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CYCLIC 3',5'-NUCLEOTIDE PHOSPHODIESTERASE

PREPARATION OF A PARTIALLY INACTIVE ENZYME AND ITS SUBSEQUENT STIMULATION BY SNAKE VENOM

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SUMMARY

1. A cyclic 3',5'-nucleotide phosphodiesterase has been partially purified from bovine brain cortices. The "purified" enzyme showed little activity unless preincubated with snake venom. Phosphodiesterase as extracted from the cortices was maximally active but gradually lost activity as purification proceeded. Activation by venom was seen only after phosphodiesterase had been purified and was not due to protection by proteins in venom.

2. The venom presumably contained a stimulatory factor(s), which activated the purified but not the crude enzyme. Since 5'-adenosine monophosphate did not affect phosphodiesterase activity, stimulation was not due to relief of product inhibition by 5'-nucleotidase (EC 3.1.3.5) of venom. Based on stability studies, the stimulatory factor could be distinguished from 5'-nucleotidase of venom.

3. The stimulatory factor was labile to extreme pH's and elevated temperatures, but part of the activity survived these treatments. It was not dialyzable and appeared to be a protein. Although venom exhibited tryptic activity, neither its tryptic nor stimulatory activity was affected by trypsin inhibitor. Activation of phosphodiesterase by venom appeared to be a monomolecular reaction and was not dependent on Mn^{2+} at a concentration optimal for phosphodiesterase activity.

INTRODUCTION

Adenosine 3',5'-monophosphate (cyclic AMP) occupies a unique position in cellular biochemistry. Besides being an intracellular second messenger mediating many of the actions of a variety of different hormones, it is involved in many other seemingly unrelated effects. A comprehensive review covering the myriad effects of this cyclic nucleotide has appeared recently[1].

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Cyclic 3′,5′-nucleotide phosphodiesterase is known to hydrolyze cyclic AMP to 5′-AMP (ref. 2). Its tissue distribution[3], subcellular localization[4,5], and general properties[6,7,8] have been reported. Recently, a preliminary note described the isolation of a partially inactive phosphodiesterase and its subsequent activation by snake venom[9]. The present communication describes in detail the preparation of the enzyme and some aspects of the activation phenomenon.

MATERIALS AND METHODS

Chemicals

Cyclic [8-^{14}C]AMP was obtained from Schwarz BioResearch, (New York). Crystalline bovine serum albumin, dried snake venoms and coarse DEAE-cellulose with capacity 0.9 mequiv/g were purchased from Sigma (St. Louis). DEAE-cellulose was washed in 0.5 M NaOH, 0.5 M HCl to remove some yellow color and then exhaustively in 20 mM Tris–HCl (pH 7.5) before use.

Phosphodiesterase by chemical assay

The method in principle is essentially that of BUTCHER AND SUTHERLAND[3], employing snake venom as a source of 5′-nucleotidase to convert the product of phosphodiesterase, *viz.* 5′-AMP, into P_i and adenosine. P_i was determined according to FISKE AND SUBBAROW[10]. The reaction usually was run in 1 ml containing 40 mM Tris–HCl (pH 8.0), 1 mM cyclic AMP, 0.1 mM $MnCl_2$, about 100 μg phosphodiesterase and 100 μg snake venom (*Crotalus atrox* was used unless otherwise stated). After thermal equilibration, the reaction was started by addition of substrate and incubated for 10 min at 30°. The reaction was stopped with 0.1 ml of 55% trichloroacetic acid, and P_i released was measured. This is referred to as the one-state assay.

To demonstrate the stimulatory effect of snake venom, a two-stage incubation was used. The reaction mixture was the same as above except that it was preincubated with or without snake venom for 10 min before starting the reaction with cyclic AMP. Incubation was for 10 min and enzymic activity was terminated by boiling for 4 min. The system was brought back to 30° and more snake venom (100 μg) was added to incubate for another 10 min. The reaction was stopped with trichloroacetic acid and P_i was measured as before. All assays were done in duplicate and the data had been corrected for a control without phosphodiesterase. 1 unit of phosphodiesterase activity is defined as the amount of protein which hydrolyzes 1 μmole cyclic AMP/min at 30°.

Phosphodiesterase by ^{14}C

For experiments using cyclic [8-^{14}C]AMP, the reaction mixture was 0.4 ml and the conditions were identical to the one-stage assay described above except that 3 M $HClO_4$ was used instead of trichloroacetic acid. Solid $KHCO_3$ was added for neutralization. Denatured proteins and insoluble $KClO_4$ were removed by centrifugation and an aliquot of the supernatant fluid was put on Whatman No. 1 paper for descending chromatography as described previously[8].

