Enzymes From Snake Venom
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Phosphodiesterases, Ribonucleases and Deoxyribonucleases

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1. INTRODUCTION

Enzymatic components which hydrolyze nucleic acids and derivatives are found in many different venoms from front-fanged snakes. However, with several notable exceptions, relatively little work has been done in recent years to characterize these near-ubiquitous venom components. After a flurry of research activity in the 1970s and 1980s, primarily with regards to venom phosphodiesterases, toxinological interest in these enzymes seems to have greatly decreased, though their use as probes of nucleic acids remains prevalent (e.g., Bykov et al., 1995; Liu and Kahn, 1995; Nara et al., 1995; Chen et al., 1994; Mao et al., 1993; Kawai et al., 1993; Mans et al., 1992; Auron et al., 1982). Still, very little is known about the contribution of these enzymes to sequelae of envenomation, *in vivo* activity, amino acid or cDNA sequence, degree of homology of enzymes from various species of snakes and the absolute relation of exonuclease and endonuclease activities. Even for venom phosphodiesterases, the best-studied venom nucleases, there are significant gaps in our understanding of basic biochemical properties of these enzymes (cf. Tables 1 and 2). It is clear that much work remains to be done on venom nucleases, and it is hoped that this review will stimulate renewed interest in the biochemical characterization and comparative analysis of these enzymes.

Several reviews of snake venom components, including phosphodiesterases, DNases and RNases, were published in the late 1970s (Tu, 1977;

Elliott, 1978; Iwanaga and Suzuki, 1979). It is not the intention of the present chapter to duplicate the efforts of these earlier works; rather, this review will primarily summarize work which has appeared since this time, with less extensive reference to earlier works concerning snake venom phosphodiesterases and endonucleases.

1.1 A Definition of Nucleases in Venoms

A review of the literature indicates that a consistent definition of nuclease activities has not always been closely adhered to during the analysis of crude venoms and/or characterization of purified components. Multiple enzymes with nucleolytic activity appear to copurify even after multiple-step isolation procedures; in addition, purified components may have several activities. It seems likely that some earlier reports of specific venom nucleases actually contained several discrete proteins with different activities, and this problem likely plagued commercially available preparations as well. For the purposes of this chapter, the following definitions will apply:

A. Phosphodiesterase (Oligonucleate 5'-nucleotidohydrolase; EC 3.1.15.1)

This nuclease is perhaps most abundant in snake venoms and has been best characterized. Although there is good reason to accept the suggestion that the enzyme be referred to as "exonuclease" (Laskowski, 1971; Tu, 1977), this name has not been used commonly, and it seems that the trivial name of phosphodiesterase will remain in the literature. The term should at least be prefaced so as to indicate source (i.e., venom phosphodi-esterase) and to distinguish it from phosphodiesterases from other tissues and from bacterial sources. In addition, an identifier which indicates the species of origin would be useful (e.g., Cmp venom phosphodiesterase from *Crotalus mitchelli pyrrhus* venom; see below), because differences in physical parameters of these enzymes from various species sources are likely and remain largely uncharacterized. Species-specific variation in relative amounts of various nucleases has also been noted (Richards et al., 1965).

Numerous names exist in the literature for the same activity, often from the same venom. The following names, when used in relation to venom-derived products, are here considered synonymous with venom phosphodiesterase: exonuclease, 3'-exonuclease, 5'-exonuclease, 5'-phosphodiesterase, 5'-nucleotide phosphodiesterase, phosphodiesterase I and 2-5A phosphodiesterase. The terms DNase and ADPase have also been applied to proteins which appear to be venom phosphodiesterases (Sittenfeld, 1991; Ouyang and Huang, 1986).

Venom phosphodiesterases catalyze the hydrolysis of phosphodiester bonds in a progressive fashion, beginning at the 3' end (hence the use of the

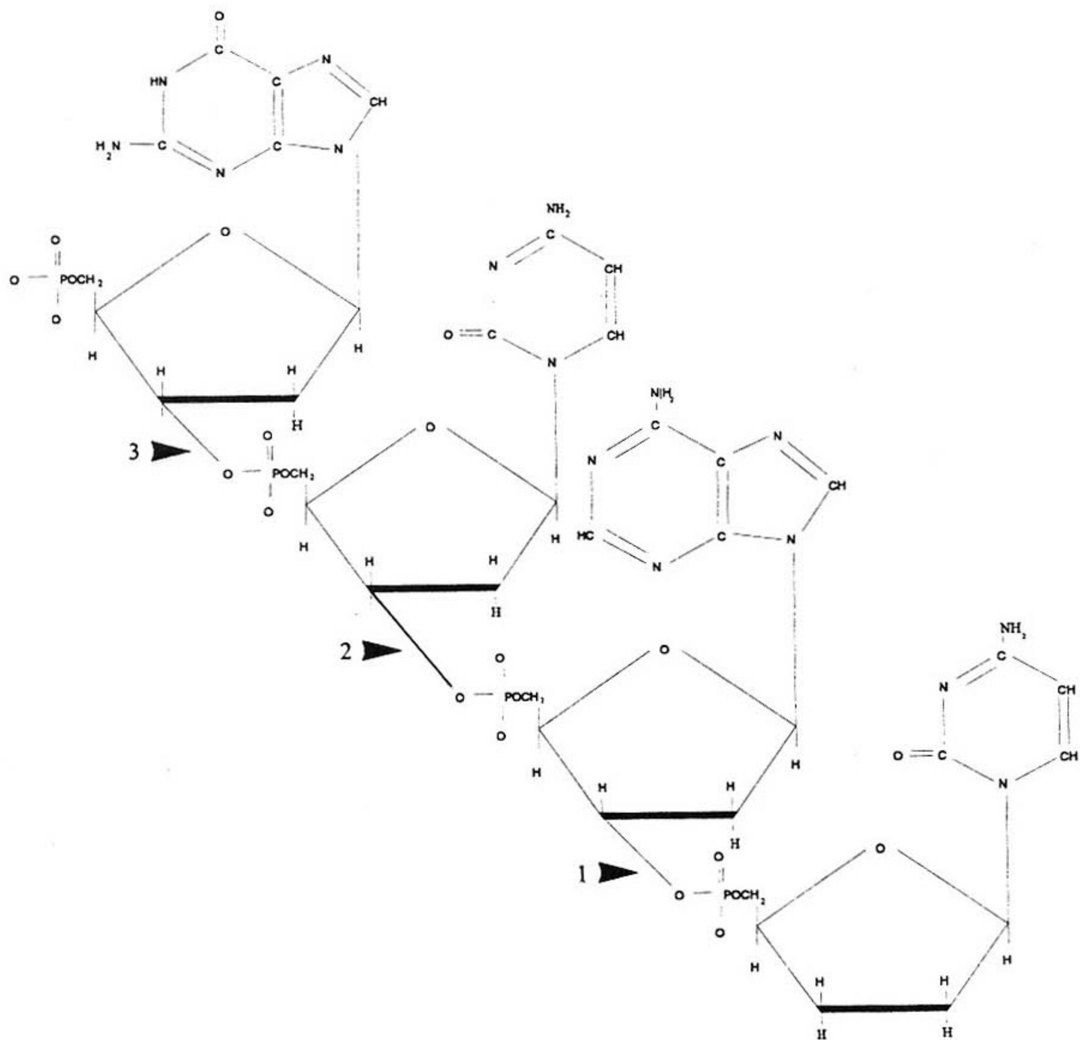

Figure 1. Hydrolysis of DNA by venom phosphodiesterase. Enzyme action (arrowheads) on native substrates is exonucleolytic, catalyzing hydrolysis stepwise from the 3' end and liberating 5'-nucleotides.

term "exonuclease"; Fig. 1). Many native and synthetic substrates are hydrolyzed by the enzyme (see below), but free 5'-mononucleotides are the product of oligonucleotide hydrolysis.

B. Ribonuclease

Many snake venoms also catalyze the hydrolysis of RNA molecules of varying length, and venom RNases have been considered endonucleases (Fig. 2). However, experimental evidence demonstrating the presence of a unique enzyme is limited to venom of a single species, *Naja naja oxiana* (central Asian cobra; Vassilenko and Babkina, 1965; Vassilenko and Ryte, 1975; see below). It is highly unlikely that specific RNases are limited to the venom of a single species, and a thorough investigation of venoms from other snakes, especially the numerous cobra species, is warranted. The "non-specific" ribonuclease activity present in many venoms is likely due to venom phosphodiesterase activity (and possibly non-specific endonuclease activity).

C. DNase

Snake venoms usually contain one or more components capable of catalyzing the hydrolysis of native or denatured DNA of various length, and internal hydrolysis of a DNA molecule or oligonucleotide would indicate endonuclease activity of a venom DNase (Fig. 3). The precise nature of this activity is unclear, and more specific characterization of venom nucleases needs to be undertaken to determine whether unique endonucleases truly exist in venoms (see below) or whether this is simply an additional activity of venom phosphodiesterase. Many phosphodiesterases have been shown to catalyze the hydrolysis of DNA (Williams et al., 1961; Laskowski, 1971; Iwanaga and Suzuki, 1979; Mori et al., 1987). However, the acidic pH optimum of described DNase activity (pH 5.0; Georgatsos and Laskowski, 1962; also summarized in Iwanaga and Suzuki, 1979) suggests either a bifunctionality for one of the venom nucleases or the existence of a true endonuclease. Very little recent work has been directed toward venom DNases, and this area of toxinology also needs attention. A recent report has shown that DNase activity is an inherent property of the phosphodiesterase from *Crotalus adamanteus* venom (Stoynov et al., 1997).

D. Phosphomonoesterase (Adenosine Triphosphatase, Non-specific Phosphatase; Orthophosphoric monoester phosphohydrolase; EC 3.1.3.2)

These enzymes catalyze the hydrolysis of inorganic phosphate from nucleotides, synthetic substrates and nucleic acid fragments; these enzymes are not the subject of this review and are covered in chapter 13.

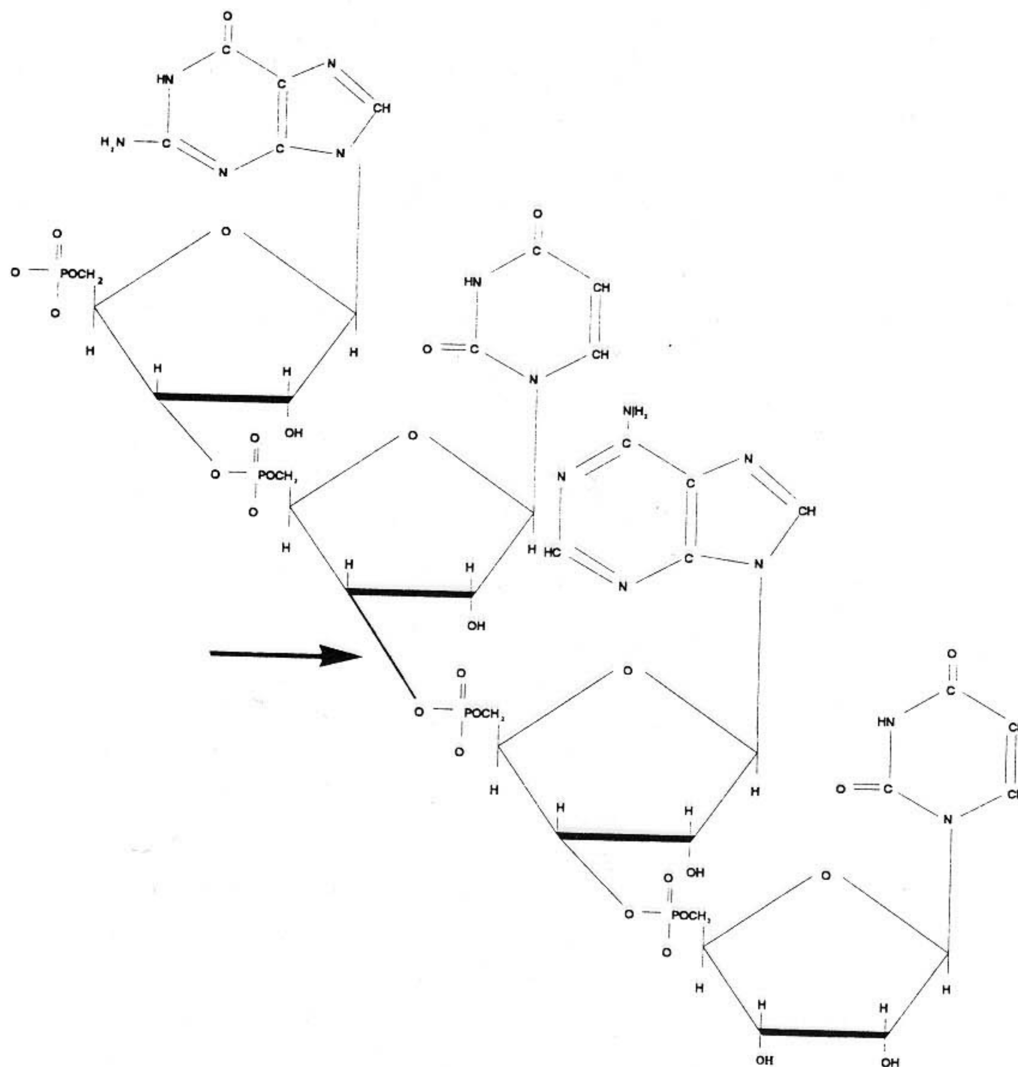

Figure 2. Hydrolysis of RNA by venom RNase. Enzyme action (arrow) is endonucleolytic, liberating oligonucleotides and/or 5'-nucleotides. RNase V_1 (*N. n. oxiana* venom) shows a high degree of specificity for double-stranded regions.

