Biochemical Pharmacology, 1964, Vol. 13, pp. 793–795. Pergamon Press Ltd., Printed in Great Britain.

The action of *Bothrops* venoms on the Keilin–Hartree heart-muscle preparation

(*Received* 22 *November* 1963; *accepted* 7 *January* 1964)

SNAKE VENOMS (*Naja naja*, *Crotalus terrificus terrificus*, etc.) inactivate the $NADH_2$–oxidase and succin-oxidase systems from animal tissues, and the inhibitory power is related to the phospholipase contained by the venoms.[1–4] Similar observations are reported now with the venoms of *Bothrops neuwiedii* and *B. alternatus*, two snake species often found in the north and northeast regions of Argentina. A selective action of venoms on the quinol oxidase activity of the heart muscle preparation has been demonstrated.

Keilin–Hartree heart-muscle suspensions (20 to 25 mg/ml, dry weight) were incubated in 0·1 M phosphate buffer, at pH 7·4, with *Bothrops* venoms (7 μg venom/mg preparation) for 80 min at 30°. For comparative purposes, similar heart-muscle samples were treated as with *N. naja* and *Crotalus t. terrificus* venoms. The venoms were added dissolved in 0·05 M phosphate buffer (pH 5·9). Heat-treated venoms were kept for 10 min at 100° in a boiling-water bath and centrifuged to remove the coagulated protein. Incubation samples (1 ml) of the venom-treated preparations and the respective controls were diluted with 9 ml of 0·1 M phosphate buffer (pH 7·4) and centrifuged for 30 min at 15,000*g* at 0°. The residues were washed once in the centrifuge with 10 ml of phosphate buffer and resuspended in 1 ml of the same solution.

As shown in Table 1 (experiments A, B, F), treatment with *B. neuwiedii* and *B. alternatus* venoms produced significant inhibition of the $NADH_2$–oxidase, $NADH_2$–cytochrome c reductase and

TABLE 1. EFFECT OF VENOMS ON THE CATALYTIC PROPERTIES OF THE KEILIN–HARTREE HEART-MUSCLE PREPARATION*

In experiments A–F the reaction rates were measured spectrophotometrically at 31° (cf. Ref. 10–11) and in experiments G and H manometrically at 30°. Reaction mixtures (2·95 ml) were made up as follows. *Expt. A*: 0·25 mM $NADH_2$, 0·04 mM cytochrome c, and 0·13 M phosphate buffer (pH 7·4). *Expt. B*: 1 mM KCN; oxidant, 0·07 mM cytochrome c and other conditions as in Expt. A; *Expt. C*: oxidant, 0·13 mM Q_0; other conditions as in Expt. B. *Expt. D*: conditions as described by Minakami *et al.*[12] *Expt. E*: 50 μM K_3H_2, 0·04 mM phosphate buffer (pH 6·24) and 1 mM EDTA. *Expt. F*: 30 mM succinate, 0·04 mM cytochrome c, and 0·13 M phosphate buffer (pH 7·4). *Expt. G*: conditions as described in Ref. 13. *Expt. H*: 10 mM *p*-phenylendiamine, 0·04 mM cytochrome c, and 60 mM phosphate buffer (pH 7·4). All samples contained 0·10 to 0·15 mg of heart-muscle suspension treated as described in text. *B. neuwiedii*, *B. alternatus*, and *Crotalus t. terrificus* venoms were from the Instituto Nacional de Microbiologia, and *N. naja* venom was from the Haffkine Institute, Bombay, India. Final pH of the reaction mixtures is indicated in parentheses.

Expt.	*Reaction*	*Specific activity of control preparation*	*Venom*							
			B. neuwiedii		*B. alternatus*		*Crotalus t. terrificus*		*N. naja*	
			C	*H*	*C*	*H*	*C*	*H*	*C*	*H*
			(inhibition of activity, %)							
A	$NADH_2 \rightarrow O_2$	0·215	84	79	54	40	87	43	84	79
B	$NADH_2 \rightarrow$ cyt. c^{3+}	0·024	100	100	83	79	100	63	100	96
C	$NADH_2 \rightarrow Q_0$	0·185	48	44	36	27	48	34	59	52
D	$NADH_2 \rightarrow Fe(CN)_6^{3-}$	10·3	−1	−1	−4	−1	−1	3	−6	2
E	$K_3H_2 \rightarrow O_2$	3·1	94	87	35	35	100	45	100	100
F	Suc. $\rightarrow O_2$	0·641	92	87	44	35	90	21	97	97
G	Suc. $\rightarrow$ PMS	0·330	−12	0	− 6	−9	−9	−3	0	−36
H	Cyt. $c^{2+} \rightarrow O_2$	0·392	58	44	17	14	27	35	61	45

