Studies on the Insecticidal Action of Nereistoxin,
4-N, N-Dimethylamino-1, 2-Dithiolane

IV Role of the Anti-Cholinesterase Activity in the Insecticidal
Action to Housefly, *Musca domestica* L.

(Diptera : Muscidae)

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The cholinesterase (ChE) inhibition by nereistoxin hydrogen oxalate in adult male housefly, *Musca domestica* L., was investigated to reveal the role of the inhibitory action in the insecticidal action of this compound. The injected nereistoxin (100 μ g/g) caused only a slight depression of ChE activity in the fly head. The experiments *in vitro* using the fly-head homogenate as a ChE preparation showed nereistoxin was a weak, competitive, reversible and unprogressive inhibitor of ChE. The known amount of acetylcholine in the insect nervous tissue, the active dosage of nereistoxin in its insecticidal action and the results of the present experiments indicated that the anti-ChE activity of this compound did not play the fundamental role in the insecticidal action. However, it was estimated that high dosages, more than 200 μ g/g, exerted 50 per cent inhibition of the ChE activity. Therefore the anti-ChE action by such a high dosage might be able to play an additional effect in the toxic action.

INTRODUCTION

It has been demonstrated that nereistoxin blocks the acetylcholine (Ach)-receptor so as to suppress the contraction by Ach in the rectus abdominis muscle of frog (SAKAI, 1966 b). This fact suggests that the insecticidal action of nereistoxin (SAKAI, 1964 b) is the result of neuro-junctional block. On the other hand, a preliminary experiment by the author revealed that the compound is a weak inhibitor of cholinesterase (ChE) in the housefly, *Musca domestica* L. (SAKAI, 1964 a).

Although there may be difference in the active surfaces between Ach-receptors and ChE's, it has been generally thought that the surfaces of both the proteins possess more or less affinity to protonized moieties, especially in amine compounds. Therefore it is not unnatural that nereistoxin, a tertiary amine compound, competes with Ach in forming an ion-pair with the anionic site of ChE molecule.

Probably, the main mechanism of an extraordinary nervous excitation produced in nereistoxin-poisoned insects (SAKAI, 1966 a) will be explained in such a way that the compound acts as an antagonist or a depolarizant against Ach-receptors. However, if the anti-ChE activity of nereistoxin is of an appropriate strength, the activity can play at least a part in the toxic action of nereistoxin.

The purpose of the present experiments is to reveal the mode of anti-ChE

action of nereistoxin and to discuss whether the role of this action is important in the insecticidal action.

MATERIALS AND METHODS

Insects Adult male housefly (Lab-em-7-em strain, susceptible to insecticides), which was reared at the room temperature of $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$, was used throughout the experiments.

Chemicals Nereistoxin hydrogen oxalate synthesized by the method of HAGIWARA *et al.* (1965) and, as a comparison, DDVP (98 per cent purity) which is a potent ChE inhibitor were used.

Determination of cholinesterase activity After an appropriate enzymatic reaction was finished, the remaining substrate, acetylcholine bromide (Ach.Br), in the reaction mixture was determined by the colorimetric method of HESTERINE (1949). The colorization was finalized by adding 2 ml of the mixture of 3.5 N NaOH and 2 M hydroxylamine hydrochloride (1:1 in volume), 1 ml H_2SO_4 solution (conc. H_2SO_4 was diluted with distilled water by 10.6 times in volume) and 1 ml 0.37 M FeCl_3 (in 0.1 N HCl) in this order. The absorbance of the colored solution at $530\text{m}\mu$ was measured with Hitachi EPU-2A-type spectrophotometer. Henceforth, the colorimetric procedure was same as described above except a few modifications.

