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SUSCEPTIBILITY OF CERTAIN ENZYMES OF THE CENTRAL NERVOUS SYSTEM TO TETRODOTOXIN

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The neurotoxic poison from the roe of Fugu, a globe fish living in the waters of Japan, was investigated with reference to its effect on the main oxidation-reduction systems, acetyl-cholinesterase and choline-acetylase in the brain of the guinea-pig.

It was found that the poison inhibited the choline-acetylase activity, even in a concentration of 4.4×10^{-7}. Acetyl-cholinesterase and cytochrome-c-oxidase activities were also inhibited in concentration of 1.3×10^{-5} and 0.8×10^{-5} respectively.

The Fugu, a poisonous kind of globe fish living in the home waters of Japan, causes several hundreds of cases of lethal poisoning every year, because the inhabitants eat it with relish, in spite of the high toxicity of some of its organs, particularly the ovaries. Hypaesthesia and paraesthesia are the first symptoms of the poisoning; motor paralysis follows; the patients die from paralysis of the respiratory centre and of the respiratory muscles, especially the diaphragm.

As it is clear from the symptoms that the poison, tetrodotoxin, affects the nervous system, its pharmacological actions on this system have been extensively studied (Kuriaki, Hiyoshi and Mochizuki, 1956). Yamagata (1940) claimed that tissue respiration and dehydrogenase activity in the brain of the cock and the mouse were inhibited by low concentrations and stimulated by higher concentrations of tetrodotoxin. Torikai (1944) observed that 0.3% of tetrodotoxin stimulated the activity of the succinic acid dehydrogenase of the rabbit heart and that xanthine dehydrogenase was markedly stimulated after a latent period. Some results of our studies on the biochemical aspects of its action are reported here.

METHODS

The poison of Fugu has now been successfully purified and has been obtained in crystalline form. The minimum lethal dose in mice is 0.01 to 0.001 mg./kg. body weight. The empirical formula is $C_4H_7O_3N$ (Yokowo, 1947; Tsuda and Kawamura, 1952). However, as the crystalline poison was not available to us, we used tetrodotoxin, a concentrate prepared by Sankyo Co. The method of preparation was precipitation of the concentrated aqueous extract of Fugu roe by lead acetate in an alkaline medium. Lead acetate in methanolic ammonia was then added to the concentrated solution of the precipitate in acidified water (10% H_2SO_4). The preparation was centrifuged and methanolic ammonia was added to the supernatant. The precipitate was dissolved in water and the solution treated with hydrogen sulphide and filtered. The filtrate was evaporated to dryness *in vacuo*. 1 g. of this concentrate corresponded in its toxic potency for the mouse to about 3 mg. of the pure crystalline poison.

In order to compare the effects of the poison on various enzymes and metabolites in the same tissue, all experiments were performed on homogenates of adult guinea-pig brain, except those on the yellow enzyme, which was derived from pig heart.

For determination of dehydrogenase activity, triphenyltetrazolium chloride (TTC) was employed in place of methylene blue, because, in contrast to the latter reagent, TTC when reduced gives a distinct red colour which is stable in the presence of oxygen. Sodium lactate, sodium succinate, sodium citrate, sodium pyruvate, and sodium β-glycerophosphate were used as substrates. One part of the brain of the guinea-pig was homogenized by means of a Potter homogenizer with five parts of phosphate buffer solution (0.2 M, *p*H 7.4). Each test-tube contained 1.0 ml. of the solution of tetrodotoxin, or 1.0 ml. of distilled water in the control group, 1.0 ml. of the solution of a substrate, 1.0 ml. brain homogenate, 1.0 ml. of 0.5% TTC reagent, and 2.0 ml. phosphate buffer solution. The test-tubes were filled with coal gas, tightly stoppered, and incubated at 38° for 2 hr. The reaction was stopped by addition of 1/10 volume of 20% trichloracetic acid. The triphenylformazan dye produced was extracted with 7.0 ml. of ethylacetate and the optical density was measured spectrophotometrically at a wavelength of 480 mμ.