Assay of 5′-nucleotidase

1 ml of reaction mixture contained 40 mM Tris–HCl, pH 8.0, 0.1 mM $MnCl_2$, and an aliquot of snake venom. The reaction was started with 0.5 mM 5′-AMP and

incubated for 10 min at 30°. Next, 0.1 ml of 55% trichloroacetic acid was added to stop enzymic activity. P_i was determined as usual.

Protein determination

In the initial stages of purification, proteins were measured with the Biuret reagent containing sodium deoxycholate. Bovine serum albumin was used as a standard. Proteins at later stages usually were estimated according to WARBURG AND CHRISTIAN[11].

Preparation of a partially inactive phosphodiesterase

Fresh bovine brains, obtained from a local slaughterhouse, were cleaned in water and the cortices were isolated at room temperature. These cortices were used fresh or were stored at −20° until use. No appreciable change of activity was noted in using either the fresh or frozen cortices. Subsequent operations were carried out at 0–4° and glass-distilled water was used throughout. The tissue was homogenized in 3 volumes of chilled water in a Waring Blender, Model CB-5, first at low speed for 1 min, and then at high speed for another min with a 5-min interval between the two speeds. The homogenate, which had a pH of 6.4, was filtered through a double layer of cheesecloth to remove tissue debris. The filtrate was adjusted to pH 5.9 with 6 M acetic acid and then centrifuged at 13 000 × *g* for 30 min. The supernatant fluid was filtered through glass wool to remove lipid material and then fractionated with solid $(NH_4)_2SO_4$, the solution being maintained at pH 7.0 by addition of 1 M NH_4OH. The 30–50% saturation fraction was collected and dissolved in a minimal volume of 20 mM Tris–HCl, pH 7.5 (used throughout unless otherwise stated) and was dialyzed against the same buffer with several changes. After dialysis the protein concentration was adjusted to 10 mg/ml with Tris buffer. Calcium phosphate gel (38 mg dry wt./ml) prepared according to the method of KEILIN AND HARTREE[12] was added slowly to the protein solution to reach a final ratio of 0.8 g gel to 1 g protein. After stirring for 2 h, the gel was collected by centrifugation and the supernatant fluid discarded. The gel was washed with a volume of Tris–HCl equal to the supernatant fluid. The absorbed protein was eluted with a similar volume of 0.5 M $(NH_4)_2SO_4$, pH 7.5. Elution was repeated once and the two eluates were combined. Solid $(NH_4)_2SO_4$ was added to 100% saturation. After stirring overnight the precipitate was collected by centrifugation and dissolved in Tris–HCl. $(NH_4)_2SO_4$ was removed by dialysis against the same buffer. A DEAE-cellulose column (2 cm × 33 cm) was charged with about 150 mg of the dialyzed protein. The column was washed with 200 ml of Tris–HCl and eluted with an exponential gradient of $(NH_4)_2SO_4$ with a lower reservoir containing 130 ml Tris–HCl and an upper reservoir containing 0.5 M $(NH_4)_2SO_4$ in Tris–HCl. Phosphodiesterase emerged in a single peak after 100 ml of eluate had been collected. Peak activity tubes were pooled and dialyzed in Tris–HCl buffer. The preparation at this stage, referred to as the "purified" enzyme, had little activity unless preincubated with venom.

RESULTS

Purification of phosphodiesterase

Brain phosphodiesterase is partly soluble and partly particulate[4,5]. We have shown earlier that Triton X-100 unmasked considerable latent activity in the crude

TABLE I

PREPARATION OF A PARTIALLY INACTIVE CYCLIC 3',5'-NUCLEOTIDE PHOSPHODIESTERASE

Data presented in Table I were based on 1 kg of frozen bovine brain cortices. The pH 5.9 supernatant accounted for about 40% of the total homogenate activity assayed in the absence of Triton X-100. Under this condition, the crude homogenate had a specific activity of 20 m, units. Previous studies showed that Triton X-100 unmasked considerable latent phosphodiesterase in the crude homogenate[8]. All fractions were preincubated with snake venom before enzymic assay by the one-stage procedure.