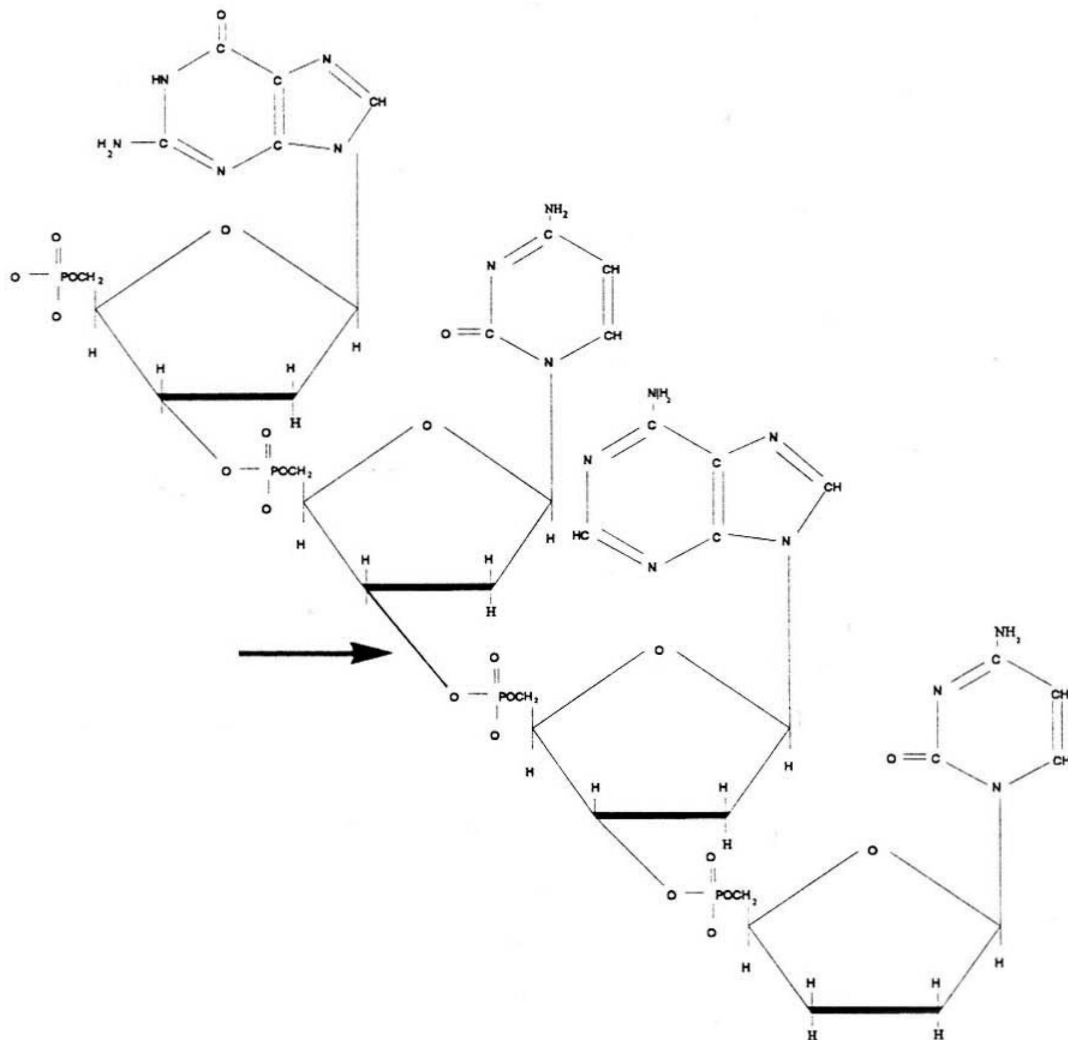

Figure 3. Hydrolysis of DNA by venom DNase. Enzyme action (arrow) toward native DNA is endonucleolytic, liberating oligonucleotides.

E. 5'-Nucleotidase (5'-Ribonucleotide Phosphohydrolase; EC 3.1.3.5)

This venom component catalyzes the hydrolysis of 5' mononucleotides, releasing nucleoside and inorganic phosphate. This activity is also the subject of chapter 13 and will not be discussed here.

2. EXONUCLEASES: PHOSPHODIESTERASES

2.1 Occurrence of Phosphodiesterase in Snake Venoms

Venoms from most front-fanged snakes which have been analyzed thus far show at least low levels of venom phosphodiesterase activity (Kocholaty et al., 1971; Tu, 1977; Iwanaga and Suzuki, 1979; Mebs, 1970), but absolute amounts of phosphodiesterase activity in a given species' venom and amounts relative to other nucleases vary considerably. Most sea snake (family Hydrophiidae) venoms showed no phosphodiesterase activity (Tan and Ponnudurai, 1991a; but see Mackessy and Tu, 1993; Tu and Toom, 1971, and Setoguchi et al., 1968), while phosphodiesterase was prevalent in venoms from snakes of the family Crotalidae (Kuch et al., 1996; Tan and Ponnudurai, 1991b,c; Mori et al., 1987; Sugihara et al., 1986; Mackessy, 1985). Unlike metalloprotease and phospholipase A_2 activities, venom phosphodiesterase activity in Pacific rattlesnakes (*Crotalus viridis helleri* and *C. v. oreganus*) did not show ontogenetic changes in activity (Mackessy, 1988). Venoms from snakes of the family Elapidae typically showed levels of phosphodiesterase activity which were 1-2 orders of magnitude lower than venoms from crotalid snakes (Tan and Ponnadurai, 1990a,1992; Tan et al., 1991; Aird and da Silva, 1991), while venoms from viperid snakes showed intermediate levels (e.g., Tan and Ponnadurai, 1990a,b). Venom from *Azemiops feae* (Feae's viper), a monotypic genus in the viperid subfamily Azemiopinae, had moderate phosphodiesterase activity, comparable to other viperid venoms (Mebs et al., 1994).

Venoms from snakes in the family Colubridae (rear-fanged or opisthoglyphous) are poorly characterized, but at least seven species (Weinstein and Kardong, 1994) produced a venom with detectable levels of phosphodiesterase activity. These species include *Boiga irregularis* (brown tree snake; Vest et al., 1991), *Coluber ravergieri* (variegated racer; Ovadia, 1984), *Leptodeira annulata* (cat-eyed snake; Mebs, 1968), *Malpolon monspessulanus* (montpellier snake; Ovadia, 1984), *Natrix tesselata* (dice snake; Ovadia, 1984), *Sphalerosophis diadema cliffordi* (diadem snake; Rosenberg et al., 1985) and *Thelotornis kirtlandii* (African twig snake; Kornalik et al., 1978), The present author's lab has extracted and assayed venoms from several species of colubrid snakes (Hill and Mackessy, 1997; Hill and Mackessy, in prep), and none demonstrated phosphodiesterase activity using calcium-bis-nitrophenyl phosphate as substrate. Venoms tested

were from the following species: *Amphiesma stolata* (Chinese keeled water snake), *Diadophis punctatus* (ringnecked snake), *Hydrodynastes gigas* (false water cobra), *Hypsiglena torquata* (night snake), *Thamnophis elegans vagrans* (wandering garter snake) and *Trimorphodon biscutatus lambda* (Sonoran lyre snake). However, using p-nitrophenyl thymidine-5'-phosphate as substrate, venoms from the following species showed low but detectable levels of phosphodiesterase activity (Hill and Mackessy, in prep): *D. punctatus, Heterodon nasicus nasicus* (western hognose snake), *H. n. kennerlyi* (Mexican hognose snake) and *T. e. vagrans*.

2.2 Characterization of Phosphodiesterase from Snake Venoms

A. Native Substrates for Venom Phosphodiesterase

Phosphodiesterases from snake venoms appear to be able to catalyze the hydrolysis of numerous native substrates including double and single-stranded DNA, ribosomal and transfer RNA, oligonucleotides (Iwanaga and Suzuki, 1979) including polyadenylic acid (Philipps, 1976) and cyclic nucleotides (Suzuki et al., 1960). From native RNA and DNA substrates, 5'-nucleotides are typically released via exonucleolytic cleavage initiated at the 3' end (Laskowski, 1971). However, other phosphodiester linkages are hydrolyzed by venom phosphodiesterases; V_{max} decreased in the following order: 3',5' > 5',5' > 2',5' (Richards et al., 1967). The present author's lab has also isolated venom phosphodiesterases, from *Crotalus viridis helleri* (southern Pacific rattlesnake) and *C. m. pyrrhus* (southwestern speckled rattlesnake), which hydrolyze cyclic 3'-5'-AMP to form 5'-AMP (Perron et al., 1993; Mackessy, 1989). The site of hydrolysis of cyclic 3',5'-AMP catalyzed by venom phosphodiesterases is shown in Fig. 4. It seems likely that other cyclic nucleotides would be subject to hydrolysis by venom phosphodiesterases. However, cyclo-pTpT was hydrolyzed by *C. adamanteus* (eastern diamond-back rattlesnake) venom phosphodiesterase at a 100-fold lower rate than pTpT, and 2',3'-cUTP was hydrolyzed at a rate 1000-fold lower than the thymidine dinucleotide substrate (Razzell and Khorana, 1959).

A single-chain phosphodiesterase isolated from *Agkistrodon acutus* (hundred pace snake) venom also hydrolyzed ADP and inhibited ADP-dependent platelet aggregation (Ouyang and Huang, 1986). Venom phosphodiesterases (*C. v. helleri* and *C. m. pyrrhus*) also hydrolyzed ATP and ADP, producing 5'-AMP (Perron et al., 1993; Mackessy, 1989). Thymidine dinucleotide to heptanucleotide substrates were readily hydrolyzed by venom phosphodiesterase from *Crotalus adamanteus* venom (Razzell and Khorana, 1959). Based on competitive inhibition assays with p-nitrophenyl thymidine-5'-phosphate, the longer thymidine oligonucleotides were better substrates.

Phosphodiesterase from *Bothrops atrox* (fer de lance) venom was long ago shown to degrade thymus DNA to mononucleotides by "essentially"

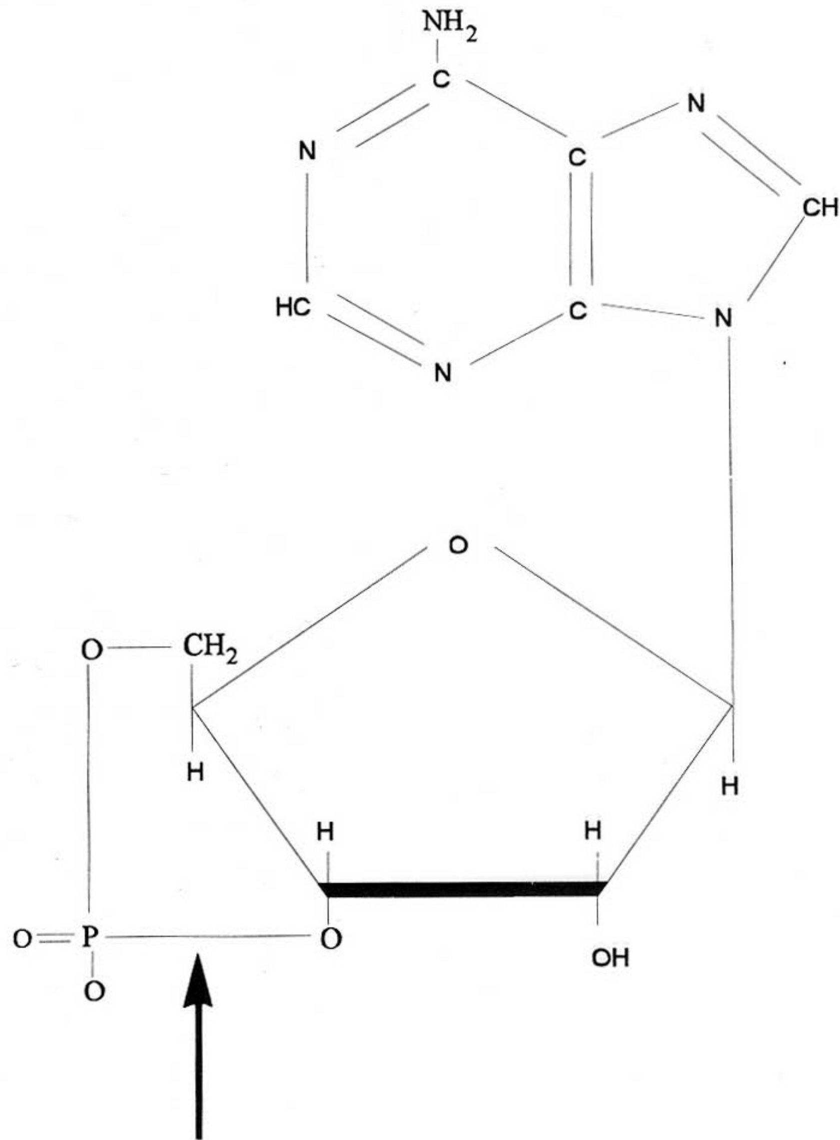

Figure 4. Hydrolysis of 3',5'-cAMP by venom phosphodiesterase. Enzyme action (arrow) typically liberates 5'-AMP.

exonucleolytic action (Williams et al., 1961). A purified phosphodiesterase obtained from the venom of *C. ruber ruber* (red diamond rattlesnake) catalyzed the hydrolysis of native DNA and RNA substrates as well as synthetic substrates (Mori et al., 1987). A more recent report on the DNase activities of venoms from several crotalid snakes, including several *Bothrops* species (Sittenfeld et al., 1991), is most likely due to the action of venom phosphodiesterases on calf thymus DNA utilized in the assay.

