. The major reason, however, was our survey (Richards *et al.*, 1965), which led to the conclusion that *C. adamanteus* venom fairly closely resembles the venom of *B. atrox*, previously used in this laboratory. Another serious candidate was *Agkistrodon piscivorus*. The species is common in North America and its venom is the cheapest available. The snake easily survives captivity and could be farmed, should the demand for its venom be high. We discarded the species reluctantly because the amount of exonuclease in its venom is low, and because our attempts to apply the purification procedures of Björk (1963) were discouraging. It is known from recent studies of Björk[7] that step 1 of his 1963 procedure does not work with the venom of *Haemochatus hemochatus*. Purification of exonuclease from the venom of *A. piscivorus* similarly might require major changes in the methods previously used in our laboratory.

On the whole, the hope that only small modifications would be required for the adaptation of previous methods to the venom of *C. adamanteus* was justified. The only potential difficulty was the elimination of the endonuclease, the starting level of which is threefold higher in *Crotalus* than in *Bothrops* venom. Exonuclease prepared from *Crotalus* venom as described here has a higher specific activity than any of our previous preparations, and is equally free of contaminating monophosphatases. However, it appears to be contaminated with endonuclease to a higher extent than the preparation from *Bothrops* venom. A direct comparison using the present viscometric method cannot be made because the *Bothrops* preparation is no longer available to us. The problem of endonuclease contamination in the exonuclease preparation can be very serious when high molecular weight substrates are studied. On the other hand, the slight residual endonuclease activity detected at pH 5 undoubtedly would be even lower at the higher pH used for the exonuclease digestion. Because it is not possible to make reliable measurements on very low levels of endonuclease at high pH with techniques presently available to us, we are not able to settle this question directly.

In determining values for V_m and K_m the choice of the ionic media was made partly arbitrarily. With DNA as substrate Bowman (1959) showed that Ca^{2+} and Mg^{2+} were equally effective activators of exonuclease. This was confirmed by Williams *et al.* (1961), who also found that the optimal concentration of bivalent cation was dependent on the concentration of monovalent cation. With the low molecular weight substrates, Razzell and Khorana (1959) found activation with high concentrations of NaCl alone, which we now confirm. In the present work rC3′p5′rC was used to establish the composition of the ionic media because it was the only dinucleoside monophosphate substrate available commercially. It was assumed that the same conditions are applicable to other substrates. With rC3′p5′rC the high salt medium gave better Lineweaver–Burk plots than the low salt medium, but with other substrates good straight lines were obtained with both media. Besides the concentration, the two media also differed in the nature of activating ion. Mg^{2+} (6.7×10^{-4} M) was used with the low salt medium and Ca^{2+} (10^{-2} M) with the high salt medium. In the latter medium acetates were used instead of chlorides because they allowed a higher activity. Close similarity of V_m and K_m values obtained with the two strikingly different media strengthens the general conclusion of this work that the highly purified venom exonuclease hydrolyzes the derivatives of all three sugars with rather similar rates.

[7] W. Björk, personal communication quoted in Cantoni and Davies (1966).

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