Fraction	*Protein (mg)*	*Activity (munits)*	*Specific activity (munits/mg protein per min)*	*Yield (%)*
pH 5.9 supernatant	11430	1710 000	150	100.0
30–50% $(NH_4)_2SO_4$	3420	870 000	255	50.8
Calcium phosphate gel eluate	1170	369 000	316	21.6
DEAE-cellulose eluate	335	188 000	562	11.0

homogenate[8]. The latent activity was associated with a microsomal fraction and was not released upon repeated freezing and thawing. The activity in the pH 5.9 supernatant represented about 40% of the total homogenate activity assayed in the absence of the detergent[4]. Table I summarizes the recovery and purification of phosphodiesterase from a typical preparation starting with 1 kg of frozen bovine brain cortices. This procedure is concerned with the soluble enzyme in the pH 5.9 supernatant fluid. The recovery of phosphodiesterase activity was 10% and the purification from the pH 5.9 supernatant was 4-fold. If calculation is based on the crude homogenate, which had a specific activity of 20 munits, purification was about 30-fold.

Effect of snake venom on phosphodiesterase activity

Crude phosphodiesterase as extracted from the brain cortices was fully active

TABLE II

EFFECT OF SNAKE VENOM ON ACTIVITY OF CYCLIC 3',5'-NUCLEOTIDE PHOSPHODIESTERASE AT DIFFERENT STAGES OF PURIFICATION

An appropriate aliquot of each fraction was assayed by the two-stage procedure. Note the increasing dependence of phosphodiesterase on venom for optimal activity as purification proceeded.

Fraction	*Preincubation conditions*		*Ratio (b)/(a)*
	With venom (a)	*Without venom (b)*	
	Activity (absorbance/tube)		
Homogenate	0.398	0.408	102
pH 5.9 supernatant	0.196	0.189	96
30–50% $(NH_4)_2SO_4$	0.420	0.304	72
Calcium phosphate gel eluate	0.448	0.342	76
DEAE-cellulose eluate	0.817	0.123	15

TABLE III

EFFECT OF BOVINE SERUM ALBUMIN ON THE ACTIVITY OF CYCLIC 3′,5′-NUCLEOTIDE PHOSPHODIESTERASE

1 ml of reaction mixture contained standard compositions *plus* additions as indicated in Table III. "Purified" phosphodiesterase was 100 μg. The enzyme was preincubated with albumin and/or venom for 10 min before the reaction was started with the substrate. Phosphodiesterase was assayed according to the two-stage procedure.

Tube No.	*Additions*	*Activity (absorbance/tube)*
1	None	0.104
2	100 μg albumin	0.105
3	200 μg albumin	0.104
4	1000 μg albumin	0.114
5	100 μg venom	0.495
6	100 μg venom + 100 μg albumin	0.504

but lost activity upon purification. Table II presents the activities of phosphodiesterase at different stages of purification assayed with or without preincubation with snake venom. In the homogenate as well as the pH 5.9 supernatant fluid, phosphodiesterase did not depend on venom for maximal activity. When the enzyme was purified through a DEAE-cellulose column, the activation of venom on phosphodiesterase activity became pronounced. At this stage, the activity of the "purified" enzyme varied from 10–20% of its activity after activation by venom.

Effect of bovine serum albumin

Since the amount of protein added in the form of snake venom was of the same order of magnitude as the "purified" phosphodiesterase, the possibility existed that the stimulation afforded by snake venom might be a protein-stabilizing phenomenon. In Table III, bovine serum albumin was used as a source of protein to test this hypothesis. In this experiment, albumin was added comparable to (Tube 2) or in excess over (Tubes 3 and 4) phosphodiesterase in the preincubation mixture of the control (Tube 1). The activity obtained from the tubes receiving different amounts of albumin was the same as the control receiving no albumin. Tube 5 with the usual amount of venom gave the expected increase. Also, albumin added to a system containing venom in the preincubation period failed to give any additional increase (Tube 6). These data showed that serum albumin affected neither the activities of the nonactivated nor the activated enzymes, and that the activation of the "purified" enzyme by snake venom could not be mimicked by serum albumin.

Effect of 5′-adenosine monophosphate

As has been pointed out earlier, venom was added as a source of 5′-nucleotidase. Stimulation of the "purified" phosphodiesterase was achieved after preincubation with snake venom. In an experiment designed to rule out the possibility that stimulation is not due to a release of product inhibition by 5′-AMP, cyclic [8-^{14}C]AMP was used as a substrate and unlabeled 5′-AMP as an "inhibitor". At the end of an incubation, 5′-AMP or adenosine (when venom was present) was isolated by paper chromatography and counted for radioactivity. Expt. A in Table IV shows that the addition of 0.1 mM–

TABLE IV

EFFECT OF 5′-ADENOSINE MONOPHOSPHATE ON THE ACTIVITY OF CYCLIC 3′,5′-NUCLEOTIDE PHOSPHODIESTERASE

Reaction mixture of 0.4 ml contained 40 mM Tris–HCl, pH 8.0, 2 mM cyclic [8-^{14}C]AMP (15 nC/μmole), 0.1 mM $MnCl_2$, 200 μg of "purified" phosphodiesterase in Expt. A, 95 μg phosphodiesterase in Expt. B, and 5′-AMP or snake venom as indicated. Enzyme used in Expt. B had not been purified through the DEAE-cellulose step and this preparation was not sensitive to venom stimulation. Phosphodiesterase was assayed as described under *Phosphodiesterase by* ^{14}*C*.

