The Relationship of Certain Enzymes in Cobra and Rattlesnake Venoms to the Mechanism of Action of these Venoms

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Several authors have reported enzymic activities of cobra venom which they believe to be connected with the toxic action of this venom. In particular, it was reported that the lecithinase activity of cobra venom disrupts mitochondria, thereby changing the spatial relationship of biochemically important enzyme systems (Braganca & Quastel, 1953). A correlation of the toxicity with the ribonuclease activity of various venoms including cobra venom has been reported (Taborda, Williams & Elvehjem, 1952). Chain (1939) found that black tiger-snake venom hydrolyses diphosphopyridine nucleotide. Slotta (1955) and Zeller (1948) suggested that the adenosine triphosphatase of snake venoms is concerned with their toxicity.

Preliminary observations led us to conclude that *Naja flava* (cobra) venom is composed of a relatively simple mixture of proteins which could be separated into a highly toxic fraction and a less toxic fraction. It appeared that such a fractionation might be useful in a study of the relationship of enzyme activity to toxicity. It would be expected that if a given enzyme action of snake venom causes its toxicity, then this enzyme activity should be most marked in the most toxic fraction.

METHODS

Fractionation of *Naja flava* venom. The venom (2 g.) was dissolved in 150 ml. of water in a 600 ml. flask, which was immersed in a mixture of ice and salt, and 300 ml. of 95% (w/v) ethanol at -5° was slowly (15 min.) stirred in. Stirring was continued for a further 15 min. The mixture was then centrifuged; the supernatant solution was separated and kept at -5° . The precipitate was dissolved in cold water (25 ml.) and again precipitated by slow addition of cold 95% ethanol (50 ml.) as before. After it had stood for 15 min. the mixture was centrifuged and the supernatant removed, and combined with the previous supernatant and maintained at -5° . The precipitate was dissolved in the minimum quantity of water and freeze-dried. With the temperature maintained below freezing, the combined supernatant solutions were evaporated to 25 ml. in a flash evaporator and then freeze-dried. The supernatant (1.6 g.) was designated fraction 1, and the precipitate (0.4 g.) was designated fraction 2.

Electrophoresis. A Spinco model R paper-strip-electrophoresis apparatus was used. All runs were 6 hr. in duration at a constant current of 15 ma. The proteins were

stained with bromophenol blue according to the instructions given with the apparatus.

Determination of lecithinase A activity. Lecithinase A activity was determined as follows: Sørensen's phosphate buffer, pH 7.1 (1 ml.), was mixed with 1 ml. of an egg-yolk solution (10 g. of fresh yolk diluted to 1 l.), 2 ml. of a 3% (v/v) suspension of packed human erythrocytes in isotonic NaCl soln. and 1 ml. of an isotonic NaCl soln. containing the venom. This mixture was incubated at 40° for 90 min. The extent of haemolysis of the erythrocytes produced by the lysolecithin was determined by centrifuging the whole erythrocytes from the mixture and reading the density of the clear supernatant solution at 545 m μ . The optical density of the blank (enzyme omitted) was deducted from all readings. When enzyme was present but no substrate, the optical density was indistinguishable from that of the blank.

Determination of succinic dehydrogenase activity. Succinic dehydrogenase activity was measured by a slight modification of the method of Kun & Abood (1949). The following were placed in a test tube: 0.5 ml. of Sørensen's 0.1M-phosphate buffer, pH 7.4, 1 ml. of the enzyme preparation (a homogenate of rat brain in 6 vol. of buffer), 0.5 ml. of 0.2M-sodium succinate, 1 ml. of a freshly prepared solution (0.1%) of triphenyltetrazolium chloride and 0.2 ml. of the solution being tested as enzyme inhibitor. This reaction mixture was incubated at 38° for 15 min., then 7 ml. of acetone was added, and the tube stoppered and well shaken and the contents were filtered. Optical-density measurements were made at 480 m μ . The density of the blank solution containing no enzyme was subtracted from the density reading of the experimental determinations. Solutions which contained enzyme but no substrate did not differ significantly from the blank. Inhibition (%) was taken as the corrected density of the reaction mixture with inhibitor, divided by the corrected density of the reaction mixture without inhibitor, multiplied by 100.