For experiments on yellow enzyme, flavin adenine dinucleotide (FAD) was prepared from the heart

TABLE I

EFFECT OF TETRODOTOXIN ON ACTIVITIES OF DEHYDROGENASES FROM GUINEA-PIG BRAIN *IN VITRO*

Activity is expressed as amount of triphenylformazan dye produced in μg./g. wet weight of the brain.

No. of Expts.	Substrates	Conc. of Tetrodotoxin					
		0	$1{\cdot}7\times10^{-3}$	$1{\cdot}7\times10^{-4}$	$1{\cdot}7\times10^{-5}$	$1{\cdot}7\times10^{-6}$	$1{\cdot}7\times10^{-7}$
7	Succinate ..	10·57±2·99	12·15±2·7 ($0{\cdot}8>P>0{\cdot}7$)	11·6±3·4 ($0{\cdot}9>P>0{\cdot}8$)	10·5±2·45 ($0{\cdot}9>P>0{\cdot}8$)	7·8±2·3 ($0{\cdot}4>P>0{\cdot}3$)	7·35±1·65 ($0{\cdot}4>P>0{\cdot}3$)
7	Lactate ..	9·25±2·55	15·0±5·8 ($0{\cdot}5>P>0{\cdot}4$)	8·0±2·0 ($0{\cdot}8>P>0{\cdot}7$)	8·25±2·2 ($0{\cdot}8>P>0{\cdot}7$)	8·5±4·15 ($0{\cdot}9>P>0{\cdot}8$)	8·25±2·3 ($0{\cdot}8>P>0{\cdot}7$)
7	Pyruvate	8·27±2·44	14·5±6·0 ($0{\cdot}4>P>0{\cdot}3$)	6·0±2·45 ($0{\cdot}6>P>0{\cdot}5$)	7·8±1·65 ($0{\cdot}9>P>0{\cdot}8$)	8·35±4·45 ($0{\cdot}9>P>0{\cdot}8$)	7·15±2·85 ($0{\cdot}8>P>0{\cdot}7$)
7	Glycero-phosphate	9·26±2·92	11·0±2·25 ($0{\cdot}7>P>0{\cdot}6$)	10·3±3·35 ($0{\cdot}8>P>0{\cdot}7$)	6·15±2·1 ($0{\cdot}5>P>0{\cdot}4$)	8·5±3·1 ($0{\cdot}9>P>0{\cdot}8$)	8·5±3·2 ($0{\cdot}9>P>0{\cdot}8$)
7	Citrate	7·43±2·59	8·8±2·3 ($0{\cdot}8>P>0{\cdot}7$)	8·45±3·2 ($0{\cdot}8>P>0{\cdot}7$)	9·9±4·45 ($0{\cdot}7>P>0{\cdot}6$)	6·25±3·8 ($0{\cdot}9>P>0{\cdot}8$)	6·3±2·05 ($0{\cdot}9>P>0{\cdot}8$)

muscle of the pig according to the description of Lockhart (1930) and von Euler and Hellström (1938). Preparation of reduced diphosphopyridine nucleotide (DPN) was performed according to Green, Dewan, and Leloir (1937). The experimental procedure of Greig (1946) was followed, except that TTC was used instead of methylene blue as the hydrogen acceptor.

Cytochrome oxidase activity was determined by a simple method which consisted of immersing pieces of sliced and frozen guinea-pig brain in a solution of Nadi reagent for 15 min. and comparing their colour density macroscopically with that of pieces put in the Nadi reagent containing tetrodotoxin (Becker, 1949).

The method of Feldberg (1950) was followed for determination of choline-acetylase activity, and effect *in vitro* of the poison on the enzyme was studied. The procedure of von Muralt (1942) with the frog rectus muscle was used for the evaluation of acetylcholine production.