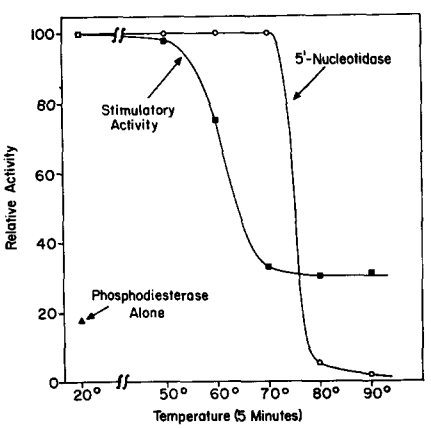
Expt. No.	*Additions*	*Activity (counts/min per aliquot)*		
		5′-AMP	*Adenosine*	*5′-AMP + adenosine*
A 1	None	304	—	304
2	0.1 mM 5′-AMP	300	—	300
3	1.0 mM 5′-AMP	315	—	315
4	10.0 mM 5′-AMP	311	—	311
5	40 μg venom	79	1499	1578
B 1	None	924	—	924
2	0.1 mM 5′-AMP	979	—	979
3	1.0 mM 5′-AMP	1059	—	1059
4	10.0 mM 5′-AMP	1048	—	1048
5	40 μg venom	66	926	992

10 mM 5′-AMP did not decrease the formation of labeled 5′-AMP from cyclic [8-^{14}C]-AMP (compare Tubes 2–4 with Tube 1). When venom was present (Tube 5), adenosine was the major product. Radioactivity recovered from adenosine and 5′-AMP amounted to 5 times the control (Tube 1). The concentration of 5′-AMP in Tube 1 at the end of the experiment was about 0.3 mM. This experiment demonstrated that the "purified" enzyme was not subject to product inhibition by 5′-AMP over a wide range of concentrations. In Expt. B, conditions were comparable to those in Expt. A, except that the phosphodiesterase used here was not activated by venom. Again, 5′-AMP had no effect on the rate of cyclic AMP hydrolysis (compare Tubes 2–4 with control Tube 1). Tube 5 had been preincubated with snake venom and no stimulation was noted, as expected. From these data it was concluded that the stimulation caused by venom was not due to relief of product inhibition by 5′-nucleotidase.

Dissociation of stimulatory factor of snake venom from its 5′-nucleotidase activity

Evidence has been presented that the stimulatory effect of snake venom is not due to product inhibition released by 5′-nucleotidase. Experiments described in Fig. 1 and Fig. 2 showed that the stimulatory factor in venom could be distinguished from its 5′-nucleotidase. In Fig. 1 the venom was exposed to different temperatures for 5 min before use. 5′-Nucleotidase was not affected after exposure of the venom to 70°, but the stimulatory activity was greatly suppressed. At 90°, 5′-nucleotidase activity was abolished, but part of the stimulatory activity survived the heat treatment.

Fig. 2 presents the effect of pH on the stimulatory and 5′-nucleotidase activities of snake venom. Both 5′-nucleotidase and stimulatory activities decreased to less than half of their maxima after exposure of the venom to pH 5 for 90 min. 5′-Nucleotidase activity was essentially zero at pH 3. The stimulatory effect, on the other hand, re-



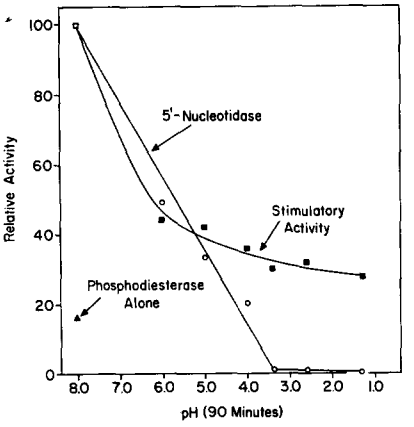


Fig. 1. Dissociation of stimulatory factor in snake venom from its 5′-nucleotidase activity by temperature. For each temperature, a set of 2 pairs of tubes was used. Each tube in a final volume of 0.8 ml contained 40 mM Tris–HCl, pH 8.0, 0.1 mM $MnCl_2$, and 100 μg *C. atrox*. These tubes were incubated for 5 min at the temperatures indicated and then transferred to an ice bath. One pair was assayed for its 5′-nucleotidase activity. The other pair was measured for its stimulatory effect by preincubating for 10 min with 60 μg "purified" phosphodiesterase. Phosphodiesterase was determined according to the two-stage procedure.

