Insensitive Cholinesterase in the Nakagawara Strain of the Green Rice Leafhopper, *Nephotettix cincticeps* UHLER (Hemiptera: Cicadellidae), as a Cause of Resistance to Carbamate Insecticides

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The Nakagawara strain of the green rice leafhopper is resistant to both carbamate and organophosphorus insecticides. Activity and sensitivity of cholinesterase, and toxicity of 10 substituted phenyl N-methylcarbamates for the resistant and susceptible strains were investigated and compared. Inter-strain difference in cholinesterase activity was found to be not significant, but the cholinesterase in the resistant strain was relatively insensitive to all the inhibitors tested as compared with the susceptible strain. Inter-strain ratio of $I_{50}$ among inhibitors ranged from 3 to 120. Relationships of anticholinesterase activity and toxicity of carbamate insecticides tested for the two strains showed good correlations. Resistance spectrum showing especially high resistance to bulky alkyl or alkoxy phenyl carbamates were sufficiently well explained by the sensitivity of cholinesterase. Some considerations were given to the relationships between the structure of carbamates and the anticholinesterase activity in relation to resistance.

INTRODUCTION

The resistance of the green rice leafhopper, *Nephotettix cincticeps* UHLER, to organophosphorus insecticides first appeared in Shikoku Island, in 1961, and since then the organophosphorus resistant leafhoppers have gradually spread to many parts of the country. Recently this insect has also been developing resistance to some carbamate insecticides in a few places. IWATA and HAMA (1971) tested the susceptibility to various insecticides of three colonies of the green rice leafhopper collected in the fields where some carbamate insecticides were not so effective against this insect as before and found that a colony collected at Nakagawara in Ehime Prefecture showed high resistance to organophosphorus and carbamate insecticides. They supposed that the resistance to carbamate insecticides by this colony was not caused by similar detoxification mechanism as in the resistant strain of the house fly, because the synergistic effect of piperonyl butoxide to carbamate insecticides was similar to that between the resistant Nakagawara and susceptible strains.

Decrease of sensitivity of cholinesterase (ChE) to organophosphorus or carbamate insecticides was found as a major cause of resistance in spider mite (SMISSAERT, 1964; Voss and Matsumura, 1964) and cattle tick (Lee and Batham, 1966; Wharton

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1 A part of this work was presented at the Annual Meeting of Jap. Soc. Appl. Ent. Zool., Tokyo, April 8, 1971.
and Roulston, 1970), but such mechanisms of resistance have not been reported in insects. We found insensitive ChE in the Nakagawara strain of the green rice leafhopper and reported preliminarily that the insensitivity of the ChE was the major cause of resistance to carbamate insecticides. In this paper we describe in more detail the insensitive ChE of the Nakagawara strain in relation to resistance to carbamate insecticides.

MATERIALS AND METHODS

Insect: The resistant Nakagawara strain of the green rice leafhopper, Nephrotettix cineticeps Uhler, was collected in fields at Nakagawara in Ehime Prefecture, during the spring of 1970, and shall be referred to as the N strain hereinafter. The susceptible strain (S) used was collected from fields in Miyagi Prefecture in 1969. Additional two colonies collected from fields in Ōzu and Uwajima of Ehime Prefecture, in the spring of 1971, were also used in an experiment for comparing ChE activities. The insects were reared on rice seedlings at 27±1.5°C and 16 hr illumination per day without insecticidal pressure throughout successive generations. Adults of 3 to 8 days after emergence were used for each experiment.

Insecticide: Although all of the following carbamate insecticides were used for toxicity tests, 7 of them except Hopcide, Macbal and BASSA were used as inhibitors of ChE. Purity of the insecticides except Hopcide (93%), Macbal (96.2%), and BASSA (97%) was more than 98%.

- Hopcide® (2-chlorophenyl N-methylcarbamate)
- Tsumacide® (3-methylphenyl N-methylcarbamate)
- Meobal® (3,4-dimethylphenyl N-methylcarbamate)
- Macbal® (3,5-dimethylphenyl N-methylcarbamate)
- carbonolate (2-chloro-4,5-dimethylphenyl N-methylcarbamate)
- Mipcin® (2-isopropylphenyl N-methylcarbamate)
- BASSA® (2-octylphenyl N-methylcarbamate)
- propoxur (2-isopropoxyphenyl N-methylcarbamate)
- Hydrol® (4-diallylamino-3,5-dimethylphenyl N-methylcarbamate)
- carbaryl (1-naphthyl N-methylcarbamate)

Determination of ChE activity: Ten adults were homogenized in a glass homogenizer with 1 ml of 1/15 M phosphate buffer (pH 7.2). The homogenate was filtered through a nylon cloth and the filtrate was used as the source of ChE. For the determination of ChE activity, 1 ml homogenate was incubated with 1 ml of 0.004 M acetylcholine bromide in a phosphate buffer for 40 min at 37°C. The residual acetylcholine was determined by the method of Hestin (1949).