B. Assays of Venom Phosphodiesterase

A variety of assays are available for venom phosphodiesterase, and the choice of a substrate depends on the type of information desired and the availability of suitable equipment. Several spectrophotometric assays are commonly used during the isolation and initial characterization of the enzyme, and most are based on quantification of the nitrophenolate chromophore (at 400 nm) released at hydrolysis. The method of Björk (1963) is routinely used in the present author's lab for comparative analyses of phosphodiesterase activity of crude venoms and during chromatographic isolation of venom phosphodiesterase (e.g., Mackessy, 1985). This substrate, calcium bis-p-nitrophenyl phosphate, is widely available commercially, is inexpensive and produces satisfactory results. A second substrate, p-nitrophenyl thymidine-5'-phosphate (Razzell, 1963), is a better substrate for the enzyme and is useful for comparative kinetic analyses of venom phosphodiesterases; venom phosphodiesterases also show a 1000-fold higher V_{max} toward this substrate (Laskowski, 1980). The phosphodiesterase from *C. r. ruber* venom was shown to have a K_m of 8.3 mM at pH 8.9 (rather weak affinity) for the latter substrate (Mori et al., 1987). Two related substrates, thymidine 3'-(2,4-dinitrophenyl)phosphate and thymidine 5'-(2,4-dinitrophenyl)phosphate (von Tigerstrom and Smith, 1969), are also available and can be used to distinguish substrate specificity of phosphodiesterases from sources such as venom or bovine spleen.

Hydrolysis of cyclic mononucleotides by venom phosphodiesterases has been followed by thin layer chromatography of reaction products (Suzuki et al., 1960; Perron et al., 1993). Products of hydrolysis can be quantified following chromatography by phosphate analysis of separated spots, but this method can be replaced by quantitative HPLC analysis. A coupled assay utilizing adenylate kinase, pyruvate kinase and lactate dehydrogenase which followed the production of NAD^+ was recently described for a 3':5' cyclic nucleotide phosphodiesterase from the bacterium *Vibrio fischeri* (Callahan et al., 1995), and this procedure should be adaptable to assay venom phosphodiesterases (at least partially purified) as well.

Fluorescent nucleotide triphosphate analogs have been utilized in a sensitive spectrofluorometric assay (Pollack and Auld, 1982). Hydrolysis of the substrates by venom phosphodiesterase (*C. adamanteus*; Worthington

Biochemical Corp.) releases the diphosphorylated fluorophore, 1-aminonapthalene-5-sulfonate diphosphate, and a nucleotide monophosphate. Substrate excitation occurred at 320 nm and emission was measured above 410 nm (depending on substrate); hydrolysis of pyrimidine substrates resulted in enhancement of fluorescence 2- to 6-fold, while hydrolysis of purine substrates resulted in a 1.2- to 1.4-fold quenching of fluorescence. Various derivatized nucleotide triphosphates were utilized in steady-state kinetic assays, and Ca venom phosphodiesterase showed k_{cat} values of 200-600/sec and K_m values of 5-30 mM at 20°C and pH 8.0.

Venom phosphodiesterases (Sigma: *C. durissus*; tropical rattlesnake) can also be analyzed electrophoretically via a zymogram assay using a phosphonate monoester, 5-iodoindoxyl-3-phenylphosphonate (Gangyi, 1990). However, the results shown indicate that this method is grossly qualitative and is not particularly suited for comparative analysis of venom phosphodiesterases.

An isotachophoretic assay was proposed by Bruchelt et al. (1991). Using an LKB Tachophor 2127, the hydrolysis of 2'-5'-triadenylate (ApApA) was followed using either conductivity or absorbance at 254 nm. Phosphodiesterase from snake venom (using 0.01 U; Sigma Chemical Co., source species not given) showed a turnover rate of 1.96 nmol/min at 37°C. Venom phosphodiesterase was used to calibrate the system for analysis of 2-5A phosphodiesterase from mononuclear blood cell lysates. A high degree of sensitivity and reproducibility was obtained, and this assay represents a significant advantage over other assays available for phosphodiesterases involved in the interferon-induced 2-5A system (Schmidt et al., 1979; Nilsen and Baglioni, 1979). However, its utility for assays of venom phosphodiesterases is limited unless capillary electrophoretic equipment is readily available.

Radiolabeled substrates have also been employed, primarily during investigations of the reaction mechanism of venom phosphodiesterases (see Section 2.3.A below). For example, using thymidine 5'[a-^{32}P]triphosphate as a substrate, a phosphorylated threonine residue was identified as the covalent intermediate of the venom phosphodiesterase-catalyzed reaction (Culp and Butler, 1986). Incubation of venom phosphodiesterase (*C. durissus terrificus*; Boehringer-Mannheim) with a 1-napthyl ester of 5'-[methyl-^{3}H]thymidylic acid, followed by reaction termination and thin layer chromatographic separation of products, showed that products were 1-napthol, 5'-thymidylic acid and thymidine (Rugevics and Witzel, 1982). Again, the general utility of labeled substrates for assaying crude venoms or for following purification is somewhat limited because of the additional methodology and precautions required.

An agar gel enzymodiffusion assay described for venom DNase (Sittenfeld et al., 1991) was likely actually assaying venom phosphodiesterase activity toward native DNA. This assay used calf thymus DNA co-

polymerized in agar gels (pH 7.4 or 9.0); venom was introduced into wells, and after staining with ethidium bromide, activity was visualized as a clear area which did not stain significantly. Measurement of cleared areas at several dilutions and comparison with known nucleases (DNase I, bovine pancreas, Sigma; DNase II, bovine spleen) allowed for relative quantification of activity. Using this procedure, phosphodiesterase from *C. adamanteus* venom showed significant activity. The pH of the assay (usually 7.4; some assays 9.0) and the observation that most "venom DNases" in this study showed basic pIs (see Table I) were both consistent with venom phosphodiesterases being the primary activity detected by this assay. Although this method is useful for demonstrating the activity of a venom (or lack thereof) toward native DNA, it is more difficult to quantify than the use of chromogenic substrates designed for phosphodiesterases.

C. Multiple Forms of Venom Phosphodiesterases

Using electrophoretic and zymogram methods, multiple forms of phosphodiesterase were isolated from *Vipera palastinae* (Palestine viper) venom (Levy and Bdolah, 1976). The isoenzymes had an approximate molecular mass of 130 kD and basic isoelectric points. When the isolated preparation was electrophoresed and reacted with a stain specific for phosphodiesterase activity (Sierakowska and Shugar, 1963), at least 4 bands of apparent phosphodiesterase activity were apparent. However, from the chromatogram in the original report, it appears likely that this preparation also contained other protein components as well as phosphodiesterase.

Purification of four isoenzymes from the venom of *Trimeresurus flavoviridis* (Habu) was achieved using three chromatographic steps (Kini and Gowda, 1984). Crude venom was first fractionated on CM-Sephadex; the non-bound phosphodiesterase (termed PDE-I) was then chromatographed on QAE-Sephadex. Two other phosphodiesterases (termed PDE-II and PDE-III) were separated from contaminants on Sephadex G-100, and the fourth phosphodiesterase (PDE-IV), which eluted with 75 mM phosphate, was not further purified. Carbohydrate content of three of the isoenzymes varied from 3.8-23.8%, but molecular mass was not determined. All four iso-enzymes also showed some phosphomonoesterase activity and were inhibited by EDTA. Since the carbohydrate content was variable, the four isoenzymes may simply differ from one another by extent of carbohydrate moiety. The molecular mass of venom phosphodiesterase was suggested to be somewhat variable depending on the method of isolation used (Laskowski, 1980).

D. Isolation of Venom Phosphodiesterases

Older methods for the isolation and purification of venom phospho-diesterases included one to several solvent precipitation steps (e.g., Razzell

and Khorana, 1959; Williams et al., 1961; Björk, 1963) followed by several chromatographic steps. More recent methods (Perron et al., 1993; Mackessy, 1989; Mori et al., 1987, Setoguchi et al., 1986) relied exclusively on chromatographic steps, usually involving size exclusion, anion exchangers (often DEAE functionalities) and cation exchangers (such as carboxymethyl resins). Other resins, including phosphocellulose P-11 and hydroxyapatite (Philipps, 1975, 1976), have been included in purification steps. Another method, designed to eliminate completely all other nuclease activities, involved acetone and ammonium sulfate fraction and several affinity ligand chromatography steps (Laskowski, 1980).

Most methods required several ion exchange steps with different functionalities in order to remove contaminating nucleases from phosphodiesterases. As an example of a multiple step chromatographic scheme, a method utilized by the present author's lab will be given (Perron et al., 1993). Crude venom from *C. m. pyrrhus* was initially fractionated using a size-exclusion column of BioGel P-100 (2.8 cm x 100 cm, 10 mM HEPES/ 60 mM NaCl pH 6.8). Phosphodiesterase activity eluted in the front portion of the first peak (Fig. 5A). This material was combined, dialyzed and lyophilized, resuspended in tris buffer (10 mM, pH 7.8) and applied to a 1.0 cm x 25 cm column of DEAE-Sephacel; phosphodiesterase activity did not bind to the matrix and eluted in the first peak (Fig. 5B). This material was dialyzed, lyophilized and resuspended in phosphate buffer (30 mM, pH 7.0) and applied to a 1.0 cm x 20 cm column of CM-Sephadex; the Cmp venom phosphodiesterase (>90% pure, based on SDS-PAGE) eluted in the fourth peak (Fig 5C). Rechromatographing this material on a Sephadex G-75 column gave a single symmetric peak. The chromatographic behavior of the Cmp venom phosphodiesterase (molecular mass about 110,000 Da) was very similar to that seen for *C. r. ruber* venom phosphodiesterase (Mori et al., 1987), which had a molecular mass of about 98,000 Da.

E. Biochemical Characterization of Venom Phosphodiesterases

Venom phosphodiesterase from *Crotalus adamanteus* venom was demonstrated to be a zinc metalloenzyme (Pollack and Auld, 1982). The phosphodiesterase from *Crotalus r. ruber* venom was shown to contain 1.04 mol zinc per mol enzyme. It contains carbohydrate, has a pI of 10.5, is heat-labile, consists of two identical subunits of 49,000 Da (native molecular mass of 98,000 Da) and is composed of 886 amino acid residues (Mori et al., 1987). Inhibition of activity by p-chloromercuribenzoate indicated the presence of essential cysteine or disulfides (Mori et al., 1987).