Acetyl-cholinesterase activity of the brain homogenate and effect of the poison on it were determined manometrically according to the method of Ammon (1934). 0.5 ml. of 0.0025 M acetylcholine chloride was placed in the side arm. Homogenate of 0.3 g. brain of the guinea-pig in 3.0 ml. of 0.025 M Ringer-bicarbonate solution and 0.5 ml. of tetrodotoxin solution of various concentrations were placed in the main compartment of the flask.

Determination of high energy phosphates was carried out as follows: the brain of the normal guinea-pig and that of the guinea-pig administered sub-lethal dose of tetrodotoxin (0.45 mg.) were homogenized at a temperature below 0° by cooling from outside the homogenizer with dry ice and methanol. The high energy phosphates were fractionated by paper chromatography, as described by Fleckenstein (1952, 1953) and Gerlach, Weber, and Döring (1955). Extracts of the paper were treated as described by Berenblum and Chain (1938), and the optic densities were determined spectrophotometrically.

RESULTS

As Table I indicates, the Fugu poison in concentrations of 10^{-7} to 10^{-3} did not have any significant effect on activities of the dehydrogenases when incubated with various substrates *in vitro*.

Experiments were also performed on the dehydrogenases *in vivo*, injecting a guinea-pig first with 0.45 mg. (sub-lethal dose) of the poison subcutaneously; then 25 min. later with 1.0 ml. of 1% TTC solution in saline in the carotid artery. The brain was excised 10 min. later and its colour density was compared with that of an untreated brain of a control animal. There was no apparent difference in the colour density of the two brains.

The yellow enzyme was not affected by the poison in concentrations of 10^{-7} and 10^{-4}, as shown in Table II.

TABLE II

EFFECT OF TETRODOTOXIN ON THE YELLOW ENZYME ACTIVITY *IN VITRO*

Activity is expressed as amount of triphenylformazan dye produced in μg./g. wet weight of the brain.

No. of Expts.	Conc. of Tetrodotoxin		*P* Value
8	0 (control)	5·13±0·36	
8	$1{\cdot}1\times10^{-7}$	5·21±0·32	$0{\cdot}9>P>0{\cdot}8$
8	$1{\cdot}1\times10^{-6}$	5·21±0·32	$0{\cdot}9>P>0{\cdot}8$
8	$1{\cdot}1\times10^{-5}$	5·25±0·32	$0{\cdot}9>P>0{\cdot}8$
8	$1{\cdot}1\times10^{-4}$	5·31±0·33	$0{\cdot}9>P>0{\cdot}8$

On cytochrome oxidase, the poison was ineffective in concentrations of 10^{-8} to 10^{-6}, but effective in concentrations of 10^{-5}, depressing the activity. In concentrations of 10^{-4} to 10^{-3}, inhibition was marked, as seen in Table III.

TABLE III.

EFFECT OF TETRODOTOXIN *IN VITRO* ON CYTOCHROME OXIDASE ACTIVITY OF GUINEA-PIG BRAIN

Activity was estimated as degree of coloration of Nadi reagent and intensity is proportional to the number of symbols +.

No. of Expts.	Conc. of Tetrodotoxin	
8	0 (control)	+++
8	$0{\cdot}8\times10^{-8}$	+++
8	$0{\cdot}8\times10^{-7}$	+++
8	$0{\cdot}8\times10^{-6}$	++
8	$0{\cdot}8\times10^{-5}$	–
8	$0{\cdot}8\times10^{-4}$	–
8	$0{\cdot}8\times10^{-3}$	–

Choline-acetylase activity was inhibited by the poison in concentrations of 10^{-6} and 10^{-7}, 0.128 and 0.133 mg. of acetylcholine being produced respectively/g. acetone powder of the guinea-pig brain, in contrast to 0.221 mg./g. acetone powder in the control. In concentrations of 10^{-9} to 10^{-8}, the poison had no significant effect on the enzyme (Table IV).

TABLE IV

EFFECT OF TETRODOTOXIN *IN VITRO* ON CHOLINE-ACETYLASE ACTIVITY OF GUINEA-PIG BRAIN

Activity is expressed as amount of acetylcholine produced in mg./g.