Expts. A–D: μmole of $NADH_2$ oxidized/min/mg preparation. *Expt. E*: k'/min $^{-1}$ (mg prep./ml) (k', constant of the first-order equation). *Expts. F and G*: μmole of succinate oxidized/min/mg preparation. *Expt. H*: μatoms of oxygen consumed/min./mg preparation. Dry weights (at 100°) are used throughout to express protein concentration.

* Abbreviations: Q_0: 2, 3-dimethoxy-5-methylbenzoquinone; K_3H_2: menadiol; PMS: phenazine methosulfate. C = crude; H = heated.

succinoxidase systems. The venom of *B. neuwiedii* (either crude or heat treated) was apparenly as potent as the cobra venom and more effective than the *Crotalus* or the *B. alternatus* venoms. Heat treatment scarcely affected the effectiveness of the venoms on the $NADH_2$- and succinate-oxidizing systems, excepting *Crotalus* venom the action of which was significantly reduced. On the other hand, neither $NADH_2$-dehydrogenase (experiment D) nor succinic dehydrogenase (experiment G) was inhibited by the venoms assayed, and eventually the latter enzyme was somewhat activated, particularly with heated *N. naja* venom.

Quinones mediate electron transport between dehydrogenases and the cytochrome system,[5, 6] and therefore oxidation of a quinol (K_3H_2) and reduction of a quinone (Q_0) by venom-treated preparations was measured. The K_3H_2 oxidase system (experiment E) was strongly inhibited by *B. neuwiedii*, *N. naja*, and *Crotalus* venoms, and a lesser effect was obtained with *B. alternatus* and boiled *Crotalus* venom. The $NADH_2$-Q_0 reductase system was also inhibited by all the venoms assayed (experiment C) but less than was the quinol oxidation. Inhibition of quinone (quinol) reduction (oxidation) could be explained by diminution of the quinone content of the heart-muscle preparation. However, the values obtained with venom-treated preparations (3·1 to 3·6 μmole ubiquinone/g protein) did not differ significantly from the untreated controls. Ubiquinone was determined according to Pumphrey and Redfearn.[7]

Bothrops venoms contain phospholipase (Table 2). Compared with *N. naja* and *Crotalus* venoms, the phospholipase activity of the heat-treated samples can be graded in the following order: *N. naja* > *B. neuwiedii* > *B. alternatus* > *Crotalus t. terrificus*. If inactivation of succinoxidase is taken as a standard of comparison, the effect of venoms (heated) on electron transport fits in well with the respective phospholipase activities.

TABLE 2. PHOSPHOLIPASE ACTIVITY OF HEAT-TREATED SNAKE VENOMS

Venoms were dissolved in 0·05 M phosphate buffer (0·1 mg/ml), at pH 5·9, and heated for 10 min at 100° in a boiling-water bath. After cooling, the precipitate was discarded by centrifugation and the phospholipase activity measured in the supernatant according to Habermann and Neumann[14]. The proteolytic activities of the heat-treated venoms, assayed according to Kunitz,[15] were negligible.

Venom	*Incubation with egg yolk (min)*	T_c* *(min)*	*Increase of* T_c (%)
B. neuwiedii	10	7·5	274
B. neuwiedii	20	14·0	600
B. alternatus	10	3·5	70
B. alternatus	20	6·0	200
Crotalus t. terrificus	20	3·5	70
N. naja	10	13·0	550
N. naja	20	40·0	1,900
None	10	2·0	
None	20	2·0	

* T_c, coagulation time of egg yolk.

