LII. INHIBITION OF DEHYDROGENASES BY SNAKE VENOM

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(*Received 9 December 1938*)

It has been reported previously [Chain, 1937, 1] that a substance, present in a number of different snake venoms, inhibits in very small concentrations the glycolysis and fermentation of cell-free extracts. The inhibitory effect of this substance is neutralized by the same quantity of antivenin as will neutralize the lethal effects of the venom *in vivo* [Chain, 1937, 2]. The chemical behaviour and mode of action of the inhibitory substance suggested an enzymic action causing the destruction of some integral part of the glycolysing and fermenting complex [Chain & Goldsworthy, 1937].

The following communication deals with the effect of snake venom on oxidation-reduction enzymes. This study was undertaken in the hope of throwing some light on the mechanism of the inhibitory action of the venom on glycolysis and fermentation, since oxidation-reduction enzyme systems play a predominant part in these processes.

Experimental

As in the previous experiments, dried venom of the Australian black tiger snake (*Notechis scutatus*) was used. The effect of this venom on various dehydrogenases and oxidases was studied.

Preparation of enzyme solutions. The lactic, malic, β-hydroxybutyric and succinic dehydrogenases and the cytochrome oxidase were obtained from pig's heart muscle. The muscle was minced twice through a Latapie mincer, washed exhaustively with tap water, ground with sand and $M/15$ phosphate buffer in a mortar and the solution strained through muslin [Ogston & Green, 1935]. The solution was brought to *p*H 7 and centrifuged for a short time. For some experiments a dried heart muscle preparation was used, prepared by dehydrating the minced washed pig's heart muscle with ice-cold acetone and evaporating off the acetone in a desiccator over P_2O_5. The dried powder (1·3 g.) was ground with sand and $M/15$ phosphate buffer *p*H 7·4 (20 ml.) in a mortar, the solution strained through muslin and centrifuged for a short time. The glycerophosphate dehydrogenase was prepared from rabbit's muscle [Green, 1936]. For xanthine oxidase untreated milk was used. The uric acid oxidase was prepared from sheep liver which was minced through the Latapie mincer, dried in a shallow dish *in vacuo* over P_2O_5 and ground in a mortar to a fine powder. The powder (1 g.) was extracted with borate buffer (10 ml.) *p*H 8·7 for 1 hr. at 37° and the suspension centrifuged. Amino-acid dehydrogenase was prepared from pig's kidney [Krebs, 1935].

Manometric experiments. The effect of the venom on the oxidative enzymes was tested in all cases by measuring O_2 uptakes in Warburg vessels at 37° in

O_2. In addition to 0·3 ml. 40 % KOH for CO_2 absorption in the middle chamber, the vessels contained the following solution:

For lactic acid dehydrogenase:

Side bulb: 0·5 ml. 10 % Na lactate.

Main space: 0·5 ml. 0·1 % cozymase.[1]
0·3 ml. 5 % semicarbazide hydrochloride, neutralized.
0·2 ml. 1 % methylene blue.
1–2 ml. enzyme, according to activity.

For malic and β-hydroxybutyric acid dehydrogenases:

Side bulb: 0·5 ml. substrate (15·6 % Na malate or 6·3 % Na β-hydroxybutyrate)

Main space: 0·5 ml. 0·1 % cozymase.
0·3 ml. *M*/10 Na pyrophosphate.[2]
0·3 ml. *M*/2 hydrazine sulphate, neutralized.
0·3 ml. 1 % methylene blue.
1–2 ml. enzyme, according to activity.

For succinic and α-glycerophosphate dehydrogenases and cytochrome oxidase:

Side bulb: 0·5 ml. substrate (10 % Na succinate, 4 % ammonium α-glycerophosphate, 1 % phenylenediamine hydrochloride, neutralized).

Main space: 0·5 ml. 0·1 % cozymase.
0·2 ml. 1 % methylene blue.
0·5–2 ml. enzyme, according to activity.

For xanthine oxidase:

Side bulb: 0·3 ml. 0·1 % xanthine.

Main space: 1·7 ml. milk.
0·5 ml. *M*/5 phosphate buffer *p*H 7·4.

For amino-acid dehydrogenase:

Side bulb: 0·1 ml. 10 % alanine.

Main space: 1 ml. water.
1 ml. enzyme.

For uric acid oxidase:

Side bulb: 0·3 ml. of a 1 % suspension of uric acid in borate buffer *p*H 8·7.

Main space: 1 ml. water.
1 ml. enzyme.

Four Warburg vessels were used for each experiment—two control vessels containing the above solutions and two vessels containing snake venom in addition to these solutions. The amount of venom used was usually 1 mg. dissolved in 0·2 ml. water and it was placed together with the other solutions in the main space of the vessels.