Fig. 2. Dissociation of stimulatory factor in snake venom from its 5′-nucleotide activity by pH. *C. atrox* was dissolved in 20 mM Tris–HCl, pH 8.0, at 1.0 mg/ml. Solid cysteine·HCl or concentrated HCl was used to adjust the pH to the appropriate values. Both control and acidified venoms were left at 22° for 90 min. Solid $KHCO_3$ was added for final neutralization and 5′-nucleotidase and stimulatory activities were assayed as described in Fig. 1.

mained significantly higher than the control containing phosphodiesterase alone, indicating again that the stimulatory factor was distinguishable from 5′-nucleotidase.

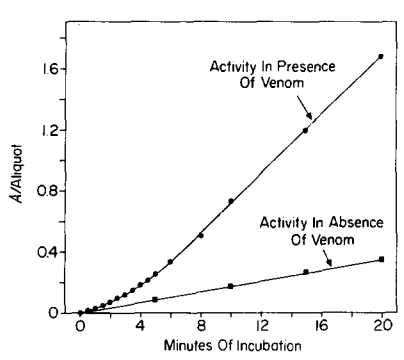
Time course of cyclic AMP hydrolysis in presence or absence of venom

Experiments described so far concerned the static events after phosphodiesterase had acted on cyclic AMP over a definite incubation period. In Fig. 3, an attempt was made to follow the kinetics of cyclic AMP hydrolysis throughout the incubation. In the presence of venom, there was an initial lag lasting about 5 min; thereafter the rate was linear. When venom was omitted in the incubation mixture, no such lag was observed and the rate of hydrolysis was linear throughout the course of the experiment. At any given time, the amount of cyclic AMP hydrolyzed in the presence of venom was greater than that in its absence. At the end of 20 min, the ratio was roughly 5:1.

Kinetics of activation of purified phosphodiesterase by snake venom

The initial lag of phosphodiesterase activity in the presence of venom (Fig. 3) suggested that the process of activation was time dependent. This was illustrated clearly in Fig. 4. In this experiment, phosphodiesterase was exposed to snake venom for various times before assay. The rate of activation was linear up to 5 min; thereafter it slackened off and reached a plateau in 15 min.

The kinetic aspect of activation was examined in more detail in another experiment in which both the time of preincubation and the concentration of venom was varied (Fig. 5). In the presence of 10 μg venom, the rate of activation was linear and



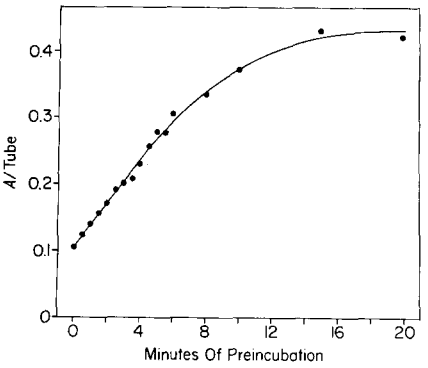


Fig. 3. Time course of cyclic AMP hydrolysis by "purified" phosphodiesterase in presence and in absence of snake venom. Reaction mixture of 18 ml contained 40 mM Tris–HCl, pH 8.0, 0.1 mM Mn^{2+}, 3.6 mg of "purified" phosphodiesterase and when present, 1.8 mg of snake venom. There was no preincubation and the reaction was initiated with 2 mM cyclic AMP. At times indicated, 1-ml aliquot was withdrawn and injected into a tube containing 0.1 ml of 55% trichloroacetic acid. Phosphodiesterase was assayed with the one-stage procedure. In the control experiment which received no venom, 1-ml aliquot was withdrawn and injected into a tube partly immersed in a boiling-water bath. The tube was brought back to 30° and 100 μg venom was added for a further incubation of 10 min. Reaction was stopped with 0.1 ml trichloroacetic acid and phosphodiesterase was determined according to the two-stage procedure.

Fig. 4. Kinetics of activation of "purified" phosphodiesterase by snake venom. Reaction mixture contained 40 mM Tris–HCl, pH 8.0, 0.1 mM Mn^{2+} and 400 μg "purified" phosphodiesterase. After thermal equilibrium, 100 μg of snake venom was introduced and the system was preincubated for various lengths of time as indicated in Fig. 4. 2 mM cyclic AMP was added and incubation was continued for another 2 min. To stop the reaction 0.1 ml 55% trichloroacetic acid was added. Phosphodiesterase was determined according to the two-stage procedure.

