PHYSIOLOGICAL ACTIVITIES OF NEREISTOXIN*

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The pharmacological activity of nereistoxin, which was isolated from a marine annelid, *Lumbriconereis heteropoda* MARENZ, was formerly investigated by NITTA¹⁾, using about 10 species of test animals from monkey to fly, and it was concluded that nereistoxin is a neurotoxic substance. HIRAYAMA *et al.*²⁾ reported that fish are more susceptible than warm-blooded animals to nereistoxin and the toxicity is considerably enhanced when the medium is adjusted to the alkaline side.

In spite of the well-known fact that flies die from contact with the dead body of the worm (PL. 1), the insecticidal activity of nereistoxin itself has been only insufficiently examined thus far. In the light of its chemical structure which has been recently proved to be 4-N, N-dimethylamino-1, 2-dithiolane by the present authors³⁾ and the fact that some organic sulfur compounds have been widely used as insecticides, it seemed to be very interesting to examine the insecticidal activity of nereistoxin.

The present paper deals with the results of tests on some noxious insects, together with biological assay method of nereistoxin. The lethal dose to mice and the lack of growth-promoting activity to *Streptococcus faecalis* IAM 1119 are also reported.

PLATE 1

Nereistoxin, 4-N, N-dimethylamino-1, 2-dithiolane

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Experimental and Results

1) Biological assay of nereistoxin

It was found by HIRAYAMA *et al.*²⁾ that the toxicity to killifish, *Oryzias latipes* TEMMINCK *et* SCHLEGEL, is enhanced by adjusting the medium to the alkaline side and by elevating the water temperature. This was also recognized by the present authors. On the basis of results of these preliminary experiments, the biological assay was carried out as follows.

After adjusting the test solution to pH 7.6 with sodium hydroxide solution, 3 ml of M/15 SÖRENSEN'S phosphate buffer (pH 7.6) is added and the volume is made to 50 ml with water. The water temperature is then maintained at 25°C during the experiment. Five individuals of adult fish, weighing 300~400 mg, are kept in this solution. The fish which does not show any response to being taken out from water with forceps is assumed to be dead, and the death time is recorded.

The dose-death time relationship is shown in Fig. 1, and the curve is expressed as follows:

$$C = 28.6 T^{-1.19},$$

where C is concentration of nereistoxin (mg/l) and T death time (min.).

Fig. 1 Dose-death time relationship in killifish

The assay is more accurate in the range of concentration between 0.5 and 2.5 mg/l. The minimum lethal dose of nereistoxin to killifish is about 0.28 mg/l, and this value is only slightly influenced by the size of test animals.

2) Concentration of nereistoxin in the worm

The concentration was estimated by the above-mentioned biological method on the crude water extracts.

Ten or twenty grams of the live worms was homogenized with a half weight of sea sand in a mortar. Three grams of the homogenate was extracted with 25 ml of 0.1 N hydrochloric acid in a boiling water bath for 15 minutes. After centrifugation, the residue was washed with a small amount of water, and the washing was combined to the supernatant. After dilution to about 50 ml, the solution was adjusted to pH 7.6 with sodium hydroxide solution. Amorphous precipitates appeared were eliminated by centrifugation. Six milliliters of the phosphate buffer were added to the supernatant which was finally made to 100 ml. Biological assay was carried out on the 50 ml portion of this solution.

Results obtained on the two batches of sample, each of which was assorted into three groups by size to be assayed separately, are shown in Table 1. The figures clearly show that the concentration is considerably different according to the size of worms and the values are higher for the smaller worms. This may coincide with the finding of HIRAYAMA *et al.*²⁾, that nereistoxin is located only in the skin of worms.

3) LD₅₀ of nereistoxin to mice

Lethal doses of nereistoxin hydrogen oxalate to rabbits and mice in subcutaneous injection were reported by NITTA¹⁾ to be 1.8 and 38 mg/kg, respectively. The present authors examined the LD₅₀ to mice by intravenous administration of the aqueous solution of nereistoxin hydrogen oxalate. Male mice, weighing from 16 to 20 g, were used. The result is given in Table 2, and LD₅₀ calculated according to the LITCHFIELD-WILCOXON method is 33.6 mg/kg as hydrogen oxalate.

4) Activity to *Streptococcus faecalis* IAM 1119

Among the natural organic sulfur compounds so far reported, α -lipoic acid has a dithiolane moiety in common with nereistoxin and the former is known as a growth factor for some microbes, such as *Streptococcus faecalis* IAM 1119, and as a cofactor of pyruvate oxidation system. This close similarity led us to examine the action of nereistoxin to this microorganism, by using the media reported by WADA *et al.*⁴⁾

Even on addition of nereistoxin up to 1900 μ g/l the growth of the microbe did not occur so far as α -lipoic acid was omitted. Moreover, in the presence of 0.6 or 1.8 μ g/ml of α -lipoic acid, nereistoxin exerted neither a growth-promoting action on

Table 1. Concentration of nereistoxin in *Lumbriconereis heteropoda*

Body weight Range (mean) g	Mean death time (min.)	Nereistoxin mg/100 g
2.8~3.7 (3.3)	13.8	68
2.1~2.5 (2.2)	11.9	80
0.5~0.9 (0.7)	9.1	105
2.8~3.3 (3.0)	15.8	57
1.9~2.3 (2.0)	10.5	93
0.4~1.0 (0.6)	9.1	105

Table 2. Intravenous injection of nereistoxin hydrogen oxalate to mice

Dose mg/kg	27.0	30.0	33.3	35.1	37.0
Survivor	5	4	3	1	0
Dead	0	1	2	4	5

the bacterium nor an antagonizing one to α -lipoic acid in the range of concentration from 0.25 to 1900 $\mu\text{g/ml}$.

