CYCLIC 3',5'-NUCLEOTIDE PHOSPHODIESTERASE: PRONOUNCED STIMULATION BY SNAKE VENOM

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Sutherland and Rall (1958) discovered a cyclic nucleotide phosphodiesterase in beef heart. A similar enzyme has been described by Drummond and Perrott-Yee (1961) from rabbit brain, by Nair (1966) from dog heart, and by Cheung (1966, 1967) and Cheung and Salganicoff (1967) from rat brain. A method generally used to follow the activity of phosphodiesterase is outlined below:

$$\text{cyclic AMP} \xrightarrow{\text{phosphodiesterase}} \text{5'-AMP} \xrightarrow[\text{(snake venom)}]{\text{5'-nucleotidase}} \text{Pi + adenosine}$$

This assay (Butcher and Sutherland, 1962) can be carried out with phosphodiesterase and 5'-nucleotidase incubated together (one stage), or it can be terminated at the 5'-AMP level and then incubated further with snake venom (two stages). Inorganic phosphate released by 5'-nucleotidase is measured colorimetrically. It has been the general experience that the amount of inorganic phosphate formed at the end of incubation is independent of the one-stage, or two-stage procedure.

In an attempt to purify phosphodiesterase from cow brains, we noted that at some stages of purification, the enzymic activity varied widely, depending on whether the one-stage or the two-stage procedure was used.

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We found that while phosphodiesterase in the crude extract was fully active, the "purified" enzyme depended on snake venom for its maximal activity.

"Purified" phosphodiesterase was prepared by classical means. The procedure of purification consisted of extracting brain cortices with water, followed by pH fractionation, differential centrifugation, $(NH_4)_2SO_4$ fractionation, calcium phosphate gel adsorption and DEAE chromatography. The enzyme eluted from the DEAE column is dependent on venom for maximal activity and is referred to as the "purified" enzyme. Details of this method and a full account of the activation phenomenon will be published elsewhere.

Activation is achieved by incubating the "purified" enzyme with snake venom prior to enzymic assay of phosphodiesterase. Table I compares the activity of the crude and "purified" phosphodiesterase preincubated in the presence or absence of snake venom. The data show that the crude enzyme exhibits identical activity whether preincubation was performed with or without snake venom. The "purified" enzyme, on the other hand, is several times more active with venom than without it. It appears that the venom contains a factor (or factors) which activates the "purified" enzyme. All venoms examined are capable of activating phosphodiesterase. It should be pointed out that snake venom itself is virtually inactive towards cyclic-AMP and that the data have been corrected for a control containing no phosphodiesterase.

Figure 1 shows that the degree of activation of "purified" phosphodiesterase is a function of the venom concentration during preincubation. There is no appreciable activation below 5 μg venom per ml of assay mixture. Under our conditions, 20 μg venom causes 50% stimulation whereas 100 μg gives maximal activation. Venom in excess of 100 μg does not give any more activation. Indeed, when

Enzyme	Preincubation Conditions	
	With venom	Without venom
	Activity (O.D./sample)	
Crude Phosphodiesterase	0.625	0.621
"Purified" Phosphodiesterase	0.476	0.061

TABLE I. Effect of snake venom on the activation of "purified" phosphodiesterase. The two-stage procedure was used for the assay. One ml of reaction mixture contained 40 mM Tris HCl, pH 8.0, 1 mM cyclic AMP, 0.1 mM $MnCl_2$, and 100 µg snake venom (Crotalus atrox). Crude phosphodiesterase (10,000 g supernatant of brain homogenate), 1.2 mg protein; "purified" phosphodiesterase, 0.1 mg protein. After having been preincubated with or without snake venom for 10 minutes at 30°C, the reaction was started by the addition of substrate and the incubation was allowed to continue for 10 more minutes. Enzymic activity was terminated by boiling for 4 minutes and the reaction mixture was cooled. More snake venom was then added and the incubation continued for another 10 minutes. The reaction was stopped with 0.1 ml of 55% trichloroacetic acid. Inorganic phosphate was measured according to Fiske and Sabbarow (1925). The protein was estimated after Warburg and Christian (1941).