Determination of ribonuclease, diphosphopyridine nucleotidase, and adenosine triphosphatase activities. These activities were determined by incubating a mixture of the enzyme (venom), buffer and substrate at 37° and measuring the rate of change in pH produced by the liberation of free phosphoric acid. The buffer used was 0.2 mM- KH_2PO_4 -1.8 mM- Na_2HPO_4 , pH 7.9. The substrates used were diphosphopyridine nucleotide (2 mg./ml.), adenosine triphosphate (2 mg./ml.), or ribonucleic acid (5 mg./ml.) in isotonic saline. The reaction mixture consisted of 1 ml. of the buffer solution, 1 ml. of substrate and 0.5 ml. of the enzyme (venom) solution in isotonic saline. Measurements of pH were with an electronic pH meter (Photovolt model 110) at 30-60 min. intervals, the solution being returned to the reaction tube after each measurement. The cup assembly

of the pH meter was kept close to incubation temperature with heating tape. When either the substrate or the enzyme was omitted from the reaction mixtures, no pH change was observed. Fig. 1 shows the quantitative response obtained with *Crotalus t. terrificus* venom with adenosine triphosphate as a substrate.

Materials. The snake venoms were obtained from the Miami Serpentarium, Miami, Florida, U.S.A. Diphosphopyridine nucleotide, triphosphopyridine nucleotide and ribonucleic acid were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

RESULTS

Electrophoretic studies and chemical properties of the fraction of cobra venom. Fig. 2 presents the results obtained when cobra venom was subjected to electrophoresis by the paper-strip method. Sodium diethyl barbiturate buffer, pH 8.6, I 0.06, was used. The amount of material (venom, etc.) applied in each case was 0.01 ml. of a 10% (w/v) solution. The paper strip obtained indicates the presence of five proteins, labelled *A*, *B*, *C*, *D* and *E* in Fig. 2. Very similar results were obtained when the venom was subjected to electrophoresis with a Sørensen phosphate buffer of pH 6.8, I 0.06. Electrophoresis of fraction 1 (see Methods) indicates that it is composed of proteins *A* and *B*, and fraction 2 contains proteins *C*, *D* and *E*. Electrophoretic separation at pH 10 with a glycine-sodium hydroxide buffer, I 0.065, revealed that proteins *A* and *B* were still migrating towards the

cathode, indicating that the isoelectric point of these proteins is above 10. Included in Fig. 2 is a similar electrophoretic study at pH 8.6 of *C. t. terrificus*. The paper strip obtained reveals what appears to be a single protein migrating toward the anode. Fraction 1, containing proteins *A* and *B*, represents approx. 80% of the total weight of the venom, and fraction 2 makes up 20%. It should be emphasized that the fractionation process is a simple precipitation procedure in which nothing was discarded.

Both fractions were subjected to the Molisch test for carbohydrates: fraction 1 was negative but fraction 2 was positive, indicating the possible presence of glycoprotein.

Toxicity of fractions 1 and 2 of *Naja flava* venom. The approximate lethal doses (Deichmann & LeBlanc, 1943) of fraction 1 and fraction 2 were respectively 0.33 and 1.5 mg./kg. intravenously in mice, 0.4 and 1.6 mg./kg. subcutaneously in rats and 0.1 and 0.4 mg./kg. subcutaneously in the dog. Thus it is evident that in these animals, by these routes of administration, fraction 1 is about four times as toxic as fraction 2.

The recovery of toxicity obtained in the fractionation procedure based on intravenous administration to mice is shown in Table 1. Since nothing is discarded, total recovery of the toxicity was to be expected unless denaturation occurred. The total 'units' of toxicity (weight divided by the approximate lethal dose in mg./kg.) recovered in the two fractions add up to slightly more than the initial activity, probably a reflexion of the error of the approximate lethal-dose determination. The term toxicity, when used in this instance, means the number of kilograms of mice which would be killed by the intravenous administration of the entire fraction, or the total number of approximate lethal doses in the fraction.

Fig. 1. Quantitative response obtained with *Crotalus t. terrificus* venom with adenosine triphosphate as substrate (substrate concentration was 2 mg. in 2.5 ml.). The highest concentration of venom used (○) was 1.0 ml. of 0.1 mg./ml. of solution (100 μg. in 2.5 ml.), the lowest concentration (▲) was 0.1 ml. of the 0.1 mg./ml. of solution (10 μg. in 2.5 ml.). Intermediate concentrations are: ●, 50 μg. and △, 20 μg./2.5 ml.

Fig. 2. Electrophoresis of venoms and venom fractions on paper at pH 8.6. For details see text.