[1] I am greatly indebted to Prof. R. A. Peters, Dr S. Ochoa and Dr L. A. Stocken, Department of Biochemistry, Oxford, for gifts of a cozymase preparation of approximately 90 % purity.

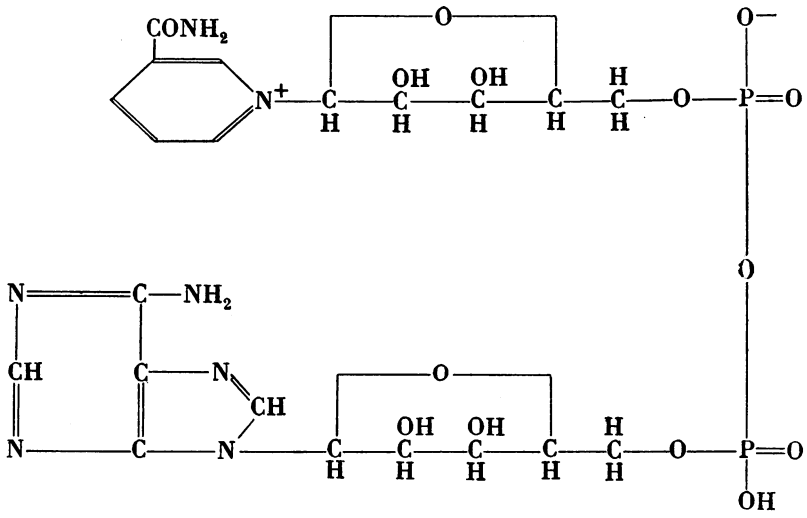
[2] In confirmation of the statement of Green *et al.* [1937] Na pyrophosphate was found to extend considerably the linear period of O_2 uptake with malic and β-hydroxybutyric dehydrogenases.

The results of the tests are summarized in Table I, the figures representing typical values of at least three sets of experiments.

Table I. *Effect of snake venom on oxidative enzymes*

Enzyme	O_2 uptake in 30 min. (μl.) Without venom	With venom	% inhibition
Lactic dehydrogenase	280	0	100
Malic dehydrogenase	180	0	100
β-Hydroxybutyric dehydrogenase	210	0	100
Amino-acid dehydrogenase	142	0	100
Succinic dehydrogenase	600	300	50
α-Glycerophosphate dehydrogenase	85	61	28
Cytochrome oxidase	417	276	34
Xanthine oxidase	167	175	0
Uric acid oxidase	296	308	0

It appears from Table I that lactic, malic, β-hydroxybutyric and amino-acid dehydrogenases are completely inhibited by the venom; the other enzymes are not or only partially inhibited. The four inhibited dehydrogenases have one characteristic in common which differentiates them from the non-inhibited enzymes, namely the need for a coenzyme for their activation. The conclusion therefore naturally suggested itself that the inhibitory effect of the venom might be due to some action on the coenzymes. The need of a coenzyme for the lactic, malic and β-hydroxybutyric dehydrogenases has been known since the systematic study of their properties; it was found to be identical with coenzyme I, which according to Euler & Schlenk [1937] has the structural formula:



The existence of a coenzyme for amino-acid dehydrogenase was discovered only recently by Warburg & Christian [1937] who isolated it in form of its Ba salt and found it to be a flavin-adenine-nucleotide [1938, 1, 2].

By the following experiment it could be shown that coenzyme I is inactivated by black tiger snake venom. Coenzyme I (6 mg.) was dissolved in phosphate buffer *p*H 7·2 (2 ml.) and venom (2 mg.) in water (0·2 ml.) was added. The mixture was incubated for 1½ hr. at 37°. It was then brought to *p*H 1·5 with a

few drops of *N* HCl and incubated with cryst. pepsin (1 mg.) for 1 hr. at 37° in order to destroy the venom. The solution was then brought back to *p*H 7·2 and made up to 3 ml. A solution of coenzyme I (3 mg.) in phosphate buffer (2 ml.), serving as control, was incubated without the addition of venom for 1½ hr. at 37°, then adjusted to *p*H 1·5, incubated with cryst. pepsin (2 mg.) for 1 hr., brought back to *p*H 7·2 and made up to 3 ml. The coenzyme I activities of these solutions were tested with the malic acid dehydrogenase system. The arrangement of the test and its results are shown in Table II.