Experiment on ChE inhibition in vivo The houseflies were injected with nereistoxin solution prepared with 0.9 per cent NaCl into the thoraces at the dosage of $100\text{ }\mu\text{g/g}$. The volume of injected solution was $1.2\text{ }\mu\text{l/fly}$. The injected flies were decapitated and the collected heads were homogenized in 0.1 M phosphate buffer (pH 7.4) by a glass homogenizer at the rate of 6 mg heads per 1 ml of the buffer. To 0.5 ml of the homogenate, 0.5 ml of $9 \times 10^{-3}\text{ M}$ Ach.Br in a saline (0.15 M NaCl and 0.04 M MgCl_2) and 0.5 ml of the equivoluminous mixture of the phosphate buffer and the saline (buffered saline) were added. The ChE reaction was held at 30°C for 15 min shaking the reaction mixture in a water bath. The nereistoxin-injected flies were compared with the flies injected with 0.9 per cent NaCl in the hydrolytic activity of ChE.

In this experiment, the flies were kept at the room temperature of $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$, and the heads from 15 flies were homogenized each time after the injection. The ChE activity was determined on 3 tubes of the same homogenate.

Experiments on ChE inhibition in vitro The enzymatic preparation employed in the experiments was the homogenate containing 3 mg of the fly-heads per 1 ml of the buffered saline. Ach.Br and inhibitor solution were prepared with the buffered saline.

Before starting the enzymatic reaction, the ChE preparation and the test tubes containing the mixed solution of the substrate and the inhibitors (1 ml/tube) were kept separately at 30°C in a water bath for approximately 5 min. At the start of the reaction, 0.5 ml of the homogenate was added to the tube. Because the accuracy of the enzymatic reaction period was required, the homogenate was added to each tube at 30 sec intervals.

The reaction period, the time from the addition of the enzyme to the suppression of the enzymatic reaction by the addition of NaOH-hydroxylamine HCl mixture, was varied with the concentrations of the substrate and the enzyme; *vis.*

it was so adjusted that the hydrolytic velocity was approximately constant. Detailed descriptions on this matter will appear later.

Since it was difficult to determine Ach.Br of a high concentration directly, when the Ach.Br concentration was 5×10^{-3} M at the start of the enzymatic reaction, 0.5 ml of the reaction mixture was pipetted into the solution containing 2 ml NaOH-hydroxylamine HCl mixture and 1 ml distilled water at the end of the reaction.

To test the reversibility of the inhibition, a dilution method was performed for nereistoxin. After 1.5 ml solution containing 1×10^{-3} M Ach.Br, 1×10^{-3} M nereistoxin and ChE (1.5 mg heads) was kept at 30°C for 15 min, 1.5 ml of 1×10^{-3} M Ach.Br was added to dilute nereistoxin to 5×10^{-4} M. Thus, this dilution kept the substrate concentration at approximately 1×10^{-3} M after the dilution. The diluted reaction mixture was kept for the following 25 min at 30°C . The resultant inhibition rate in the diluted series was compared with those of undiluted 1×10^{-3} M and 5×10^{-4} M nereistoxin.

In the experiment in which the volume of enzymatic reaction mixture was 3 ml, the addition of 1.5 ml H_2SO_4 solution was successful to eliminate a precipitation which was obtained by the addition of 1 ml H_2SO_4 solution and interfered with the colorimetric measurement.

The progression of the inhibition was investigated with the prolonged incubation of ChE with the inhibitors. A test tube containing 1 ml buffered saline with 7.5×10^{-4} M nereistoxin or 1.5×10^{-3} M DDVP, and ChE (1.5 mg heads) was kept at 30°C in a water bath. After the incubation of the inhibitor with the enzyme for a certain period, 0.5 ml of 3×10^{-3} M Ach.Br was added (the final concentration of Ach.Br was 1×10^{-3} M) and the hydrolysed Ach.Br in 15 min was determined.

Throughout these experiments *in vitro* three tubes were used for each series of the enzymatic reaction.