Cvo venom phosphodiesterase is a basic protein consisting of 2 (likely) identical subunits with apparent molecular masses of 57,000 Da each; the native enzyme is a homodimer of approximately 114,000 Da (Mackessy, 1989). Cmp venom phosphodiesterase, likely a closely homologous protein,

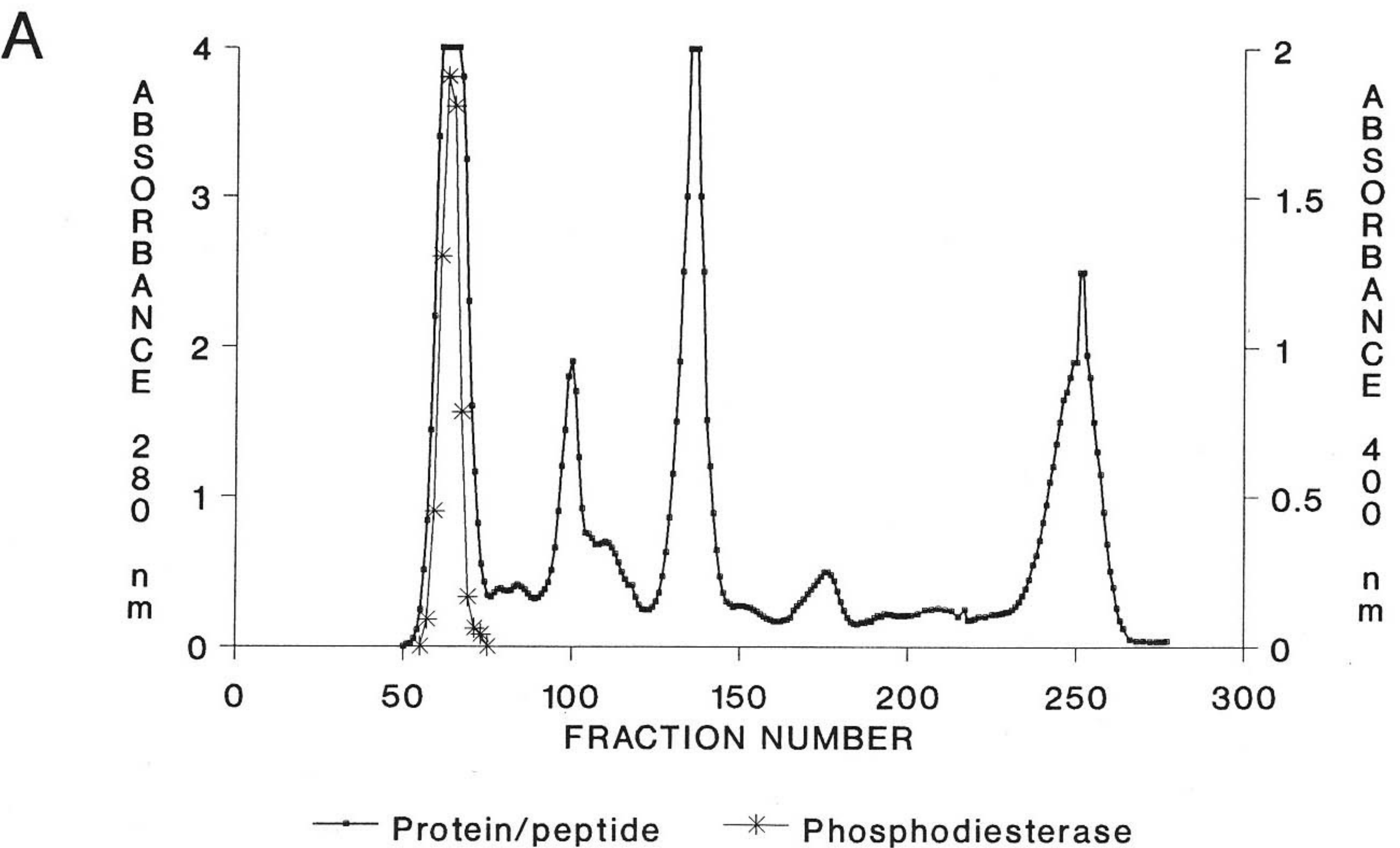

Figure 5. An example of the isolation of phosphodiesterase from the venom of *Crotalus mitchelli pyrrhus*. A. Size exclusion chromatography of crude venom on a 2.8 cm x 100 cm column of BioGel P-100. B. DEAE-Sephacel anion exchange chromatography of BioGel peak I; phosphodiesterase activity eluted with unbound proteins in the first peak. C. CM-Sephadex cation exchange chromatography of DEAE peak I; phosphodiesterase activity eluted in peak IV.

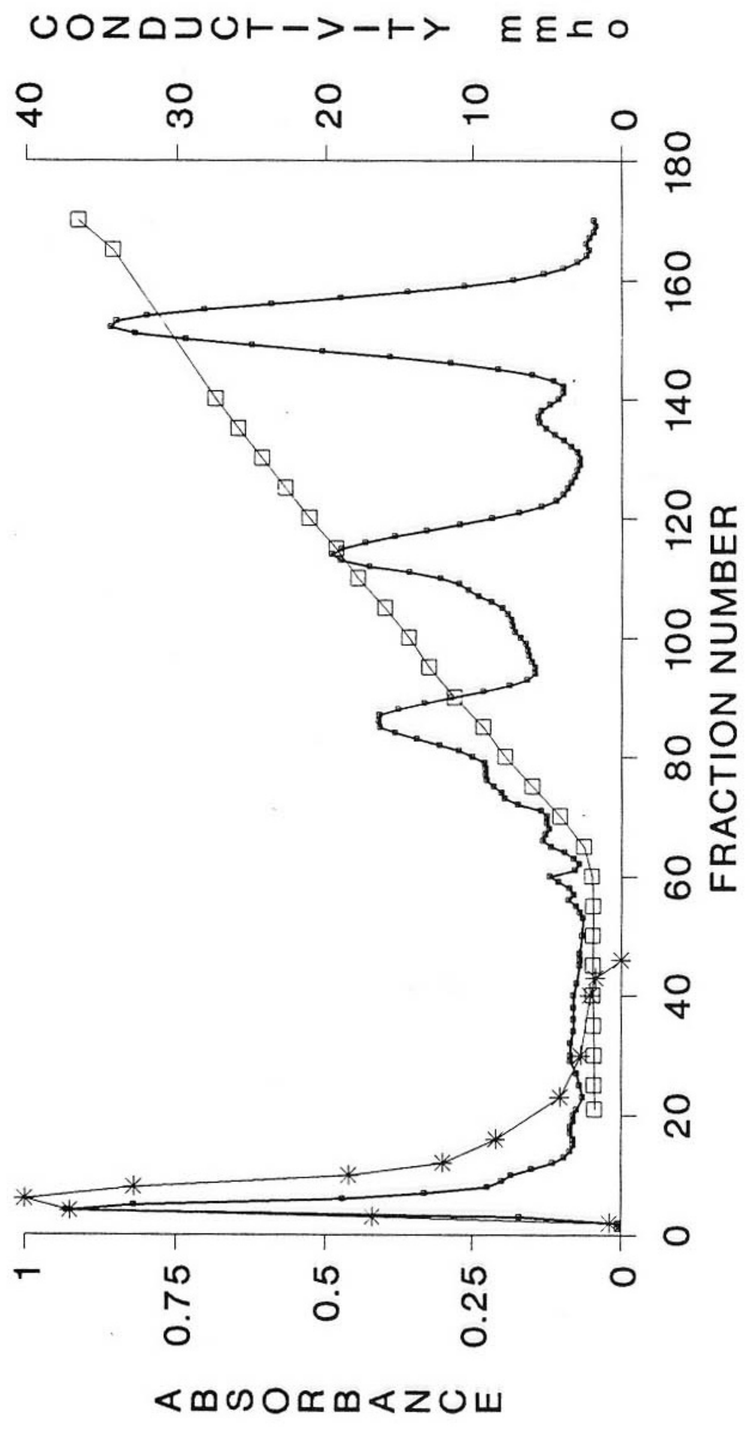

Fig. 5B

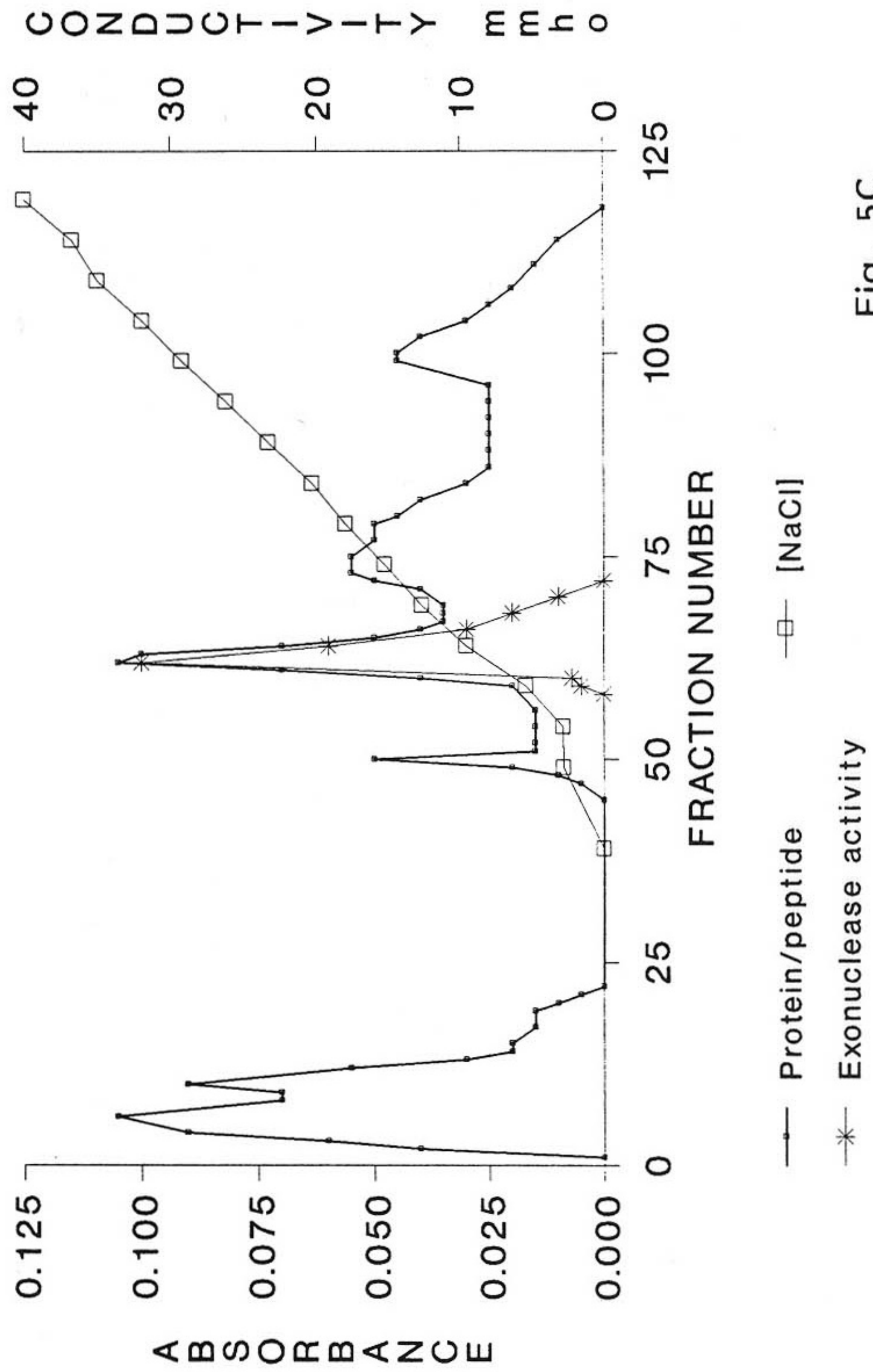

Fig. 5C

is also basic, consists of two subunits of 55,000 Da and has a native molecular mass of approximately 110,000 Da; it also has a pH optimum toward calcium bis-p-nitrophenyl phosphate of about pH 8.5 and catalyzes the hydrolysis of cAMP (Perron et al., 1993). The activity of this enzyme showed a rather narrow dependency on pH when calcium bis-p-nitrophenol phosphate was used as a substrate, and activity fell below 50% at pH <7>10 (Fig. 6). At pH 5.5, the pH of expressed venom, activity was less than 12% maximal activity.

A phosphodiesterase isolated from the venom of *Bothrops atrox* is also a basic glycoprotein and has an apparent molecular mass of 130,000 Da (Frischauf and Eckstein, 1973). Venom phosphodiesterases with native molecular masses of 140,000 Da have been isolated from *Agkistrodon acutus* venom (Sugihara et al., 1984) and *Trimeresurus mucrosquamatus* (Chinese habu) venom (Sugihara et al., 1986). Relative molecular masses were not determined for four isoenzymes isolated from the venom of *Trimeresurus flavoviridis* (Kini and Gowda, 1984), but all four phosphodiesterases were characterized as metalloglycoproteins.

Although zinc has been shown to be the metal cofactor for several venom phosphodiesterases, it can also be inhibitory (Mori et al., 1987). Other divalent cations, specifically magnesium or calcium, appear to be necessary for maximal activity (Williams et al., 1961). These authors also showed that NaCl was inhibitory at 0.11 M.

Properties of several isolated/purified venom phosphodiesterases are summarized in Table 1. The venom enzymes are generally greater than 100,000 Da in molecular mass, have basic pIs and a pH optimum of pH 8.5-9.2; most are homodimers, but one venom phosphodiesterase, isolated from *Agkistrodon acutus* venom ("ADPase"; Ouyang and Huang, 1986), was reported to be a single polypeptide of 94,000 Da.. Preparations of venom phosphodiesterase are generally heat-labile, and storage at -20°C in 5 mM tris-acetate, pH 8.8 containing .005% Triton X-100 and 50% glycerol preserved activity for at least one year (Laskowski, 1980).