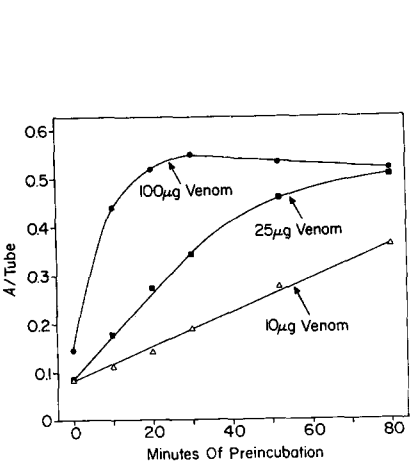
activation still was incomplete at the end of 80 min. As the concentration of venom was increased the rate of activation was accelerated. In the presence of 25 μg and 100 μg of venom, full activation was obtained in 80 min and 20 min, respectively. The data in Fig. 5 were replotted in Fig. 6 with the log percent of the nonactivated species expressed as a function of the time of preincubation. The slopes were linear and proportional to the concentration of venom in the preincubation mixture, indicating that the activation process was monomolecular.

Examination of stimulatory factor in various snake venoms

Crotalus atrox was used routinely as a source of 5'-nucleotidase. A preliminary survey of other snake venoms showed that some activated "purified" phosphodiesterase while others were ineffective (Table V). In fact, one of them, *Vipera russelli*, was slightly inhibitory in this experiment.

Effect of Mn^{2+} on activation of phosphodiesterase by snake venom

In all experiments described so far, Mn^{2+} was present at an optimal concentration in the preincubation mixtures. The question arose as to whether Mn^{2+} was necessary for the activation process. Table VI summarizes an experiment in which Mn^{2+} was omitted from the preincubation mixture and the activity of phosphodiesterase (Tube 5) was compared to that containing Mn^{2+} (Tube 4). The two activities were comparable, indicating that Mn^{2+} added after preincubation was as effective as that added prior to



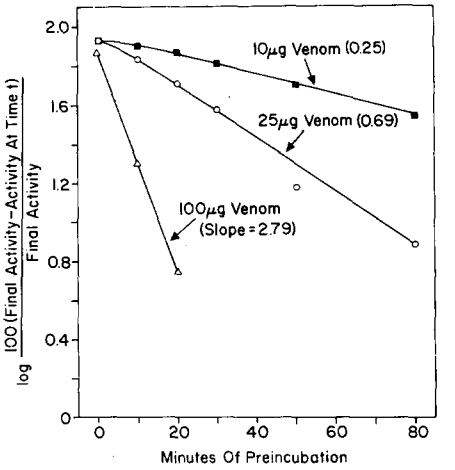


Fig. 5. Effect of venom concentration and time of preincubation on the activity of phosphodiesterase. Reaction mixture contained 40 mM Tris–HCl, pH 8.0, 0.1 mM Mn^{2+} and 396 μg of phosphodiesterase. Time of preincubation varied from 0 to 8 min and snake venom from 10 to 100 μg. After preincubation, 1 mM cyclic AMP was added and the reaction was continued for 3 min before enzymic activity was arrested by boiling. Phosphodiesterase was determined according to the two-stage procedure.

Fig. 6. Effect of venom concentration and time of preincubation on the activity of phosphodiesterase. Data were taken from Fig. 5 and were plotted with the log per cent of the nonactivated species expressed as a function of the time of preincubation.

preincubation. In the absence of Mn^{2+} (Tube 3), the activity was approximately half that in tubes containing Mn^{2+} (Tubes 4 and 5). This suggests that although Mn^{2+} was not necessary for activation, the activated phosphodiesterase depended on Mn^{2+} for maximal activity. Similarly, the nonactivated enzyme in the absence of Mn^{2+} (Tube 1) was also about 50% as active as that containing Mn^{2+} (Tube 2).

The fact that Mn^{2+} added after activation of phosphodiesterase was as effective as that added before activation was further illustrated in an experiment depicted in

TABLE V

EFFECT OF VARIOUS SNAKE VENOMS ON THE ACTIVITY OF 3′,5′-NUCLEOTIDE PHOSPHODIESTERASE

1 ml of reaction mixture contained standard compositions and 100 μg of one of the snake venoms in the preincubation system. The two-stage procedure was used. During the second stage of the incubation, 100 μg of *Crotalus atrox* was added to all tubes as source of 5′-nucleotidase.