No. of Expts.	Conc. of Tetrodotoxin		*P* Value
10	0 (control)	0·221	
10	$4{\cdot}4\times10^{-6}$	0·128	$0{\cdot}02>P>0{\cdot}01$
10	$4{\cdot}4\times10^{-7}$	0·133	$0{\cdot}05>P>0{\cdot}02$
10	$4{\cdot}4\times10^{-8}$	0·194	$0{\cdot}8>P>0{\cdot}7$
10	$4{\cdot}4\times10^{-9}$	0·209	$0{\cdot}9>P>0{\cdot}8$

Acetyl-cholinesterase was noticeably inhibited by tetrodotoxin in high concentrations of 1.3×10^{-5} and 1.3×10^{-4}. The Q_{CO_2} was 14.43 and 9.46 ml./mg./hr. respectively, whereas it was 26.67 ml./mg./hr. in the control. In 1.3×10^{-6} of tetrodotoxin, the Q_{CO_2} was 19.72, but the difference from that of the control was not statistically significant. 1.3×10^{-7} and 1.3×10^{-8} were no more effective, Q_{CO_2} being 28.31 and 32.72 respectively (see Table V).

Contents of high energy phosphates in normal guinea-pig brain were compared with those in the brain of the guinea-pig given a sub-lethal dose of tetrodotoxin (0.45 mg.). It can be seen from Table VI that the content of creatine phosphate, adenosine triphosphate (ATP), adenosine diphosphate (ADP), and inorganic phosphate in guinea-pig brain was not significantly altered by a sub-lethal dose of tetrodotoxin.

TABLE V

EFFECT *IN VITRO* OF TETRODOTOXIN ON ACETYL-CHOLINESTERASE ACTIVITY OF GUINEA-PIG BRAIN

Activity is expressed as amount of CO_2 evolved from $NaHCO_3$ in consequence of acetic acid formation by the enzyme.

No. of Expts.	Conc. of Tetrodotoxin	Q_{CO_2} μl./mg./hr.	*P* Value
10	0 (control)	26·67	
10	$1{\cdot}3\times10^{-4}$	9·46	$0{\cdot}02>P>0{\cdot}01$
10	$1{\cdot}3\times10^{-5}$	14·43	$0{\cdot}05>P>0{\cdot}02$
10	$1{\cdot}3\times10^{-6}$	19·72	$0{\cdot}2>P>0{\cdot}1$
10	$1{\cdot}3\times10^{-7}$	28·31	$0{\cdot}8>P>0{\cdot}7$
10	$1{\cdot}3\times10^{-8}$	32·71	$0{\cdot}7>P>0{\cdot}6$

TABLE VI

EFFECT OF TETRODOTOXIN *IN VIVO* ON THE AMOUNT OF HIGH ENERGY PHOSPHATES IN THE GUINEA-PIG BRAIN

No. of Expts.	Procedure (See Text)	Brain of Guinea-pig Injected 0·45 mg. of Tetrodotoxin (μg./g.)	Control (μg./g.)	*P* Value
6	CP	35·48	37·67	$0{\cdot}8>P>0{\cdot}7$
6	ATP and ADP	65·49	56·71	$0{\cdot}7>P>0{\cdot}6$
6	Inorganic phosphate	263·99	241·61	$0{\cdot}2>P>0{\cdot}1$

DISCUSSION

It follows from these results that, of those enzymes studied, choline-acetylase was most sensitive to the poison. This fact may be noteworthy, even though these *in vitro* experiments may not lead to any direct conclusions on the mechanism of action of the poison. It resembles Botulinus toxin, which is assumed by Burgen, Dickens and Zatman (1949), Ambache (1949, 1951), Brooks (1954) and others to attack the end of the cholinergic nerve fibre, reducing the production of acetylcholine there. The concentration of the poison, 4.4×10^{-7}, which depresses the enzyme activity *in vitro*, would easily be attained *in vivo* in cases of poisoning. Fukuda and Tani (1941) have observed in the cases of poisoning that a dose of 10 g. of Fugu ovaries was a lethal dose for man. As 1 g. of ovary can kill 20 kg. of mice and the minimum lethal dose of the crystalline poison is 0.01 μg./g., the amount of toxin present in ovary is of the order of 1 part in 5,000. Thus, the minimum lethal dose for man can be computed as approximately 0.03 μg./g. Since 1 g. of the poison concentrate which we used would correspond to about 3 mg. of the crystalline poison in its toxicity for mice, the threshold concentration of 4.4×10^{-7} would be attained in the body.