Neither amino acid sequence nor cDNA sequence data is yet available for venom phosphodiesterases. A fluorescence study of *C. adamanteus* venom phosphodiesterase indicated the existence of at least two different types of tryptophan residues and indicated that about 60% of the tryptophans were found internally with the rest found in a neutral surface environment (Dimitrov et al., 1983). Amino acid compositional analysis of venom phosphodiesterases from *T. mucrosquamatus*, a basic protein (pI = 7.94), and from *C. r. ruber*, also a basic protein (pI = 10.5), indicated a relatively high proportion of aspartate/asparagine and glutamate/glutamine residues (about 21%; Sugihara et al., 1986; Mori et al., 1987); Crr venom phosphodiesterase also contained a large number of cysteine/half-cystine residues (66). Unlike most venom phosphodiesterases, Tm venom phosphodiesterase did not contain carbohydrate. The total numbers of residues

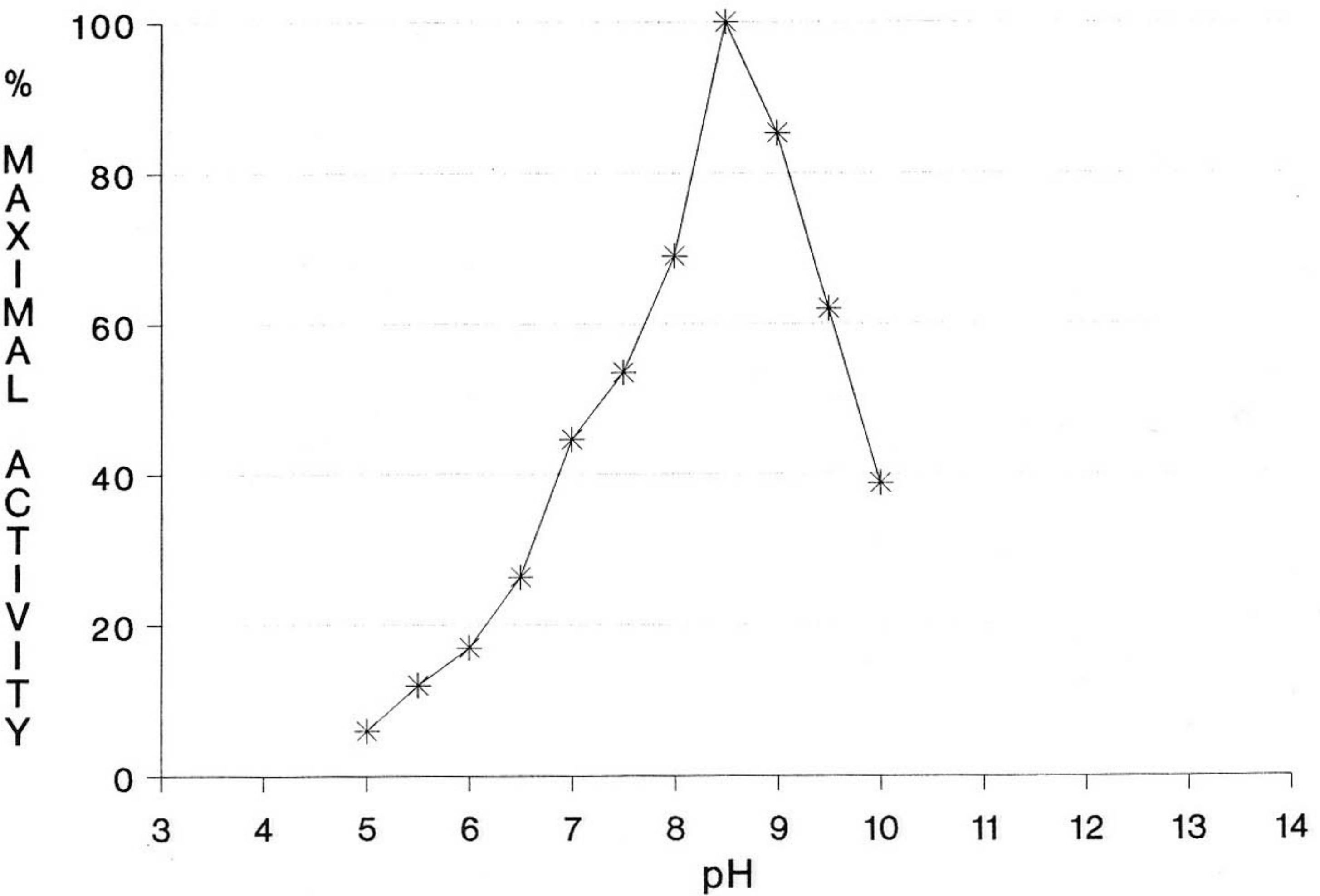

Figure 6. Effect of pH on activity of Cmp venom phosphodiesterase toward calcium bis-nitrophenyl phosphate. Note the rather narrow pH profile; optimum activity occurred at pH ~8.5.

Table 1. Biochemical properties of several venom phosphodiesterases

Species	Mr (kD)	pI	pH Opt.	Carbo.	Inhibitors	Reference
Agkistrodon acutus	94	basic	nd	nd	nd	Ouyang and Huang, 1986
Bothrops atrox	130	basic	9.2	+	EDTA	Frischauf & Eckstein, 1973 Philipps, 1976
Cerastes cerastes	110	nd	9.0	-	cys, GSH, DTT,2-ME, EDTA, AMP, ADP	Halim et al., 1987
Crotalus adamanteus	115; 140	~9.0	~ 8.5	nd	nd	Philipps, 1975; Stoynov et al., 1997
C. mitchelli pyrrhus	110	basic	8.5	nd	EDTA	Perron et al., 1993
C. ruber ruber	98	10.5	nd	+	EDTA, 1,10-phen., TGA, PCMB	Mori et al., 1987
C. viridis oreganus	114	basic	nd	nd	EDTA	Mackessy, 1989
Vipera palastinae	~130	basic	nd	nd	nd	Levy and Bdolah, 1976
Trimeresurus flavoviridis	nd	basic	nd	+	EDTA	Kini and Gowda, 1984
T. mucrosquamatus	140	7.94	nd	-	EDTA, 1,10-phen., TGA, PCMD, Cd^{2+}, Zn^{2+}	Sugihara, et al., 1986

Abbreviations: 2-ME, 2-mercaptoethanol; 1,10-phen., 1,10-phenanthroline; GSH, glutathione; Mr, relative molecular mass (in kilodaltons); PCMB, p-chloromercuribenzoate; TGA, thioglycolic acid; -/+, carbohydrate absent or present; nd, not determined.

were calculated to be 1398 and 886, respectively, and amino acid compositions are given in Table 2. Relative differences in proportions of amino acids, particularly cysteine, serine, phenylalanine, tryptophan and proline residues, suggest that the sequences of these enzymes are rather different.

2.3 Mechanism of Action of Venom Phosphodiesterases

A. Mechanism

Phosphodiester bonds found in nucleic acids are extremely resistant to hydrolysis, making these molecules superbly designed for maintaining the integrity of organism genomes. It has been shown that dimethyl phosphate, the simplest phosphodiester, has a half life in 1 M NaOH of approximately 15 years at 35° C (Chin et al., 1989). This extreme stability and resistance to nucleophilic attack has made the determination of the mechanism of action of phosphodiesterases and other nucleases extremely interesting; staphylococcal nuclease, a calcium-dependent bacterial nuclease which generates terminal 3' monophosphates, has been estimated to increase the non-enzyme catalyzed rate of hydrolysis of DNA by a factor of $\sim 10^{16}$ (Serpersu et al., 1987). The catalytic mechanisms of many nucleases, including venom phosphodiesterases, have been determined to a high degree of certainty, and an excellent summary of enzyme-catalyzed phosphodiester bond cleavage has been published recently (Gerlt, 1993). Hydrolysis of phosphodiesters proceeds via an S_N2 displacement reaction involving nucleophilic attack at the tetrahedral center of phosphorus opposite the leaving group, usually resulting in inversion of configuration.

The stereochemistry of the hydrolytic reactions catalyzed by venom phosphodiesterases has been investigated by several workers. Using [^{18}O]adenosine 5'-*O*-phosphorothioate-*O*-p-nitrophenyl ester, Burgers et al. (1979) demonstrated that the formation of [^{18}O]adenosine 5'-*O*-phosphorothioate by venom phosphodiesterase (*C. d. terrificus*; Boehringer-Mannheim) occurred with retention of configuration at the phosphorus, indicating that hydrolysis proceeded via a covalent nucleotide intermediate. An ATP analog, adenosine-5'-*O*-(1-thiotriphosphate), was utilized by Bryant and Benkovic (1979) to probe the stereochemistry of venom phosphodiesterase from *C. adamanteus* venom. As with the nitrophenyl ester, hydrolysis occurred with retention of configuration and indicated a covalent phosphoryl-enzyme intermediate. However, it was suggested that a nucleotidyl-enzyme intermediate was not proven unequivocally, because adjacent nucleophilic attack by water followed by a pseudorotation would give retention of configuration at the phosphorus (Jarvest and Lowe, 1981). Additionally, ambiguity of results of these experiments due to the low rate of reaction with substrate analogs was suggested (Mehdi and Gerlt, 1981).

Table 2. Amino acid composition of two venom phosphodiesterases

Amino acid	Trimeresurus mucrosquamatus [1]	Crotalus ruber ruber [2]
Asp/Asn	165	111
Glu/Gln	128	69
Lys	83	51
Arg	50	36
His	33	21
Cys	41	66
Ser	118	41
Thr	87	52
Tyr	66	34
Gly	91	106
Ala	72	65
Val	64	44
Leu	129	54
Ile	68	34
Phe	57	27
Trp	27	11
Met	23	17
Pro	96	47
Total	1398	886

Data from [1]Sugihara et al., 1986: based on M_r = 140 kD; [2]Mori et al., 1987: based on M_r = 98 kD.

Retention of configuration at phosphate following hydrolysis catalyzed by venom phosphodiesterase was conclusively shown using chirally labeled substrates. Using phosphodiesterase from *C. adamanteus* venom, the hydrolysis of adenosine 5'-[μ-^{16}O,^{17}O,^{18}O] triphosphate, in $H_2{}^{17}O$ was analyzed (Jarvest and Lowe, 1981). The authors postulated that a double displacement at the phosphorus occurred with a covalent nucleotidyl-enzyme intermediate. Evaluation of enzyme-catalyzed hydrolysis in $H_2{}^{16}O$ of the R_p diastereoisomer of thymidine 5'-(4-nitrophenyl[^{17}O,^{18}O]phosphate, a much better substrate for venom phosphodiesterase than thioesters, led to the same conclusion: since each nucleophilic displacement reaction is accompanied by an inversion of retention, and the phosphorus showed retention of configuration, a nucleotidyl-enzyme intermediate must be formed (Mehdi and Gerlt, 1981). Venom phosphodiesterase from *C. d. terrificus* was used in the latter study. Use of a tritiated substrate (1-napthyl ester of 5'-[methyl-3H]thymidine) confirmed these findings (Rugevics and Witzel, 1982). Therefore, the mechanism of hydrolysis of phosphodiester and phosphoanhydride bonds by snake venom phosphodiesterases involves a nucleotidyl intermediate covalently bound to the enzyme which is transferred to a molecule of (typically) water.

Short-term incubation of venom phosphodiesterase (*C. d. terrificus*) with tritiated substrate followed by phenolic extraction, chromatography on Sephadex G-25, isoelectric focusing and autoradiography demonstrated a protein-bound intermediate; a labeled amino acid residue was not identified, but the authors suggested that the nitrogen of histidine could be involved (Rugevics and Witzel, 1982). However, using venom phosphodiesterases from *C. adamanteus* and *C. d. terrificus*, and thymidine 5'-[μ-^{32}P]triphosphate as substrate, a threonine residue was identified in both enzymes as the active site amino acid forming the covalent intermediate during hydrolysis (Culp and Butler, 1986). The structure of the active site of venom phosphodiesterases remains unknown.

From assays with modified oligonucleotide substrates, it is probable that the base which is 3' to the phosphodiester linkage is involved in enzyme-substrate binding (Weinfeld et al., 1989; Buchko et al., 1992). Saturation of the pyrimidine ring of the thymidine of the dinucleotide d-ApT (conversion to 5,6-dihydrothymidine) had very little effect on venom phosphodiesterase-catalyzed hydrolysis (relative to d-ApT), indicating that decreased aromaticity of the base moiety did not affect enzyme-substrate binding appreciably (Weinfeld et al., 1993). Using ring-fragmented base substitutes, Matray et al. (1995) demonstrated that venom phosphodiesterase hydrolysis of (a-R-hydroxy-b-ureidoisobutyric acid)-containing oligonucleotides was inhibited at both the 3' and the 5' phosphates which linked this modified base with the rest of the oligonucleotide. Apparently, intact ring structure, regardless of small changes in aromaticity, is required for competent binding of substrate by the enzyme. Change of the b-phosphodiesters to a-phosphodiester

linkages was used to produce antisense oligonucleotides which are highly resistant to degradation by *C. durissus* venom phosphodiesterase (Vichier-Guerre et al., 1994) and this resistance to hydrolysis further demonstrated stereospecificity of the enzyme.