RESULTS

Preliminary test for the determination of the enzymatic reaction period Since the ChE activity was determined by the assay of the remaining amount of Ach.Br by the colorimetric method in the present experiments, it was impossible to keep the decrease of the substrate concentration in a negligible amount throughout the enzymatic reaction period. Therefore, when a substrate concentration was under the optimum, the reaction velocity might decrease due to the decrease of substrate concentration along with the progress of hydrolysis of the substrate. In the investigation on the kinetic relationships between enzymes, substrate and inhibitors, it is necessary that the substrate concentration should be kept almost constant, or otherwise as stated, the decrease of substrate concentration should be kept in a range where the decrease of enzymatic reaction velocity is negligible.

Fig. 1 shows the result of a preliminary experiment for the determination of the enzymatic reaction period in which the hydrolytic reaction progressed with a constant velocity. The result demonstrated that the reaction periods during which the constant velocities were obtainable were 15 min, 15 min and 9 min, when Ach.Br concentration were 5×10^{-3} M, 1.5×10^{-3} M and 5×10^{-4} M, respectively.

Accordingly, the reaction periods adopted in the following experiments were 7 min for 5×10^{-4} M Ach.Br and 15 min for Ach.Br higher than 1×10^{-3} M, when

Fig. 1. Time sequence of cholinesterase activity of housefly-head homogenate at 30°C. Dark circle : Ach.Br 5×10^{-3} M; Open circle : Ach.Br 1.5×10^{-3} M; Cross : Ach.Br 5×10^{-4} M.

ChE was 1 mg heads/ml. In the dilution method performed to investigate the reversibility of inhibition where the reaction system contained ChE of 0.5 mg heads/ml, the reaction period was settled at 25 min.

Acetylcholine concentration and ChE reaction velocity Many experimental results have revealed the optimum substrate concentration for Ach hydrolysis by ChE in insects is nearly 10^{-2} M, though it varies a little with the ionic composition of the reaction system (WOLFE and SMALLMAN, 1956). However, the colorimetric method was hardly applicable for the determination of the ChE activity under a high substrate concentration near the optimum concentration. Therefore, the reaction velocities at the Ach.Br concentrations of 5×10^{-3} M, 1.5×10^{-3} M and 5×10^{-4} M were determined in the present experiments.

As shown in Table 1, the variation in the velocity according to the substrate

Table 1. CHOLINESTERASE ACTIVITY OF HOUSEFLY-HEAD HOMOGENATE AT DIFFERENT SUBSTRATE CONCENTRATIONS AT 30°C

Enzymatic preparation No.	Ach.Br hydrolysed (μ M/mg heads/hr)		
	Substrate concentration		
	5×10^{-3}	1.5×10^{-3}	5×10^{-4}
1	2.60	1.90	1.68
2	2.60	2.31	1.42
3	3.28	2.35	1.48
4	2.40	2.00	1.80
5	3.40	2.34	1.40
Average	2.86	2.18	1.56

concentration was apparent, although there were little differences in the hydrolytic activity between the ChE preparations. From this result, the MICHAELIS-MENTEN constant (K_s) was calculated as 5.6×10^{-4} M. This value was in accordance with those obtained by WOLFE and SMALLMAN (1956) and METCALF *et al.* (1955) on the housefly ChE.

Thus, these results demonstrated that the colorimetric determination of ChE activity was satisfactorily applicable to the investigation of ChE kinetics.

ChE inhibition in vivo Table 2 shows the result of experiment *in vivo*. There occurred a weak inhibition at the initial time of the knock-down. However, this was eliminated gradually.

Table 2. INHIBITION OF HOUSEFLY-HEAD CHOLINESTERASE
BY NEREISTOXIN *in Vivo*^a

Time of decapitation after injection (min)	ChE inhibition rate (%)	Toxic symptoms of fly ^b
0	28.4	primary prostration
30	8.7	complete prostration
65	7.9	"
100	0.7	"
160	3.1	"

a The flies were injected with 100 $\mu\text{g/g}$ of nereistoxin.

b Ref. SAKAI (1966 a).