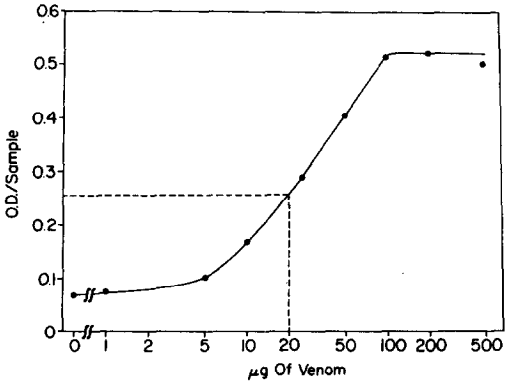

Fig. 1. Effect of venom concentration during preincubation on the activation of phosphodiesterase. Venom added to the preincubation mixture was varied as indicated. Time of preincubation, 10 minutes; "purified" phosphodiesterase, 100 µg; and other conditions as described in Table I.

500 ug venom is added, a slight decrease in activation can be noted.

Similarly, the extent of stimulation by venom can be shown to depend on the time of preincubation. A suboptimal concentration of venom can give rise to maximal activation, provided that the time of preincubation is sufficient. Kinetic studies suggest that the activation process is monomolecular. The rate of activation is not affected by 0.1 mM Mn^{++}.

Stimulation of "purified" phosphodiesterase by venom does not appear to be a protein stabilizing phenomenon, since a comparable quantity of albumin substituted for venom gives no increase of activity. To rule out the possibility that stimulation is not due to a release of product inhibition by 5'-AMP, we used cyclic AMP-8-C^{14} as substrate and unlabelled 5'-AMP (ranging from 0.1 to 10 mM) as inhibitor. At the end of the incubation, the product was isolated by paper chromatography and counted for radioactivity. The amount of 5'-AMP-8-C^{14} formed in the presence of different concentrations of 5'-AMP was the same as the control which received no exogenous 5'-AMP, thus indicating that 5'-AMP did not inhibit phosphodiesterase.

The stimulatory factor associated with the venom is non-dialyzable and is labile at acidic pH values and elevated temperatures. Similarly, 5'-nucleotidase shows loss of activity under these conditions, but its susceptibility is different from that of the stimulatory factor. For example, exposure of the venom to pH 1.3 or 100°C obliterates 5'-nucleotidase activity while some of the stimulatory activity survives these treatments, indicating therefore that the two activities can be distinguished from each other.

The fact that the crude phosphodiesterase is fully active while the "purified" enzyme depends on venom for its optimal activity suggests that the stimulatory factor originally present with the enzyme must have dissociated during the course of its purification.

Two lines of evidence support that such a dissociation has occurred. Firstly, a non-dialyzable substance has been obtained from the brain extract which shows no phosphodiesterase activity but is capable of activating the "purified" enzyme. Secondly, the activity of a mixture of the crude and "purified" enzyme is greater than the sum of the activities of the two enzymes assayed separately. Indeed, the activity of the mixture is comparable to the sum of the activities of the crude enzyme and the purified enzyme subsequent to its activation by the venom.

Recently, we noted that a minute quantity of crystalline trypsin also markedly activated phosphodiesterase, indicating that the activation of "purified" phosphodiesterase might be a consequence of proteolysis. This is reminiscent of the activation of chymotrypsinogen to chymotrypsin by trypsin (Northrop *et al.*, 1948). Whether the stimulatory effect of the venom, or of the brain extract is due to their proteolytic action similar to that of trypsin remains to be established.

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REFERENCES

Butcher, R. W., and Sutherland, E. W., J. Biol. Chem. 237, 1244 (1962).
Cheung, W. Y., Biochem. Biophys. Res. Commun., 23, 214 (1966).
Cheung, W. Y., Biochemistry 6, 1079 (1967).
Cheung, W. Y., and Salganicoff, L., Nature 214, 90 (1967).
Drummond, G. I., and Perrott-Yee, S., J. Biol. Chem. 23, 1126 (1961).
Fiske, C. H., and Subbarow, Y., J. Biol. Chem. 66, 375 (1925).
Nair, K. G., Biochemistry 5, 150 (1966).
Northrop, J. H., Kunitz, M., and Herriot, R. M., in Crystalline Enzymes, Columbia University Press, N.Y., p. 97 (1948).
Sutherland, E. W., and Rall, T. W., J. Biol. Chem. 232, 1077 (1958).
Warburg, O. and Christian, W., Biochem. Z. 310, 384 (1941).