Based on the retention of configuration at phosphorus of venom phosphodiesterase-catalyzed reactions, in contrast to the inversion of configuration seen in reactions catalyzed by all known kinases (Gerlt, 1993), it has been suggested that retention has resulted from evolutionary pressures favoring the conservation of a number of binding sites required for normal biological function (Frey, 1989). Enzymes which preserve configuration may actually act *in vivo* as nucleotide transferases rather than as nucleotide hydrolases. Evidence that venom phosphodiesterases may in fact be nucleotidyl transferases was provided by the demonstration that phosphodiesterase from *C. durissus terrificus* venom catalyzed the transfer of guanosine 5'-monophosphate to methanol, forming a GMP methyl ester (Garcia-Diaz et al., 1991).

The same venom phosphodiesterase was subsequently shown to catalyze the transfer of AMP from ATP to short-chain (poly)alcohols including 1-propanol, ethanol, methanol, ethylene glycol, glycerol, 2-chloroethanol and 2,2-dichloroethanol (Garcia-Diaz et al., 1993). Results supported a model of general acid-base catalysis in the active center of the enzyme with transient covalent binding of the substrate to a threonine residue. The rate-limiting step during nucleotide transfer to alcohols was hypothesized as the proton transfer from the alcohol to the basic group in the active center (the second nucleophilic replacement). The reaction sequence of solvolysis (hydrolysis and alcoholysis) of ATP in water/alcohol mixtures (Garcia-Diaz et al., 1993) is shown in Fig. 7. In addition to neutral alcohols, negatively charged alcohols (glycerol-2-phosphate and sn-glycerol-3-phosphate) can participate in venom phosphodiesterase-catalyzed 5'-nucleotidyl transfer reactions (Vergeles et al., 1995). At low millimolar concentrations the primary alcohol functional groups of the glycerol phosphates were much better acceptors of venom phosphodiesterase-bound AMP than was water, but this efficiency decreased abruptly when higher concentrations were used or if P_i or NaCl were present. It was postulated that a specific interaction of the phosphoryl group of the glycerols occurs with an enzyme site, perhaps a specific binding site for alkyl-CH_2OH receptors bearing negative charges.

If venom phosphodiesterases function *in vivo* as transferases, then by analogy it is likely that a regulatory component is being targeted, since many venom components act to disrupt highly regulated processes (such as the blood clot cascade reactions and control of vasotension). At present, native target sites of nucleotide transfer, if they exist, are completely unknown.

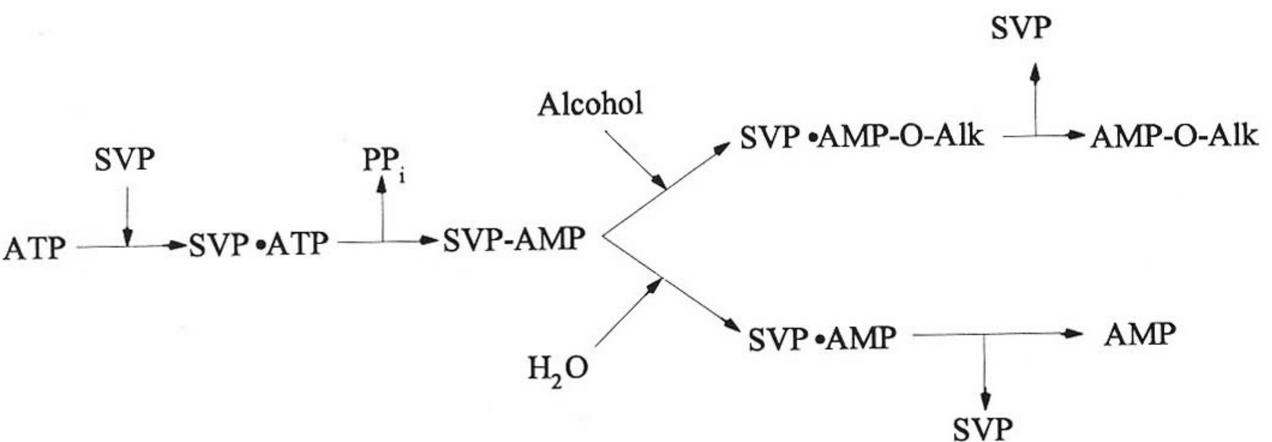

Figure 7. Proposed reaction sequences for the solvolysis of ATP by Cdt venom phosphodiesterase (Garcia-Diaz et al., 1993). In alcohol/water solvent, hydrolysis or alcoholysis (nucleotide transfer to an alcohol) can occur. Alk, alkyl group; SVP, Cdt venom phosphodiesterase; X· Y, noncovalent intermediates; SVP-AMP, enzyme-nucleotide covalent intermediate.

B. Specificity

Activity levels of various nucleases in crude venoms are commonly distinguished by the ability to catalyze the hydrolyze of specific synthetic substrates. As mentioned previously, calcium bis-p-nitrophenyl phosphate is commonly used to assay venom phosphodiesterase. Nonspecific phosphatase may be assayed in venoms using sodium p-nitrophenyl phosphate. 5'-nucleotidase is distinguished by preferential hydrolysis of 5'-mononucleotides, and endonucleases (see later) are distinguished by their ability to hydrolyze polynucleotides (often larger DNA and RNA molecules) and liberate oligonucleotide fragments. Venom phosphodiesterases show negligible activity toward p-nitrophenyl phosphate or monophosphate nucleotides, but di- and triphosphate nucleotides, DNA and RNA, and many derivatives of these native molecules can serve as substrates for phosphodiesterases.

Venom phosphodiesterases are known to hydrolyze both DNA and RNA (Laskowski, 1971), as well as ribose and deoxyribose nucleotide triphosphate analog substrates (Pollack and Auld, 1982; Mori et al., 1987). Venom phosphodiesterase from *C. adamanteus* hydrolyzed p-nitrophenyl thymidine 5'-phosphate 30-fold faster than p-nitrophenyl uridine 5'-phosphate (Razzell, 1963). Sheared *E. coli* DNA served as a substrate for venom phosphodiesterase, but chemical cross-linking of DNA greatly inhibited activity, which suggested that exonucleolytic degradation of DNA stopped at crosslinks (Pritchard and Eichinger, 1974). Supercoiled PM2 DNA (form I) can also serve as substrate for venom phosphodiesterase, and activity toward the supercoiled form is 10,000 times faster than toward the relaxed circular DNA (form I'; Laskowski, 1980). Cleavage of the supercoiled molecules may occur at the base of the supercoiled branch. After linear double-stranded fragments were generated, venom phosphodiesterase catalyzed the hydrolysis of nucleotides from both termini without leaving single-stranded ends. Using T7 viral DNA, a 5-fold greater rate of hydrolysis was noted for single stranded DNA relative to double stranded DNA (Laskowski, 1980).

Cyclic mononucleotides such as cyclic 3',5'-AMP and 2',3'-AMP are also readily hydrolyzed by venom phosphodiesterases derived from *Naja naja atra, Trimeresurus flavoviridis* and *Agkistrodon halys* venoms (Suzuki et al., 1960), liberating 3'-AMP. Based on reaction rates, cyclic 3',5'-AMP was a better substrate. The present author's lab has also shown that c3',5'-AMP is hydrolyzed by Cvo venom phosphodiesterase (*Crotalus viridis oreganus*; Mackessy, 1989) and Cmp venom phosphodiesterase (*C. mitchelli pyrrhus*; Perron et al., 1993). Oligonucleotides showing a different linkage (i.e., not 5',3'), such as 2',5'-oligoadenylates, are also recognized and hydrolyzed by venom phosphodiesterase (Bruchelt et al., 1991). As noted above, nitrophenyl derivatives of thymidine are good chromogenic substrates for venom phosphodiesterases (Björk, 1963; Razzell, 1963; von Tigerstrom and Smith, 1969).

Venom phosphodiesterase (*C. durissus terrificus*; Sigma) was shown to catalyze the hydrolysis of a tetraphosphate derivative of ATP and glyceric acid, 3-phosphoglyceroyl-g-triphospho-5'-adenosine, liberating ADP as a reaction product (Patel et al., 1991). Absolute nucleotide sequence does not seem to influence the overall rate of hydrolysis (Tu, 1977), except in a-phosphodiester-linked oligonucleotides (Vichier-Guerre et al., 1994). However, if the N-glycosylic bond between a base and the deoxyribose group is cleaved, forming an apurinic or apyrimidinic residue, hydrolysis is significantly affected (Weinfeld et al., 1989). Using modified dinucleotides, it was shown that venom phosphodiesterase (*C. adamanteus*; Sigma) fully hydrolyzed the apurinic substrate d-SpA (deoxyribose-5'-dAMP) to 5'-dAMP, while d-ApS (5'-dAMP-deoxyribose) was not hydrolyzed (Fig. 8), even if enzyme concentration was increased 10-fold and incubation time was extended 4-fold. When the apurinic deoxyribose aldehyde function was capped with methoxyamine, identical results were obtained, indicating that the aldehyde function does not influence enzyme interaction with the adjacent phosphodiester groups. If the apurinic residue was in the middle of ^{3}H-labeled poly(dA), venom phosphodiesterase produced only 5'-dAMP and d-SpA (Weinfeld et al., 1989).

These results indicated that Ca venom phosphodiesterase required a base on the 3' side of the phosphodiester bond, consistent with an enzyme-substrate interaction model which involves binding to the base of the 3'-nucleoside. Additionally, the observation that hydrolysis products of poly(dA) digestion did not include oligonucleotide fragments suggests that the Ca venom phosphodiesterase either contains endonuclease activity or has the capacity to skip over apurinic sites and continue exonucleolytic activity. Alternatively (though not overly likely), the preparation could have contained an endonuclease contaminant.

When the dinucleotide thymidylyl-(3',5')-2'-deoxyguanosine (d(TpG) was photooxidized, thymidylyl-(3',5')-4,8-dihydro-4-hydroxy-8-oxo-2'-deoxyguanosine (d(TpO) was produced (Buchko et al., 1992); neither the 4R nor the 4S diastereoisomer of d(TpO) was hydrolyzed following incubation for up to 24 hr with venom phosphodiesterase (species not given; Boehringer-Mannheim). As above, this result showed that alteration of the 3'-nucleoside site impeded the enzyme's ability to hydrolyze the phosphodiester bond. However, reduction of the aromaticity of the base 3' to the phosphodiester bond (using d-ApT) did not significantly affect hydrolysis rate (Weinfeld et al., 1993).

Stereochemical specificity of venom phosphodiesterase (*C. t. terrificus*; Boehringer-Mannheim) was demonstrated using the S_p and R_p diastereoisomers of [^{18}O]adenosine 5'-*O*-phosphorothioate-*O*-p-nitrophenyl ester (Burgers et al., 1979); only the R_p diastereoisomer was hydrolyzed. Similarly, the relative reactivity of the R and the S enantiomers of ATPμS (adenosine 5'-*O*-(1-thiotriphosphate)) to venom (*C. adamanteus*) phosphodiesterase-catalyzed hydrolysis were 70:1 (Bryant and Benkovic, 1979).