Snake venom	*Activity (absorbance/sample)*
None	0.179
Crotalus atrox (Western Diamondback Rattlesnake)	0.914
Crotalus adamanteus (Eastern Diamondback Rattlesnake)	0.179
Crotalus durrisus terrificus (Tropical Rattlesnake)	0.816
Ancistrodon piscivorus leukostoma (Western Cottonmouth Moccasin)	1.020
Anoistrodon piscivorus piscivorus (Eastern Cottonmouth Mocassin)	1.000
Naja naja (Hooded Cobra)	0.177
Vipera russelli (Russell's Viper)	0.126

TABLE VI

EFFECT OF Mn^{2+} ON ACTIVATION OF CYCLIC 3′,5′-NUCLEOTIDE PHOSPHODIESTERASE BY SNAKE VENOM

1 ml of reaction mixture contained 40 mM Tris–HCl, 100 μg "purified" phosphodiesterase and other additions as indicated. The system was preincubated for 10 min and the reaction was started with cyclic AMP, except Tube 5, which was started with simultaneous addition of substrate and Mn^{2+}. Where present, Mn^{2+} was 0.1 mM and snake venom was 100 μg/ml. The reaction was incubated for another 10 min and then stopped by boiling. Another 100 μg venom was added and the second-stage incubation was performed as usual.

Tube No.	*Other additions*	*Activity (absorbance/tube)*
1	None	0.033
2	Mn^{2+}	0.066
3	Venom	0.258
4	Mn^{2+}, venom	0.445
5	Venom (Mn^{2+} added later with substrate)	0.420

Fig. 7. Here the duration of preincubation was varied so that the effect of Mn^{2+} on phosphodiesterase could be studied at various degrees of activation. The incubation time was 3 min instead of the usual 10 min. In Curve A, Mn^{2+} was present in the preincubation system and in Curve B, Mn^{2+} was present only in the incubation stage, *i.e.*, at the end of the designated times of preincubation. Curve C represented the nonactivated phosphodiesterase activity in the presence of Mn^{2+}. The fact that Curve B virtually coincided with Curve A showed that the rate of activation was independent of Mn^{2+} in the preincubation mixture.

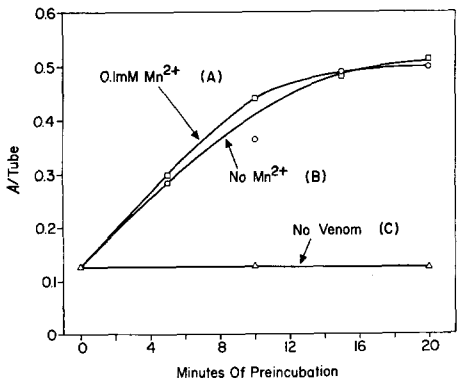


Fig. 7. Effect of Mn^{2+} on the kinetic activation of phosphodiesterase by venom. Preincubation mixture contained 40 mM Tris–HCl, pH 8.0, 400 μg "purified" phosphodiesterase, Mn^{2+} and/or venom as indicated. In Curve A reaction mixture contained 100 μg venom and 0.1 mM Mn^{2+}; in Curve B, 100 μg venom but no Mn^{2+}. In the control, Curve C, reaction mixture contained 0.1 mM Mn^{2+} but no venom. At the end of preincubation, varied as indicated, cyclic AMP was added to Curve A and Curve C; cyclic AMP and 0.1 mM Mn^{2+} were added simultaneously to Curve B. Incubation was continued for another 3 min and the enzymic reaction was stopped by boiling. Phosphodiesterase was assayed according to the two-stage procedure.

General properties of venom's stimulatory factor

It was shown in Figs. 1 and 2 that the factor was sensitive to high temperatures and acidic pH's and that part of the activity survived these treatments. Table VII shows that some of the activity even survived boiling at pH 1.2 for 5 min. Boiling at pH 11, however, caused total inactivation. Experiments not summarized here showed that activation of phosphodiesterase by the stimulatory activity remaining in the boiled venom was dependent both on the time of preincubation and the amount of venom added.