Table 3. INHIBITION OF HOUSEFLY-HEAD CHOLINESTERASE BY
NEREISTOXIN AND DDVP *in Vitro* AT 30°C

Inhibitor	Conc. of Ach. Br (M)	Conc. of Inhibitor (M)	Ach hydrolysed ($\mu\text{M}/\text{mg heads/hr}$)	Inhibition rate (%)
Nereistoxin	5×10^{-3}	0	2.82	0
		3.33×10^{-3}	0.50	82.3
		2.50×10^{-3}	1.40	50.5
		1.67×10^{-3}	1.46	48.2
		8.33×10^{-4}	2.14	24.3
	5×10^{-4}	0	1.94	0
		3.33×10^{-3}	0.18	99.0
		2.50×10^{-3}	0.50	74.4
		1.67×10^{-3}	0.68	64.9
		8.33×10^{-4}	0.92	52.6
DDVP	5×10^{-3}	0	2.50	0
		3.00×10^{-6}	0.52	79.2
		1.00×10^{-6}	0.94	62.4
		5.00×10^{-7}	1.60	36.0
	5×10^{-4}	0	1.72	0
		3.00×10^{-6}	0.32	81.4
		1.00×10^{-6}	0.69	59.9
		5.00×10^{-7}	1.03	40.1

Mode of ChE inhibition by nereistoxin in vitro The potencies of inhibitory action of nereistoxin and DDVP are shown in Table 3. Although there were some differences in the inhibitory potency according to the Ach.Br concentration, In-50 (the inhibitor concentration which produced 50 per cent inhibition of the enzymatic activity) of nereistoxin was approximately 10^{-3} M. In-50 of DDVP, a potent anti-ChE agent, was approximately 5×10^{-7} M. Thus, the ChE inhibitory action of nereistoxin is about 2,000 times less than that of DDVP.

The plots of V/V_i to the inhibitor concentrations, where V is the uninhibited ChE activity and V_i is the inhibited one, apparently show that nereistoxin is a competitive inhibitor (Fig. 2), whereas DDVP is a non-competitive inhibitor (Fig.3).

Fig. 2. Inhibition of housefly-head cholinesterase by nereistoxin. V/V_i : the ratio of uninhibited to inhibited activity; Open circle: Ach.Br 5×10^{-4} M; Dark circle: Ach.Br 5×10^{-3} M.

Fig. 3. Inhibition of housefly-head cholinesterase by DDVP. V/V_i : the ratio of uninhibited to inhibited activity; Open circle: Ach.Br 5×10^{-4} M; Dark circle: Ach.Br 5×10^{-3} M.

However, there was an anomaly in the inhibition of nereistoxin. Namely, the values of V/V_i at the inhibitor concentration of 3.33×10^{-3} M were not in a linear relationship which was obtainable at the lower inhibitor concentrations; *vis.* the values were larger than expected (Fig. 2).

The explanation of this problem would be reasonable if the acidity due to the oxalic acid in the salt of nereistoxin lowered the ChE activity, or that nereistoxin was more inhibitory under the acidic condition. These presumptions were denied by the results of the following experiments.

1) Since the pH of 3.33×10^{-3} M nereistoxin prepared with the buffered saline (pH 7.4) containing ChE (1 mg heads/ml) was 6.7, the uninhibited ChE activities at pH 6.7 and pH 7.4 to 5×10^{-4} M Ach.Br were determined and compared with each other. The result showed that the rate of activity at pH 6.7 was 85 per cent of the activity at pH 7.4.