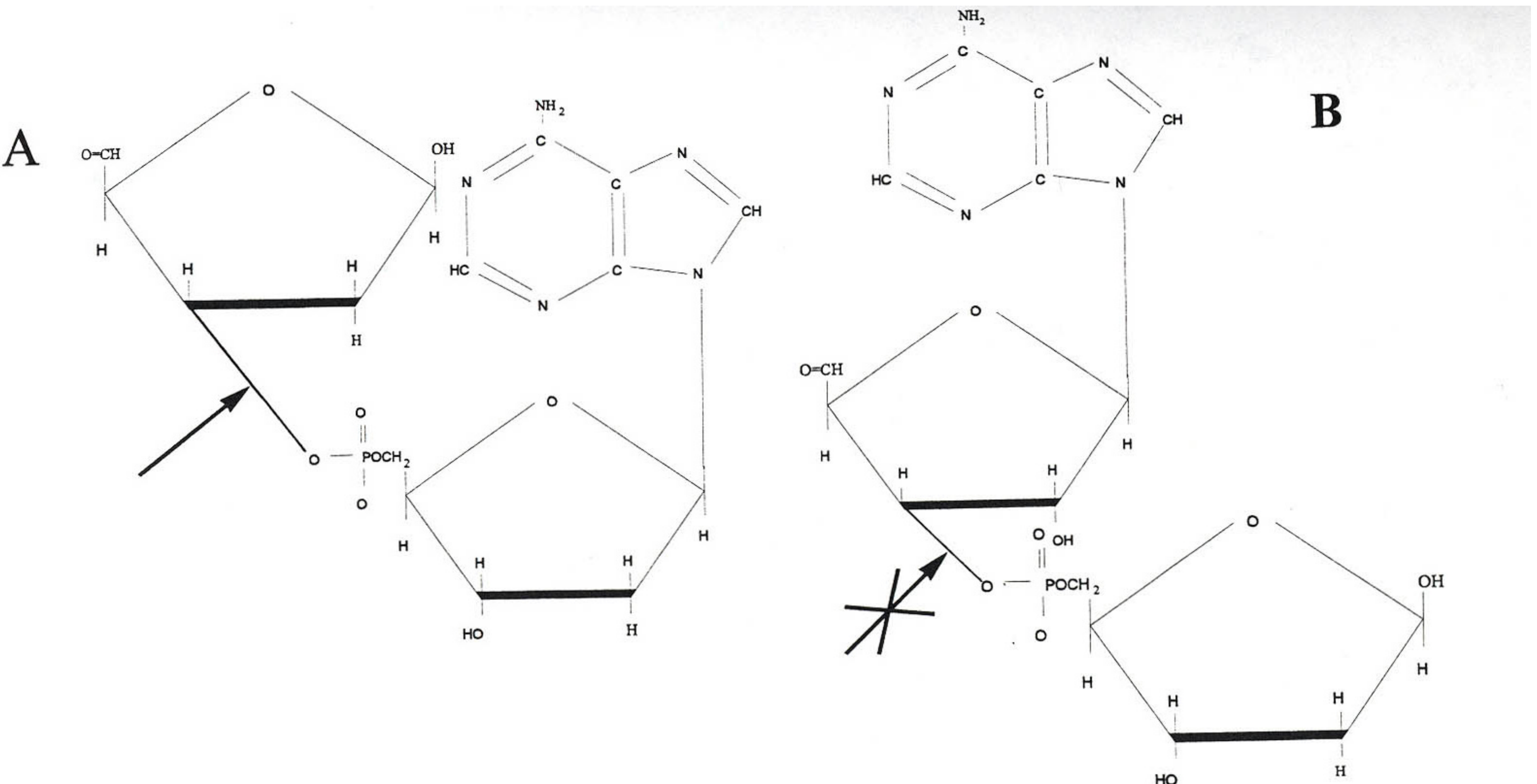

Figure 8. Effect of partial depurination of deoxyadenosine-adenosine-5'-monophosphate (d-ApA) on Ca venom phosphodiesterase activity (Weinfeld et al., 1989). Depurination on the 5' end of the phosphodiester bond had no appreciable effect on enzyme catalyzed hydrolysis (arrow), and 5'-AMP was released (A). Venom phosphodiesterase was unable to cleave the bond (X arrow) if depurination was on the 3' end (B), indicating the importance of the base component of the 3'-nucleoside.

C. Inhibitors

Venom phosphodiesterases are metalloenzymes and are sensitive to metal ion chelators such as EDTA and 1,10-phenanthroline (Pollack and Auld, 1982; Mori et al., 1987). Cysteine (at 1 mM), dithiothreitol (at 5 mM) and p-chloromercuribenzoate (at 5 mM) inhibited venom phosphodiesterase, but soybean trypsin inhibitor and benzamidine were not inhibitory (Mori et al., 1987; Sugihara et al., 1986). Zinc and cadmium ions at 5 mM were also determined by these authors to be inhibitory. Inhibition by cysteine, DTT or PCMB suggest that disulfide(s) or a reduced cysteine are essential for activity.

Citrate, an endogenous component of venoms from several (perhaps most) species of snakes, was shown to inhibit phosphodiesterase activity of *Crotalus adamanteus* venom by 75% at 100 mM (Francis et al., 1992). An older study (Razzell and Khorana, 1959) showed that at 5 mM sodium citrate, 94% of phosphodiesterase activity was retained. The high levels of citrate present in snake venoms were suggested to contribute to the inhibition of activity *in vivo* via metal ion chelation (Francis et al., 1992). High levels of NaCl (110 mM) were found to inhibit venom phosphodiesterase activity toward DNA (Williams et al., 1961) approximately 75% (compared to 10 mM NaCl). When Mg^{2+} concentrations were increased to 50 mM (from 1.0 mM) under high salt conditions, activity returned to those seen under low salt conditions. Citrate-induced inhibition could likely be reversed by high levels of magnesium.

Hydrolysis of oligonucleotides catalyzed by snake venom phosphodiesterases was shown to be inhibited by apurinic/apyrimidinic residues in the 3' side of the diester bond (Weinfeld et al., 1989). If the pyrimidine ring of thymine is opened (fragmented, producing a-R-hydroxy-b-ureidoisobutyric acid) and this modified residue is incorporated in an oligonucleotide, oligonucleotide degradation at either the 5' or the 3' end of this residue was inhibited (Matray et al., 1995). Inhibition likely occurs due to disruption of enzyme-substrate binding.

2.4 Biological Activity of Venom Phosphodiesterase

Although venom phosphodiesterase has been the subject of many studies, few have investigated the biological activity of this near-ubiquitous venom component. One of the enigmatic features of venoms in general is the presence of such a high degree of compositional complexity; if simple killing or predigestion of prey is paramount, one could envisage a venom of much simpler composition. An older study (Russell et al., 1963) of phosphodiesterases from *Crotalus adamanteus, C. atrox, C. horridus horridus, C. viridis helleri* and *Vipera russelli* venoms demonstrated an LD_{50} of 3.08-4.65 mg/kg in mice, not particularly toxic for an isolated venom component.

Symptoms of phosphodiesterase intoxication included hypoactivity, hyporeactivity, extension of the hind limbs and slight cyanosis. The role of this component to the sequelae following envenomation is unclear, but reduction of cAMP levels and production of toxic secondary metabolites may be factors contributing to prey death.

A more recent report described the platelet aggregation-inhibiting activity of venom phosphodiesterase (called an ADPase) from *Agkistrodon acutus* venom (Ouyang and Huang, 1986). This preparation caused marked inhibition of ADP-, collagen- or arachidonate-induced platelet aggregation, but a specific biological role was not assigned. It is plausible that a synergistic interaction with hemorrhagic proteases and fibrinogenases found in the same venom occurs during envenomation, interfering with normal hemostatic mechanisms and promoting blood loss and circulatory collapse.

Regulation of action of some enzymes is accomplished *in vivo* by covalent modification. Glutamine synthetase from the bacterium *Azotobacter chroococcum* was shown to be re-activated after treatment with snake venom phosphodiesterase (Boehringer-Mannheim; species not given) but *not* after treatment with alkaline phosphatase (Muñoz-Centeno et al., 1994). The authors concluded that venom phosphodiesterase catalyzed the hydrolysis of covalently-bound AMP or ADP from glutamine synthetase, and that this deadenylylation reaction lead to re-activation of the enzyme. It is at least possible that an analogous reversal of covalent regulation of an enzyme in vertebrate systems could occur. The biological role of venom phosphodiesterases in envenomation could thus result, as with many other venom enzymes, from interference with normal homeostatic mechanisms.

3. ENDONUCLEASES

3.1 Ribonuclease

As mentioned above, endonucleases from venoms are poorly characterized, lending strength to the possibility that some endonuclease activity may be due to phosphodiesterase or other venom nuclease activity. A notable exception is the double-strand-specific ribonuclease (now known as RNase V_1) isolated by Vassilenko and co-workers (Vassilenko and Babkina, 1965; Vassilenko and Ryte, 1975) from *N. n. oxiana* (central Asian cobra) venom. It is expected that this enzyme or a homolog should be present in venoms from related species of cobras, but at present no supportive data are available.

A. Isolation of Ribonuclease from Snake Venom

Isolation of the enzyme from central Asian cobra venom was first described by Vassilenko and coworkers (Vassilenko and Babkina, 1965;

Vassilenko and Ryte, 1975). Gel filtration followed by cation exchange chromatography gave a preparation with an apparent molecular mass of 15,900 Da and with double-strand-specific ribonuclease activity. The homogeneity of this preparation is somewhat in doubt.

Subsequent isolation schemes have utilized similar stepwise chromatography on Sephadex G-75 followed by cation exchange on Servacel SE23 sulfoethylcellulose (Auron et al., 1982). The material obtained by this procedure was characterized as a basic protein with an apparent molecular mass of 32,400 Da, twice that of earlier reports. However, three "minor protein contaminants" (25,700 Da, 10,000 Da and 5,900 Da) were also noted in this preparation, which was subsequently used in a comparison of tRNA structures. The homogeneity of this preparation is therefore also doubtful.

A slightly modified isolation procedure which appears to produce a single protein preparation with minimal contamination was proposed by Digweed et al. (1986). Gel filtration of *Naja naja oxiana* venom on Sephadex G-75 was followed by two sulfopropyl-Sephadex G-25 steps. RNase activity was confined to the second peak of the second SP-Sephadex step, which was subdivided into a, b and c fractions. Only fraction a appeared homogenous on SDS-PAGE, and this material (named CSV ribonuclease) was used for subsequent structural studies. However, from the original chromatogram (Digweed et al., 1986), it appears that most of the RNase activity resided in fractions b and c, suggesting one of several things. One, there may be several different ribonuclease isoforms present in the venom; or two, the first fraction of the preparation may contain the smallest proportion of several non-RNase contaminant proteins (seen on SDS-PAGE of fractions b and c). Regardless, the preparation used for structural studies was likely homogenous, but it appears that a more efficient isolation procedure may need to be developed.

B. Assays of Ribonuclease Activity

Several assays of RNase activity of crude venoms and purification step fractions have been proposed. A relatively simple method utilized by Digweed et al. (1986) involves following the hydrolysis of *Escherichia coli* 16S + 18S rRNA spectrophotometrically at 260 nm. One unit of enzyme activity was defined as the amount of enzyme producing an increase of 10^{-3} A_{260} unit/min. Hydrolyses of tRNA were conducted in 50 mM tris/HCl at pH 6.8; digests also contained 5 mM $MgCl_2$ and 50 mM NaCl. The described method utilized a one ml volume; however, the method seems amenable to adaptation for microassays, and utilization of an ELISA plate reader will allow a very rapid throughput time for numerous samples generated during purification of the enzyme. rRNA is also commercially available, further facilitating this assay. A spectrophotometric coupled-enzyme assay, utilizing 3'adenosyl dinucleoside monophosphate substrates, may be useful for

detecting and isolating snake venom RNases (Postek et al., 1992).

A more laborious assay was utilized by Auron et al. (1982) to follow the purification of ribonuclease V_1. Aliquots from column fractions were incubated with tRNA in 25 mM sodium acetate, pH 4.5, containing 5 mM $MgCl_2$ and 50 mM KCl. In this assay, the degradation of 5-^{32}P-labeled yeast tRNA was followed utilizing SDS-PAGE. Although this method may be useful for later characterization of the purified enzyme or for evaluating nuclease-susceptible sites on RNA molecules, the previous methods seem to be more efficient alternatives for scanning many fractions generated during purification procedures.

C. Specificity of Action

Ribonuclease V_1 appears to be specific for double-stranded RNA (Vassilenko and Ryte, 1975; Lockard and Kumar, 1981; Auron et al., 1982; Digweed et al., 1986). Digestion of yeast $tRNA^{Phe}$ occurred only in the regions known to be double-stranded (Fig. 9; Auron et al., 1982), whereas digestion with nuclease S1 (from the bacterium *Aspergillus oryzae*) is limited to just the exposed anticodon loop (Wrede et al., 1979a,b). When yeast $tRNA^{Phe}$ molecules complexed with aminoacyl-tRNA synthetase were digested with Rnase V_1, only the stem portion of the anticodon loop (see Fig. 9) was conferred partial protection from hydrolysis, while nearly 100% protection was conferred to the anticodon and acceptor stem of yeast $tRNA^{Val}$ (Favorova et al., 1981). 5S RNA from *Xenopus laevus* (African clawed frog) oocytes was also partially protected from hydrolysis by RNase V_1 when the 5S RNA was complexed with transcription factor IIIA (Sands and Bogenhagen, 1991). By comparison with protection conferred by a tryptic fragment of transcription factor IIIA, it was suggested that the carboxyterminal zinc fingers of the protein (8 and 9) interact with the distal portion of helix V of the 5S RNA.

A potential model for the active site region of RNase V_1 has been proposed (Auron et al., 1982). Cleavage is not associated with any particular nucleotide or nucleotide sequence, and it is hypothesized that the enzyme interacts with a helical groove in substrate regions of the tRNA duplex, particularly at position C_{28}. The "minimum active site" was represented as an L-shaped region which recognizes hydrogen bond acceptors and donors within the minor groove of the RNA molecule; nucleolytic cleavage occurs at a position which is one nucleotide removed from the recognition site (Auron et al., 1982). Cleavage sites (at pH 4.5) on the yeast $tRNA^{Phe}$ molecule are shown in Fig. 9. However, at pH 7.6, a somewhat different pattern of hydrolysis was obtained which indicated more limited RNA digestion (Butorin et al., 1982).