The venom factor was not dialyzable. 100 μg snake venom incubated with 10 μg of ribonuclease or deoxyribonuclease for 16 h at 30° retained its full stimulatory activity. In the same experiment, 20 μg trypsin decreased the stimulatory activity by 40%. Interestingly, trypsin, chymotrypsin and pronase were also stimulatory[13]. Although venom exhibited tryptic activity, as assayed with a synthetic substrate,

TABLE VII

STABILITY OF STIMULATORY FACTOR IN SNAKE VENOM

Snake venom dissolved in 20 mM Tris–HCl, pH 8.0 at 2 mg protein per ml was treated as follows: (2) no further treatment; (3) adjusted to pH 1.2 with HCl and boiled for 5 min; (4) adjusted to pH 11.1 with NaOH and boiled for 5 min. All solutions were brought back to room temperature and adjusted to pH 8 with $NaHCO_3$, if necessary. An aliquot was tested for its stimulatory activity using the usual two-stage procedure.

Tube No.	*Treatment*	*Activity (absorbance/tube)*
1	No venom	0.048
2	Venom	0.360
3	Venom boiled at pH 1.2	0.129
4	Venom boiled at pH 11.1	0.040

p-toluenesulfonyl-L-arginine methyl ester (rate of hydrolysis about 1 μmole/mg venom per min at 25°), neither its tryptic activity nor its stimulatory activity was suppressible by soybean trypsin inhibitor.

DISCUSSION

Activation of phosphodiesterase by snake venom was not due to removal of product inhibition by 5'-nucleotidase, since exogenous 5'-AMP added to the preincubation system showed no effect on enzymic activity. Bovine serum albumin, added to the preincubation mixture, did not increase phosphodiesterase activity, suggesting that activation was not a result of stabilization of labile enzymic activity by proteins in the venom. The stimulatory factor of snake venom was distinguished from its 5'-nucleotidase activity on the basis of heat and pH stability. The venom factor was not dialyzable, insensitive to ribonuclease and deoxyribonuclease and was moderately susceptible to trypsin attack. It was heat and pH sensitive but part of the activity survived boiling at pH 1.2 for several min. It remains to be seen whether the heat and pH resistant activity is attributable to some denatured activity of the native factor or to another distinct species insensitive to these drastic treatments.

Activation of phosphodiesterase was dependent on the time of preincubation and on the concentration of venom and thus appeared to follow first order kinetics. Neither the rate nor the extent of activation was affected by omitting Mn^{2+} in the preincubation system. However, Mn^{2+} was needed for optimal activity for both the activated and the nonactivated enzyme.

Snake venoms are known to contain proteolytic activities. TU *et al.*[14] have demonstrated tryptic activities in a variety of snake venoms and also have noted their resistance to soybean inhibitor. The fact that trypsin, chymotrypsin and pronase all stimulate phosphodiesterase activity[13] suggests that the stimulatory effect of venoms is probably associated with their proteolytic activities. However, the experiments reported here did not reveal the mechanism of activation.

The fact that phosphodiesterase in the extract was fully active and that as purification proceeded, the enzyme gradually became inactive suggested that an activator originally present in the extract might have dissociated during the course of purification. Two lines of evidence indicated that such a dissociation had occurred. Firstly, the activity of a mixture of crude and "purified" enzyme was greater than the sum of the activities of the two enzymes assayed separately. Indeed, the activity of the mixture was comparable to the sum of the activities of the crude enzyme and the purified enzyme subsequent to its activation by venom. Secondly, a substance was isolated from the brain extract which showed no phosphodiesterase activity but was capable of activating the "purified" enzyme[9]. The factor was insensitive to deoxyribonuclease and ribonuclease, but was inactivated by trypsin. It was nondialyzable, heat labile and appeared to be a protein[15].

Previously it has been demonstrated that phosphodiesterase was inhibited by ATP and PP_i. Other nucleoside triphosphates and inorganic polyphosphates were also highly inhibitory[8]. It was interpreted that phosphodiesterase, though present in overwhelming excess over adenyl cyclase, probably existed in a greatly inhibited state[16]. The present communidation suggests that phosphodiesterase activity may be regulated by mechanism more complicated than anticipated earlier. From the point of view of regulation of enzymic activity the isolation of a partially inactive phosphodiesterase and an activator is of special interest. It is conceivable that phosphodiesterase activity may be poised between the nucleotide inhibitors and the activator.

Attempts have been made to purify phosphodiesterase from beef heart[2], dog heart[7] and rabbit brain[6]. Because of the difference in assayed conditions, the specific activity of these purified enzymes cannot be compared directly. The enzyme, prepared according to the procedure described here, had a specific activity of about 600 munits after activation by snake venom. In this case, purification was achieved at a considerable sacrifice of the overall yield. Dissociation of the brain activator from the enzyme during the course of purification probably accounted for part of the poor recovery of activity. It is interesting to note that DRUMMOND AND PERROTT-YEE[6], working with rabbit brain, also experienced loss of phosphodiesterase during purification.

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