Selective inhibition of electron transport between the quinone and cytochrome c seems to be a common property of *B. neuwiedii*, *N. naja*, and *Crotalus t. terrificus* (crude) venoms. The accessibility of exogenous cytochrome c to the electron transport chain should also be reduced, as demonstrated by the stronger inhibition of the $NADH_2$-cytochrome c reductase system with respect to $NADH_2$-oxidase and succinoxidase systems. However, the inhibition is not strictly limited to the quinone-cytochrome c segment, since the quinone reductase and cytochrome oxidase are also affected (Ref. 8 and Table 1) and furthermore, with 45 μg venom/mg protein, boiled *B. neuwiedii* venom liberated soluble $NADH_2$-dehydrogenase. The inactivation in all probability can be attributed to the phospholipase contained by the venoms, but it remains undecided whether it is caused by the hydrolysis of the phospholipid constituents of the electron transport particle, by the action of the products of hydrolysis on the electron carriers,[4, 9] or by both.

Acknowledgement—This work has been supported by the Rockefeller Foundation and Consejo Nacional de Investigaciones Cientificas y Técnicas (Argentina). The Q_0 used was a gift of Merck Sharp & Dohme, by courtesy of Dr. K. Folkers.

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REFERENCES

1. B. M. BRAGANCE and J. H. QUASTEL, *Biochem. J.* **53**, 88 (1963).
2. A. P. NYGAARD and J. B. SUMNER, *J. biol. Chem.* **200**, 723 (1953).
3. A. P. NYGAARD, *J. biol. Chem.* **204**, 655 (1953).
4. S. W. EDWARDS and E. G. BALL, *J. biol. Chem.* **209**, 619 (1954).
5. E. C. SLATER, J. P. COLPA-BOONSTRA and J. LINKS, in *Quinones in Electron Transport* (*Ciba Foundation Symposium*, p. 161. Churchill, London (1961).
6. D. E. GREEN, *ibid.*, p. 130.
7. A. M. PUMPHREY and E. R. REDFEARN, *Biochem. J.* **76**, 61 (1960).
8. J. HATEFI, A. G. HAAVIK and D. E. GRIFFITHS, *J. biol. Chem.* **237**, 1676 (1962).
9. R. F. WITTER, A. MORRISON and G. R. SHEPARDSON, *Biochem. biophys. Acta* **26**, 120 (1957).
10. A. O. M. STOPPANI, J. A. BRIGNONE, C. C. BRIGNONE and B. N. BADANO, *Biochim. biophys. Acta* **58**, 622 (1962).
11. B. CHANCE, *J. biol. Chem.* **197**, 557 (1952).
12. S. MINAKAMI, R. L. RINGLER and T. P. SINGER, *J. biol. Chem.* **237**, 569 (1962).
13. T. P. SINGER and E. B. KEARNEY, in *Methods of Biochemical Analysis*, D. Glick, Ed., vol. **4**, p. 310. Interscience, New York (1957).
14. E. HABERMANN and W. NEUMANN, *Hoppe-Seylers Z. physiol. Chem.* **297**, 179 (1954).
15. M. KUNITZ, *J. gen. Physiol.* **30**, 291 (1946).

Biochemical Pharmacology, 1964, Vol. 13, pp. 795–797. Pergamon Press Ltd., Printed in Great Britain.

The fluorometric determination of acetylcholine

(*Received* 12 *December* 1963; *accepted* 7 *February* 1964)

PROBLEMS under investigation in this laboratory on certain aspects of acetylcholine (ACh) metabolism indicated the need for a sensitive chemical assay for the neurohumor. The principle of the method that has been devised is as follows: acetylcholine is reduced under mild conditions by the use of potassium borohydride and lithium choloride to yield ethanol and choline. The ethanol is then oxidized to acetaldehyde by alcohol dehydrogenase with the concomitant reduction of NAD (DPN) to NADH (DPNH); finally, the NADH is determined fluorometrically.

The procedure, which may be modified to accommodate any volume that is convenient, is outlined below.

Reagents. (1) Acetylcholine: a stock solution of acetylcholine chloride or perchlorate of 1 mg/ml is prepared and suitably diluted to serve as a standard. (2) Reducing solution: per milliliter, this solution contains 30 mg of potassium borohydride and 10 mg of lithium chloride and is prepared just prior to use; sodium borohydride may be used in place of the potassium salt. The borohydrides may be obtained from Metal Hydrides, Inc. (3) Glycine buffer: this solution contains 22·5 g of glycine,* 3·75 g of semicarbazide HCl and alkali to pH 9·6. The solution is freeze-dried to remove traces of alcohol in the reagents and finally diluted to 100 ml; to prevent deterioration, it should be stored in

* Most commercial samples of glycine contain a fluorescent impurity; this contaminant may be removed by treatment with charcoal.