Ribonuclease V_1 (cobra venom ribonuclease) has been used to probe the secondary and tertiary structure of 5S ribosomal RNA from several

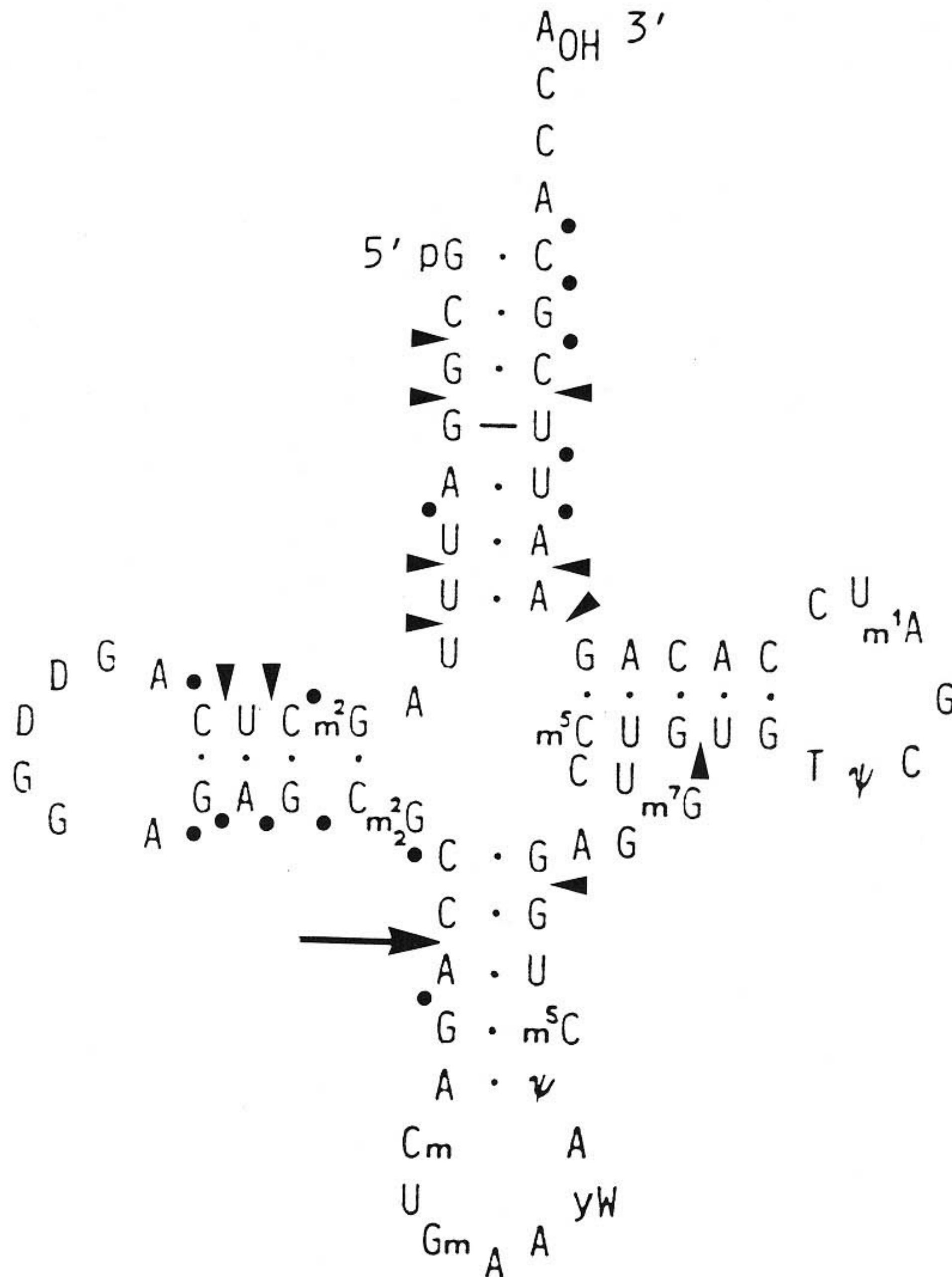

Figure 9. Ribonuclease V_1-sensitive sites on yeast $tRNA^{Phe}$ (Auron et al., 1982). RNase V_1 from cobra venom preferentially catalyzed the hydrolysis of sites in double-stranded regions. Large arrow, primary cleavage site; arrowheads, moderate intensity cleavage sites; closed circles, low intensity cleavage sites.

species (Troutt et al., 1982; Digweed et al., 1986) and tRNA-like molecules in turnip yellow mosaic virus (Mans et al., 1992). Using 5S rRNA from silk moth (*Bombyx mori*) and slime mold (*Dictyostelium discoideum*), Troutt et al. (1982) showed that these molecules were hydrolyzed differentially in the structured (double-stranded) areas. RNase V_1 (called CSV by these authors) was used to analyze the structure of 5S rRNAs from bacteria and spinach and tRNAs from yeast and bacteria (Digweed et al., 1986). For these molecules, many regions with secondary structure (double-strandedness) are susceptible to hydrolysis. Specificity of the enzyme was determined by digestion of 5'-end-labeled tRNAs from *E. coli* and yeast followed by electrophoresis on sequencing gels. RNase V_1 catalyzes the hydrolysis of tRNAs to yield oligonucleotides with 3' hydroxyl groups.

The effect of internal deoxythymidine or deoxyadenosine in oligonucleotide duplexes on patterns of hydrolysis by RNase V_1 was investigated using 14-mer oligoribonucleotide duplexes (5'-GGCCGGAUCCGCGC-3') and substituted oligonucleotides (5'-GGCCGG[**dAU** or **AdT**]CCGCGC-3') (Wyatt and Walker, 1989). Oligoribonucleotide digestion resulted in 5'-nucleotides, a dinucleotide at the 5' end and a trinucleotide at the 3' end. If dA and/or dT were incorporated, cleavage was reduced significantly at the phosphate immediately 3' of the deoxynucleotides; the rest of the cleavage sites were of unchanged intensity. Substitution of dT with dU gave the same results. These results suggested that for optimal cleavage, RNase V_1 requires two nucleotides 5' and three nucleotides 3' of the cleavage site. The enzyme may bind to the phosphates and/or sugars of these five nucleotides on one strand of a helical region which may be double-stranded RNA duplex, DNA-RNA hybrid duplex or single stranded stacked bases (Wyatt and Walker, 1989). Regardless of molecule identity, for cleavage to be efficient, the residue hydrolyzed must be a ribonucleotide.

Although RNase V_1 has had considerable use as a probe of RNA structure, the enzyme itself is rather poorly characterized. Due to its relatively small size (15,000-32,000 Da), RNase V_1 is amenable to protein or cDNA sequencing, but at present these data are not available.

3.2 Deoxyribonuclease

It seems likely that some reports of DNase activity (e.g., Sittenfeld et al., 1991) in venoms are actually due to the actions of venom phosphodiesterases, which have the capacity to hydrolyze both RNA and DNA (Stoynov et al., 1997; Iwanaga and Suzuki, 1979; Laskowski, 1971). However, endonuclease activity with a pH optimum of 5.0 has been described for several venoms (*B. atrox*: Georgatsos and Laskowski, 1962; *C. adamanteus*: Laskowski, 1980; *Naja naja oxiana*: Vassilenko and Ryte, 1975; *Trimeresurus flavoviridis*: Maeno, 1962; *Vipera russelli*: McLennan and Lane, 1968) which is in addition to venom phosphodiesterase activity (pH optimum of 8.9) in the

same venoms. If this is a unique venom protein with an acidic pH optimum, it should be considered a DNase II (deoxyribonucleate 5'-oligonucleotido-hydrolase; E.C. 3.1.21.1).

A. Isolation of Deoxyribonuclease from Snake Venoms

Isolation from snake venoms of an enzyme specific for DNA has not yet been reported. Some time ago, the isolation of an endonuclease from *Bothrops atrox* venom was described (Williams et al., 1961; Georgatsos and Laskowski, 1962). This enzyme, termed "venom endonuclease", showed activity toward both DNA and RNA as well as poly-AU (Georgatsos and Laskowski, 1962). Purification of this endonuclease was accomplished by an eight step procedure including acetone precipitation, calcium gel adsorption, ammonium sulfate fractionation and ion exchange chromatographies.

More recently, the separation of exo- and endonuclease activities of *C. adamanteus* venom which occurred at the last step of a five step isolation procedure was described (Laskowski, 1980). Crude venom was acetone-fractionated followed by acidification with acetic acid; these steps resulted in a decrease of 5'-nucleotidase activity by ~10,000-fold. The dialyzed solution resulting was chromatographed on concanavalin A-Sepharose, ultrafiltered, chromatographed on BioGel P-150 and then chromatographed on NADP-agarose. The last step resulted in separate endonuclease and exonuclease preparations which each showed 90% specific nuclease activity (Laskowski, 1980). However, the primary goal of this work was to eliminate contaminating nuclease activities from venom phosphodiesterase, and very little is known about this isolated venom DNase. A neutral DNase (pH optimum of 7) which lacks RNase and phosphodiesterase activity was isolated from a venom sac extract of *Vespa orientalis* (oriental hornet), but it is unlikely that this protein has any homology with snake venom DNases (Ring et al., 1981).

B. Assays of Deoxyribonuclease Activity

DNase activity of a phosphodiesterase preparation from *B. atrox* venom was assayed using a viscometric method (Williams et al., 1961). A spectrophotometric assay which followed the increase in absorbance at 260 nm has also been described (Georgatsos and Laskowski, 1962) and has obvious advantages over the viscometric method. This assay involved incubation of enzyme (crude venom) with calf thymus DNA at pH 5.0 and 37°C. A DNase assay described above (Section 2.2.B) is likely actually detecting venom phosphodiesterase activity, since pH 7.4 or greater was used in all experiments (Sittenfeld et al., 1991). Based on the early work in Laskowski's lab, the venom (DNA/RNA) endonucleases should have a pH optimum of 5.0, and it is likely that these enzymes would be inactive at pH 7.4 (*B. atrox* endonuclease showed activity only between pH 4 and 6; Georgatsos and

Laskowski, 1962). It would be of interest to repeat the experiments of Sittenfeld et al. (1991) at acidic pH to see if DNase activity is still detected.

An isoelectric focusing separation at a pH range of 4-6, followed by an agarose overlay containing calf thymus DNA and ethidium bromide (at pH 7.0) was used to detect DNase activity in bovine pancreatic extracts (Kim and Liao, 1982). Essentially the same procedure was used by Sittenfeld et al. (1991) as a second assay (and detection of isoenzyme variants) of venoms. Again, given the limited amount of information available about venom DNases, it appears that this assay also will not detect venom DNases with an acidic pH optimum; however, it should be possible to use this assay (with agarose at pH 5.0) to probe for true venom DNase activity.

C. Specificity of Action

The lack of information about venom DNases makes it difficult to discuss specificity (if it exists). Based on the early work with *B. atrox* venom, the venom enzyme has a pH optimum of 5.0, activity is abolished outside the range of pH 4-6, requires no divalent cations and hydrolyzes both RNA and DNA (Georgatsos and Laskowski, 1962). Trinucleotide or higher oligonucleotides were produced predominantly when DNA served as substrate, with dGpGp linkages being most susceptible to hydrolysis; products terminated in a 3'-monoesterified phosphate.

4. CONCLUSIONS

In the last 20 years significant progress has been made toward an understanding of the action of venom phosphodiesterases and RNases, but little progress has been made toward an understanding of venom DNases. However, research on these enzymes from a toxinological perspective lags behind progress made with regards to other nucleases (cf. Linn et al., 1993), and it is clear that much work remains to be done with venom nucleases. Several areas in particular require attention, including determination of amino acid and/or cDNA sequence, which in turn will allow evaluation of the degree of homology of enzymes from various species and families of snakes. A considerable literature exists on cyclic AMP-specific phosphodiesterases from other sources, and venom phosphodiesterases lend themselves well to analogous studies. For example, could calmodulin be involved in activating venom phosphodiesterases *in vivo*, as it is with several cyclic nucleotide phosphodiesterases (e.g., Repaske et al., 1992)? Can venom phosphodiesterases be phosphorylated and activated, as is the case with rat thyroid cAMP-specific phosphodiesterase (Sette and Conti, 1996)? Do venom phosphodiesterases actually function *in vivo* as nucleotidytransferases, and if so, what is the target of such a transfer?

A rigorous investigation of DNase activity in numerous venoms is required to determine unequivocally the relation of endonuclease activity to venom phosphodiesterase activity. Specific RNase activity, as found in *N. n. oxiana* venom, is likely to be more broadly distributed among elapid snakes, and a thorough analysis of venom from these species is warranted. Concerted efforts toward the isolation and characterization of these endonucleases would further our understanding of not only venom nucleases but nucleolytic enzymes in general, an important group of enzymes with powerful catalytic capacities. Venoms are rich sources of many enzymatic activites, and compared to many cell or tissue extracts, large amounts of purified materials can be obtained. A larger amount of purified material will facilitate structure/function and mechanistic studies as well.

Finally, the role of venom nucleases in the sequelae of envenomation and *in vivo* activity is poorly understood, and careful studies of nuclease effects on cells, tissues and whole organisms could provide illuminating information on their actual roles *in vivo*.

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