Table II. *Inactivation of coenzyme I by black tiger snake venom*

	Content of vessels in ml.		
	Vessel 1	Vessel 2	Vessel 3
Na malate 15·6%	0·5	0·5	0·5
Coenzyme I, 0·1% incubated as described above	0·2	0·2	—
M/10 $Na_4P_2O_7$	0·3	0·3	0·3
M/2 Hydrazine sulphate (neutralized)	0·3	0·3	0·3
Methylene blue, 1%	0·2	0·2	0·2
Coenzyme I, incubated with venom as described above	—	1·5	1·5
Water	1·5	—	—
Enzyme	1·0	1·0	1·0
O_2 uptake in 20 min. (μl.)	215	210	7

The O_2 uptake in vessel 3 is very small although it contained 1·5 ml. of the coenzyme I solution which, before incubation with the venom, contained 3·0 mg. Since 0·2 mg. of this cozymase preparation after the treatment described above was sufficient to activate the malic dehydrogenase, as shown by the O_2 uptake in vessel 1, at least 95% of it must have been inactivated by the venom during the incubation. The O_2 uptake in vessel 2 which is not essentially different from that of vessel 1 shows the effective destruction of the inhibiting substance in the venom by boiling and pepsin treatment.

An indication of the mechanism of the inactivation of coenzyme I by black tiger snake venom is obtained from the fact that CO_2 is evolved when snake venom is allowed to act on coenzyme I in $NaHCO_3$ solution.

Coenzyme I (2·4 mg.) dissolved in 0·5% $NaHCO_3$ (2·2 ml.) was placed in the main space of a Warburg vessel and snake venom (1 mg.) in water (0·2 ml.) and 0·5% $NaHCO_3$ (0·1 ml.) in the side bulb. The solutions were saturated with a gas mixture of 95% N_2 and 5% CO_2 at 37° and after establishment of equilibrium the venom solution was added to the coenzyme I solution. CO_2 evolution started immediately and ceased after 45 min. The total amount of CO_2 developed was 141 μl. and corresponds to 1·7 H^+ equivalents per mol. cozymase. The most likely explanation for this is a hydrolytic action of the venom on some of the phosphoric acid linkages in the cozymase molecule with the formation of two new acid groups. Since one of these groups is bound to be weak and therefore will not be dissociated completely at the *p*H of the solution used in the experiment only 1·7 equivalents of CO_2 are measured instead of two.

The cozymase-inactivating factor in the snake venom which is responsible for the inhibition of glycolysis, fermentation and the four dehydrogenases needing coenzymes is thus shown to belong to the group of phosphoric acid-splitting enzymes, or more specifically, to the class of nucleotidases. It has been known for some time that certain Japanese snake venoms contain a phosphomonoesterase and a phosphodiesterase [Uzawa, 1932; Takahashi, 1932] and recently Gulland & Jackson [1938, 1] in an extensive study have found these enzymes in various proportions in a considerable number of different snake

venoms. The same authors report on the occurrence of an enzyme in several snake venoms which hydrolyses adenosine-5-phosphate and inosine-5-phosphate [1938, 2], and of a nuclease which hydrolyses yeast nucleic acid [1938, 3]. They suggested that some of the toxic effects of the venom might be due to the action of the 5-nucleotidase.

At present it cannot yet be decided by which of the several theoretically possible ways the hydrolysis proceeds. It was found that only a part (approx. 25 %) of the total P is set free in the form of inorganic P during the hydrolysis, and since the cozymase preparation was not 100 % pure it is possible that this inorganic P originates partly from some impurity present in the preparation and not from the cozymase. Obviously the amount of inorganic P liberated is too small to account for the 1·7 H^+ equivalents formed in the hydrolysis. Most probably the first step of the enzymic action is the opening of the pyrophosphate linkage in the coenzyme I molecule with the formation of two mono-ortho-phosphoric esters which then are partly broken down further with the liberation of inorganic P. It is hoped to get more information on this question from a study of the specificity of the phosphatases in the black tiger snake venom.

Summary

1. The effect of black tiger snake venom on the following oxidative enzyme systems has been investigated: lactic, malic, β-hydroxybutyric, amino-acid, α-glycerophosphoric and succinic dehydrogenases; cytochrome, xanthine and uric acid oxidases.

2. The four dehydrogenases which need a coenzyme for their activation, namely the lactic, malic, β-hydroxybutyric and amino-acid dehydrogenases, are completely inhibited by small amounts of black tiger snake venom. The others are not or only partially inhibited.

3. Coenzyme I is shown to be inactivated by black tiger snake venom. The inactivation is shown to be due to enzymic hydrolysis of some of the phosphoric acid linkages in the cozymase molecule with the formation of two new acid groups. The cozymase-inactivating principle in the black tiger snake venom can therefore be classified as a phosphatase or, more specifically, as a nucleotidase.

4. The inactivation of the coenzymes by black tiger snake venom through the action of a nucleotidase is the cause of the inhibitory effect of the venom on glycolysis, on fermentation and on the four dehydrogenases mentioned above.

I am indebted to the British Empire Cancer Campaign for a personal part-time grant and a grant for apparatus.

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