Relationship between lecithinase A activity, ability to inhibit succinic dehydrogenase and toxicity. Since we have been able to achieve fractionation of cobra venom into two fractions, one of which is four times as toxic as the other, it was felt that this fractionation could be used to evaluate the relationship between lecithinase A activity, the ability to inhibit succinic dehydrogenase and toxicity. In addition, the effect on lecithinase A potency and the ability to inhibit succinic dehydrogenase of heated snake venoms was investigated. Table 2 shows the lecithinase A potency of the various fractions and whole-venom preparations, and Table 3 shows their ability to inhibit succinic dehydrogenase. Although only about one-half of

the lecithinase A activity was accounted for, fraction 2 (the less toxic fraction) is 20 times as potent in lecithinase activity as fraction 1. Fraction 2 is also many times more potent in inhibitory activity against succinic dehydrogenase than fraction 1. The effect of these fractions and venoms was greater if the dehydrogenase was preincubated for 1 hr. with the inhibitors before adding the substrate, which suggests that a direct attack is being made on the enzyme itself. Thus it seems that there is a direct correlation between lecithinase A activity and its inhibitory action against succinic dehydrogenase, but that neither property of this venom is directly related to its toxicity.

Table 1. *Recovery of toxicity in the fractions obtained from Naja flava venom*

Toxicity was measured by intravenous injection into mice. A.L.D., approximate lethal dose (Deichmann & LeBlanc, 1943).

	Crude venom	Fraction 1	Fraction 2
Wt. (g.)	2.0	1.6	0.4
A.L.D. (mg./kg.)	0.40	0.33	1.5
Wt./total A.L.D. (kg.)	5000	4850	267

Table 2. *Lecithinase A potency of venoms and venom fractions relative to that of Crotalus adamanteus and the effects of heating*

C. adamanteus venom was selected as a standard of reference. Heating was carried out at 100° for 15 min.

Venom	Amount used (μg.)	Wt. of <i>C. adamanteus</i> venom with enzymic activity (μg.)	Potency relative to <i>C. adamanteus</i> (%)	Inhibition of potency due to heating (%)
<i>Naja flava</i>	10	6.3	63	—
Fraction 1 (<i>N. flava</i>)	50	3.2	6	—
Fraction 2 (<i>N. flava</i>)	5	6.3	126	—
Heated (<i>N. flava</i>)	10	4.2	42	33
<i>Crotalus t. terrificus</i>	5	7.2	144	—
Heated (<i>C. t. terrificus</i>)	10	3.1	31	78
Heated (<i>C. adamanteus</i>)	10	4.8	48	52

Table 3. *Inhibition of succinic dehydrogenase*

In the 'immediate' series of experiments, enzyme, inhibitor (venom) and substrate were mixed and immediately incubated at 37° for 15 min. The effect of 'pre-incubation' for 1 hr. of the enzyme and inhibitor before adding the substrate was also investigated.

Venom	Venom added (mg.)	Inhibition (%)	
		Immediate	Pre-incubated for 1 hr.
<i>Naja flava</i>	0.6	0	30
	1.0	29	71
Fraction 1 (<i>N. flava</i>)	0.6	0	2
	1.0	0	12
Fraction 2 (<i>N. flava</i>)	0.6	87	37
	1.0	68	100
Heated (<i>N. flava</i>)	1.0	7	45
<i>Crotalus t. terrificus</i>	0.6	51	91
	1.0	66	100
Heated (<i>C. t. terrificus</i>)	1.0	24	48

When these venoms were heated for 15 min. at 100° (the procedure used by Braganca & Quastel, 1953), a consistent moderately destructive effect on both the lecithinase A activity and the inhibition of succinic dehydrogenase was observed. Approximately one-half of the lecithinase A potency and the inhibitory activity against succinic dehydrogenase was destroyed.

Activity of adenosine triphosphatase. Because of the importance of adenosine triphosphate in muscle metabolism and the paralysing effect on muscle of the snake venoms being investigated, the adenosine triphosphatase activity of these venoms and fractions was studied. The results obtained, when the activity of fraction 1, fraction 2 and crude *Naja flava* venom were compared on an equal-weight basis, are shown in Fig. 3. The most potent enzyme activity was obtained with fraction 2, the least with fraction 1, and the crude venom was intermediate in potency. It was considered unlikely that the inherent buffering capacity of the fractions would influence the result when only 50 µg. was used. However, to eliminate this possibility, a series of experiments was performed which showed that heated 50 µg. quantities of one fraction did not influence the change in pH produced by the other.

A similar comparison of the potency of crude *Naja flava* venom with *Crotalus t. terrificus* and *C. adamanteus* venoms was made. The results obtained, in decreasing order of potency, were: *C. t. terrificus*, *C. adamanteus* and *Naja flava*.

Activity of diphosphopyridine nucleotidase. Determination of this activity of the three venoms and the two fractions revealed results similar to the one

obtained with adenosine triphosphatase. Again fraction 2 was much more potent than either *N. flava* or fraction 1 (Fig. 3), and again the activity of *Crotalus t. terrificus* was greater than that of *C. adamanteus*, which in turn was more potent than that of *Naja flava*. However, the difference in activity between each of the three whole venoms was relatively slight.