If it is assumed that the inhibitory activity of nereistoxin is constant at various pH's, the activity of ChE at 3.33×10^{-3} M nereistoxin can be estimated to be 85 per cent of the activity which could be expected from the linear relationship in Fig. 2. From Fig. 2, the expected V/V_i 's at 5×10^{-3} M and 5×10^{-4} M Ach.Br are estimated as 2.5 and 4.8, respectively. Thus, if the expected enzymatic activity was lowered to 85 per cent of the activity at pH 7.4, the resulting inhibition rates by 3.33×10^{-3} M nereistoxin are estimated by the above calculation as 66.5 per cent and 82.5 per cent at Ach.Br concentrations of 5×10^{-3} M and 5×10^{-4} M, respectively. The empirical inhibition rates (Table 3) were higher than the calculated ones.

2) The inhibitory action of nereistoxin at different pH levels was investigated. The fly-head homogenate was prepared with the saline and it was diluted by 0.1 M phosphate buffer of different pH's. The reaction mixture consisted of the buffered saline of each pH containing 5×10^{-4} M Ach.Br and 1 mg heads/ml ChE.

The results (Table 4) revealed that the acidic condition (pH 6.5) rather lowered the inhibitory activity of nereistoxin.

Table 4. INHIBITION OF HOUSEFLY-HEAD CHOLINESTERASE BY NEREISTOXIN AT DIFFERENT LEVELS OF pH AT 30°C^a

Concentration of nereistoxin (M)	Inhibition Rate (%)		
	6.5	pH 7.4	8.0
0	0	0	0
1.67×10^{-3}	49.4	68.6	66.3
8.33×10^{-4}	34.5	59.0	49.0

^a Substrate was 5×10^{-4} M Ach.Br.

In the next experiment, the reversibility of the nereistoxin inhibition was investigated. The resultant data shown in Table 5 demonstrate that the inhibition rate in the diluted series was equal to that of the undiluted 5×10^{-4} M nereistoxin. That is, the inhibition was rapidly reversible.

The progressive inhibition was not observed on nereistoxin, whereas DDVP inhibited progressively as generally revealed on organophosphorus insecticides (O' BRIEN, 1960) (Table 6). It was noticed that the rate of inhibition of nereistoxin

Table 5. REVERSIBILITY OF INHIBITION OF HOUSEFLY-HEAD CHOLINESTERASE
BY NEREISTOXIN *in Vitro* AT 30°C^{a,b}

Dilution	Tube No.	Conc. of nereistoxin (M)	Conc. of ChE (mg heads/ml)	Ach hyd. (μ M/mg heads/hr)	Inhibition rate (%)
Not diluted	1	0	1	2.48	0
	2	1×10^{-3}	1	1.08	66.4
	3	0	0.5	2.68	0
	4	5×10^{-4}	0.5	1.40	47.8
Diluted	<i>Initial</i>				
	5	1×10^{-3}	1	—	—
		<i>After dilution</i> 5×10^{-4}	0.5	1.35 ^c	49.6

a Substrate was 1×10^{-3} M Ach.Br.

b The enzymatic reaction periods were 15 min for ChE of 1 mg heads/ml, and 25 min for 0.5 mg heads/ml.

c The value was calculated as follows; from the result in Tube no. 2, Ach in Tube no. 5 immediately before the dilution was estimated 7.3×10^{-4} M. Therefore, Ach concentration at the time immediately after the dilution (with 1×10^{-3} M Ach.Br) was $(1+0.73) \times 10^{-3} / 2 = 0.87 \times 10^{-3}$ (M). Since the resulted decrease of the substrate concentration in Tube no. 5 was determined as 0.41×10^{-3} M, Ach hydrolysed after the dilution was calculated as;

$$0.87 - (1.00 - 0.41) \times 10^{-3} \text{ M} / 0.5 \text{ mg heads} / 25 \text{ min} = 1.35 \mu\text{M/mg heads/hr.}$$

Table 6. PROGRESSION OF INHIBITION OF HOUSEFLY-HEAD CHOLINESTERASE
BY NEREISTOXIN AND DDVP *in Vitro* AT 30°C

Concentrations of inhibitor (M)		Time before addition of substrate (min)	Inhibition rate (%)
Before addition of substrate	After addition of substrate		
Nereistoxin			
		0	47
		5	48
7.5×10 ⁻⁴	5×10 ⁻⁴	10	49
		15	47
DDVP			
		0	18
		3	47
1.5×10 ⁻⁸	1×10 ⁻⁸	5	62
		15	65
		25	85

was independent of the period of incubation of enzyme-inhibitor mixture; *vis.* the inhibitory activity depended on the substrate concentration.