5) Insecticidal activity of nereistoxin

As test insects the followings were used: imagos of house fly, *Musca domestica domestica* (NAIDM strain), American cockroach, *Periplaneta americana* L, azuki bean weevil, *Callosobruchus chinensis* L, larvae of *Hyphantria cunea* DRURY and of almond moth, *Ephestia catella* WALKER. They were used regardless of sex except American cockroach in which the female was adopted.

Free nereistoxin dissolved in acetone was administered to house fly, American cockroach and larva of *Hyphantria cunea* by the topical application. To azuki bean weevil and larva of almond moth, the dry film method was applied, in which nereistoxin in acetone was poured into the petri dish of 9 cm in diameter and after elimination of acetone the insects were kept in the dish for 24 hours at 28°C.

The results of tests on these insects are summarized in Table 3. The toxicity of nereistoxin is not so strong when compared with some representative commercial insecticides, as shown in Table 4. But, nereistoxin revealed a remarkable anesthetizing activity. For example, on the administration of 23 $\mu\text{g/g}$ to American cockroach, all of them are knocked down in 10~15 minutes and then gradually recovered after 5 hours. Even in the dose of 4 $\mu\text{g/g}$ such an activity was observed. As for azuki bean weevils, too, the rate of knock down was 100% on the level of 5.1 $\mu\text{g/dish}$ and 90% even on that of 0.6 $\mu\text{g/dish}$.

Table 3. Toxicity of nereistoxin to insects*

a) House fly

Dose $\mu\text{g/g}$	7.4	20.4	37.0	55.5	74.0	104	144
Number of death	3	12	12	15	22	23	25
Death rate (%)	12	48	48	60	88	92	100
Knock down rate (%)	8	76	84	92	96	100	100

b) American cockroach

Dose $\mu\text{g/g}$	23.0	34.5	46.0	57.5	69.0
Number of death	0	4	4	7	9
Death rate (%)	0	20	20	70	90
Knock down rate (%)	100	100	100	100	100

c) Azuki bean weevil

Dose $\mu\text{g/dish}$	1.28	2.56	5.12	10.2	20.4	81.6
Number of death	0	4	3	13	16	20
Death rate (%)	0	20	15	65	80	100
Knock down rate (%)	90	95	100	100	100	100

d) Larva of *Hyphantria cunea*

Dose $\mu\text{g/g}$	27.5	37.0	52.5	75.0	150	225
Number of death	1	1	3	4	5	5
Death rate (%)	20	20	60	80	100	100

e) Larva of almond moth

Dose $\mu\text{g/dish}$	56	105	204	408	816
Number of death	1	14	37	40	40
Death rate (%)	2.5	35	92.5	100	100

* Number of death and death rate were recorded after 24 hrs and knock down rate after 2 hrs.

Table 4. Comparison of nereistoxin to some commercial insecticides

Insects	Methods	Nereistoxin	DDT	γ -BHC	Pyrethrin	Parathion
House fly ⁶⁾ *	Topical	ca. 40	8~21	3	1~259	1.4
American cockroach ⁶⁾ *	Topical	ca. 50	10	5	6	1.5
Azuki bean weevil ⁷⁾ **	Dry film	ca. 10	500	0.35	—	0.45
Almond moth larva ⁸⁾ **	Dry film	200~400	—	—	—	5.6

* LD₅₀ $\mu\text{g/g}$.

** LD₁₀₀ $\mu\text{g/dish}$

Discussion

On the toxicity to killifish similar results were obtained as those of HIRAYAMA *et al.*²⁾, and it was applicable to the biological assay method of nereistoxin.

The LD₅₀ to mice is higher than that of α -lipoic acid (264 mg/kg⁵⁾). It may be reasonably considered that the toxicity depends on the dimethylamine moiety in the molecule.

Absence of growth-promoting activity to *St. faecalis* in nereistoxin might be the natural thing to be expected, since it has been demonstrated that the microbe has a very strict requirement for α -lipoic acid and does not respond even to its closely allied homologues⁶⁾. Nevertheless, the close similarity of nereistoxin to α -lipoic acid in chemical structure may offer some physiological problems to be investigated.

The activity of nereistoxin to insects was inferior to that of some commercial insecticides contrary to expectation, but a strong anesthetizing activity and an immediate effect were noticeable. Besides to exploit such activities of nereistoxin itself, it may be worthy to survey the insecticidal activity of derivatives or allied compounds of nereistoxin.

Summary

The biological assay method of nereistoxin using killifish is described. The minimum lethal dose to killifish was about 0.3 ppm. The concentration of the toxin in the live worms was 60~105 mg%. The LD₅₀ to mice was approximately the same as reported by NITTA and was 33.6 mg/kg as hydrogen oxalate. Neither a growth-promoting action to *Streptococcus faecalis* nor an antagonizing one to α -lipoic acid was observed. Nereistoxin was not so effective as commercial insecticides, but revealed a remarkable anesthetizing activity and immediate effect on some noxious insects.

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