Activity of ribonuclease. Similar results were obtained when the ribonuclease activity of the two fractions was studied. As with the other two enzyme activities, fraction 2 was the most active (Fig. 3). Although the order of potency was the same, there was very little difference in activity between the three whole venoms. The change in pH produced by all of the venoms and fractions with ribonucleic acid was considerably less than the change produced with adenosine triphosphate and diphosphopyridine nucleotide as substrates.

DISCUSSION

The fundamental thesis of these experiments is that if a given venom is separated into a more toxic and less toxic fraction, an enzyme activity of toxicological significance should be present in higher concentration in the more toxic fraction. When several enzyme activities of the two fractions were compared on an equal-weight basis, it was found that the concentration of activity was markedly less in the more toxic fraction. It is barely conceivable that the disproportionate distribution of an inhibitor might explain these results, but it seems more likely that none of the enzyme activities tested offers an explanation for

Fig. 3. (a) Adenosine triphosphatase, (b) diphosphopyridine nucleotidase and (c) ribonuclease activities of *Naja flava* venom and fractions 1 and 2 of *N. flava* venom. Each reaction mixture contained 50 µg. of the venom or fraction. The amount of substrate used was 2 mg. of adenosine triphosphate or diphosphopyridine nucleotide and 5 mg. of ribonucleic acid. The total volume of each reaction mixture was 2.5 ml. O, Crude *N. flava* venom; ▲, fraction 1; ●, fraction 2.

the toxic action of *N. flava* venom. The correlation found between lecithinase A activity and the inhibitory action against succinic dehydrogenase is consistent with the view of Braganca & Quastel (1953) that the lecithinase A action of cobra venom is responsible for the inhibition of succinic dehydrogenase, probably by disruption of the mitochondria. However, our results indicate that both these activities are incidental to the primary toxic action.

The method of preparation by non-denaturing techniques of a fraction (fraction 1) from *N. flava* venom which has most of the toxicity of the venom but little of the enzymic activities is felt to represent an improvement over the method of heating the venom at 100° for 15 min. (Braganca & Quastel, 1953). The fraction obtained is more active, and it seems that the use of a denaturing technique such as heating to simplify a mixture of proteins is biochemically less satisfactory. It is frequently assumed that if a mixture of toxic proteins such as a snake venom is heated and no loss in toxicity is detected, the proteins primarily responsible for the toxicity of the mixture have not been affected by the heat treatment. There is no proof of this assumption. There is no reason to assume that the product obtained by heating cobra venom at 100° for 15 min., whether more, less or equally toxic, is the same biochemically as the original venom. Work is being continued in this Laboratory with this fraction in an attempt to explain its toxicity.

SUMMARY

1. *Naja flava* (cobra) venom has been separated into two electrophoretically distinct fractions, one (fraction 1) four times as toxic as the other (fraction 2).

2. Fraction 1 is composed of two highly mobile, strongly basic proteins comprising 80% by weight of the venom; fraction 2, which makes up the remaining 20% of the venom, consists of three proteins of low mobility. This fraction gives a positive Molisch test for carbohydrate.

3. Fraction 1, which is four times as toxic as fraction 2, is much less potent than fraction 2 with respect to lecithinase A, adenosine triphosphatase, diphosphopyridine nucleotidase and ribonuclease activities, indicating that none of these enzyme activities is of prime importance toxicologically.

4. Fraction 2 is also a much more potent inhibitor of succinic dehydrogenase than fraction 1. Thus although the lecithinase A activity may well be responsible for the inhibition of succinic dehydrogenase, neither action is of importance as a toxic mechanism.

5. Comparison of the adenosine triphosphatase, diphosphopyridine nucleotidase and ribonuclease activities of *Naja flava* venom with those of two *Crotalus* venoms (*C. t. terrificus* and *C. adamanteus*) revealed that *Naja flava* venom was less active in each case.

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The Effect of Various Metabolites on Incorporation *in vitro* of Labelled Amino Acids into Protein of Normal Rat Diaphragm

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Incorporation of ¹⁴C from DL-[1-¹⁴C]alanine or from uniformly ¹⁴C-labelled L-alanine into protein of the normal rat diaphragm is depressed by addition to the medium of sodium pyruvate and, to a less extent, of glucose (Sinex, MacMullen & Hastings, 1952; Manchester & Young, 1958). Manchester & Young (1958) studied the incorporation of radioactive material from another ten

labelled amino acids, but found no diminution of incorporation in the presence of glucose. In the presence of pyruvate, incorporation from several amino acids was slightly smaller, but with only alanine and aspartic acid was the diminution substantial. Sinex *et al.* (1952) considered the possibility that in the presence of pyruvate, either added to the medium or formed from glucose, a