DISCUSSION

As revealed in the present experiments, nereistoxin is a competitive inhibitor

of ChE in the housefly. This fact means that the lower Ach concentration results in the higher rate of inhibition. Therefore, to evaluate the role of anti-ChE activity in the toxic action of nereistoxin, it is required to know the concentration of nereistoxin which causes the toxic action in insects, at the same time, the actual amount of Ach, especially at the site where ChE functions.

On the active concentration of nereistoxin, the author reported that the treatment of the exposed thoracic ganglion of adult housefly with 5×10^{-5} M nereistoxin was enough to cause an abnormal nervous excitation, and that the injection of approximately 10 $\mu\text{g/g}$ (5×10^{-5} M) evoked typical toxic symptoms in the fly (SAKAI, 1966 a).

Unfortunately, it is considerably difficult to ascertain the actual concentration of Ach in the insect nervous system. COLHOUN (1958) reported that 100 μg of Ach per 1 g of tissue was found in the eserinizied and electrically stimulated abdominal nerve cord of American cockroach, *Periplaneta americana*. He also found 63 $\mu\text{g/g}$ Ach in the sixth abdominal ganglion. In housefly-heads and nerve cord of American cockroach, LEWIS and SMALLMAN (1956) reported that Ach detected was 25 $\mu\text{g/g}$ and 50 $\mu\text{g/g}$, respectively. FOWLER and LEWIS (1958) found 30 $\mu\text{g/g}$ Ach in *Calliphora*-heads.

Although it is almost impossible to know the actual concentration of Ach at the site where ChE plays its role, those data cited above seem to assure us of the presumption that Ach concentration in the insect nervous tissues is 1×10^{-4} M to 5×10^{-4} M. It is easily inferred from the results shown in Table 3 or Fig. 2 that 5×10^{-5} M nereistoxin which produces the toxic action in the fly can hardly inhibit ChE in the insect in competition with Ach in the nervous tissue. Besides, these facts that the inhibition of ChE by nereistoxin is reversible (Table 5) and unprogressive (Table 6), and that the strength of the inhibition is dependant on the substrate concentration demonstrate that the compound is too weak to evoke the toxic action by the anti-ChE activity. In fact, the experiment *in vivo* showed only a slight inhibition even at the stage of complete prostration in the fly (Table 2).

However, since 1×10^{-3} M to 2×10^{-3} M nereistoxin inhibits about 50 per cent of the ChE activity in competition with 5×10^{-4} M to 5×10^{-3} M Ach *in vitro* (Table 3), it may be considered that the compound plays a part of the toxic action with its anti-ChE activity.

The mode of anti-ChE action of nereistoxin in the fly was considerably similar to those of pilocarpine and atropine which are weak, competitive and reversible ChE inhibitors (CHADWICK, 1964), and are well known as strong Ach-receptor blocking agents. It is interesting that nereistoxin which is a potent antagonist to Ach in the Ach-receptor of frog's skeletal muscle (SAKAI, 1966 b) inhibits ChE. As another example, decamethonium, which blocks Ach-receptors by depolarization, inhibits ChE (PATON and ZAIMIS, 1949). On the other hand, probably ChE inhibitors, organophosphorus insecticides (KIMURA, 1963; KIMURA *et al.*, 1963), and a carbamate insecticide (METCALF *et al.*, 1964), bind Ach-receptors. Although the considerations on the affinity of protonized nitrogen of nereistoxin to anionic sites of the proteins are possibly necessary to investigate this problem, further discussions in this direction are not the aim of the present paper.

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