Where used in higher concentrations than in the choline-acetylase experiments, the poison also depresses the activity of cytochrome oxidase and that of acetyl-cholinesterase, but it does not affect the dehydrogenases or the yellow enzyme. Therefore, it is unlike anaesthetics, the action of which is attributed by Quastel (1951) to their inhibiting effect on flavoprotein activity. It is also unlike the bee- and snake-poisons which specifically inhibit dehydrogenase activity in markedly low concentrations (Fleckenstein and Janke, 1953). These results do not agree with those of Yamagata (1940), who claimed effects on tissue respiration and dehydrogenase activity. In any case, it is unlikely that low concentrations would inhibit and higher concentrations would stimulate. We are also unable to confirm Torikai (1944), who reported stimulation of dehydrogenases by tetrodotoxin.

Nagazawa and Nagai (personal communication) have compared the amount of acid-soluble phosphates in the brain and liver of normal rats, as well as in rats killed with tetrodotoxin ; they found no difference between normal and poisoned organs. Our results obtained by the new method agree with theirs, in that the content of high energy phosphates in the brain was not affected by the poison.

References

Ambache, N. (1949). *J. Physiol.*, **108**, 127.
—— (1951). Ibid., **113**, 1.
Ammon, R. (1934). *Pflüg. Arch. ges. Physiol.*, **233**, 486.
Becker, V. (1949). *Arch. exp. Path. Pharmak.*, **207**, 109.
Berenblum, I., and Chain, E. (1938). *Biochem. J.*, **32**, 295.
Brooks, V. B. (1954). *J. Physiol.*, **123**, 501.
Burgen, A. S. V., Dickens, F., and Zatman, L. J. (1949). Ibid. **109**, 10.
Euler, H. von, and Hellström, H. (1938). *Z. phys. Chem.*, **252**, 31.
Feldberg, W. (1950). *Methods in Medical Research*, 3, 95. Chicago: Year Book Publishers.
Fleckenstein, A. (1952). *Arch. exp. Path. Pharmak.*, **216**, 184.
—— (1953). Ibid., **219**, 531.
—— and Janke, J. (1953). *Pflüg. Arch. ges. Physiol.*, **258**, 177.
Fukuda, T., and Tani, Y. (1941). *Nippon Igaku*, **3258**, 2739.
Gerlach, E., and Weber, E. (1955). *Arch. exp. Path. Pharmak.*, **224**, 496.
—— —— and Döring, H. J. (1955). Ibid., **226**, 9.
Green, D. E., Dewan, J. G., and Leloir, L. F. (1937). *Biochem. J.*, **31**, 934.
Greig, M. E. (1946). *J. Pharmacol.*, **87**, 185.
Kuriaki, K., Hiyoshi, K., and Mochizuki, Y. (1956). *Jap. J. Pharmacol.*, **6**, 37.
Lockhart, E. (1930). *Biochem. J.*, **33**, 613.
Muralt, A. von (1942). *Pflüg. Arch. ges. Physiol.*, **245**, 604.
Quastel, J. H. (1951). *Mecanisme de la Narcose, Colloques internationaux du Centre National de la Recherche Scientifique, Paris*, p. 105.
Torikai, A. (1944). *Rinsho to Kenkyu*, **21**, 361.
Tsuda, K., and Kawamura, M. (1952). *J. pharm. Soc., Japan*, **72**, 187, 771.
Yamagata, T. (1940). *Igaku Kenkyu*, **14**, 2155.
Yokowo, A. (1947). *Riken Iho*, **24**, 136.