Inhibition tests of ChE: Pre-inhibition technique was adopted for all tests except otherwise indicated. The homogenate was incubated for 30 min at 37°C with an inhibitor applied on the inside wall of a test tube, as residual film, prior to adding the homogenate. The substrate was then added and the mixture was incubated for an additional 40 min to determine the residual ChE activity. Simultaneous inhibition of ChE was conducted by incubating a mixture of both the homogenate and the substrate for 40 min at 37°C in a test tube containing an inhibitor of residual film.

Toxicity test: A half microliter of acetone solution of insecticides was applied topically on the dorsal surface of the thorax and abdomen of female adults by means of a microapplicator. The treated insects were kept on the rice seedlings at 27±1.5°C, and their mortality was recorded 24 hr after application.

RESULTS AND DISCUSSION

Cholinesterase activity in homogenates

Activities of ChE in homogenates from various sources are shown in Table 1. In comparison to the activity of ChE per individual in whole body homogenates no significant difference appeared between S and N strains in both female and male, while the activity per body weight of N strain seemed a little higher than the S strain in both sexes. However, it has been observed that the body weight of this insect is affected by rearing conditions, especially by nutrition and population density. Therefore a little difference in ChE activities per body weight between both strains is not so significant. Activity of ChE was apparently higher in males than in females in both strains. Head homogenates showed about 10 times higher activity than the decapitated body in both strains. High ChE activity in the head of this insect was also suggested by KANEHISA (1961). Activities of ChE in Özu and Uwajima colonies of which the susceptibility to carbamate insecticides are intermediate between N and S strains were compared. No marked difference in ChE activity was found among these

Table 1. Activity of ChE in Homogenates of Several Strains of the Green Rice Leafhopper

<table>
<thead>
<tr>
<th>Strain or colony</th>
<th>Homogenate source</th>
<th>Weight mg/adult</th>
<th>μM/10 adults/40 min</th>
<th>μM/g of body weight/40 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>Whole body</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>4.35±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.12±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.7±1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>2.60±0.01</td>
<td>2.92±0.02</td>
<td>112±1</td>
</tr>
<tr>
<td></td>
<td>Head</td>
<td>0.38</td>
<td>1.08</td>
<td>284</td>
</tr>
<tr>
<td></td>
<td>Decapitated body</td>
<td>4.17</td>
<td>1.51</td>
<td>36.2</td>
</tr>
<tr>
<td>N</td>
<td>Whole body</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>3.97±0.12</td>
<td>3.00±0.04</td>
<td>75.5±0.9</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>2.30±0.0</td>
<td>2.90±0.04</td>
<td>127±3</td>
</tr>
<tr>
<td></td>
<td>Head</td>
<td>0.32</td>
<td>1.28</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>Decapitated body</td>
<td>0.40</td>
<td>1.23</td>
<td>308</td>
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<tr>
<td></td>
<td></td>
<td>3.85</td>
<td>1.37</td>
<td>35.6</td>
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<td></td>
<td></td>
<td>3.80</td>
<td>1.37</td>
<td>36.1</td>
</tr>
<tr>
<td>Özu</td>
<td>Whole body</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>4.68±0.05</td>
<td>3.25±0.03</td>
<td>69.6±0.6</td>
</tr>
<tr>
<td>Uwajima</td>
<td>Whole body</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>4.67±0.01</td>
<td>3.12±0.03</td>
<td>66.8±0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average value±standard error on 5 replicates.
two colonies and N strain. This is in contrast to the case of the spider mite and the cattle tick in which ChE of the resistant strains showed much lower activity than that of the susceptible strains (SMISSAERT, 1964; Voss and MATSUMURA, 1964; LEE and BATHAM, 1966; SCHUETZER et al., 1968; ROULSTON et al., 1968).

Inhibition of cholinesterase by carbamate insecticides

Anticholinesterase activity and toxicity of carbamate insecticides for female adults of S and N strains are shown in Table 2. It is evident that ChE of the N strain is relatively insensitive to all the inhibitors tested as compared with the S strain. Interstrain ratio of \( I_{50} \) ranged from 5 to 120. \( I_{50} \) values in N strain showed a larger variation among inhibitors, i.e., \( 4 \times 10^{-5} \) to \( 1.5 \times 10^{-3} \) M as compared with \( 1.1 \times 10^{-6} \) to \( 2.7 \times 10^{-5} \) M in S strain. These \( I_{50} \) values were obtained on crude homogenates as the source of ChE. Therefore it may be considered that some factor(s) in the homogenate affects the reactions between ChE and inhibitors.

MENGLE and CASIDA (1960) found that, when whole body homogenates of the house fly were incubated with paraoxon or malaoxon, the rate of inhibition of ChE in the homogenates was more rapid in a susceptible strain than in the other two organophosphorus resistant strains. This was not due to any difference in ChE, because when homogenates of the head were examined, ChE sensitivity was similar between susceptible and resistant strains. They suggested that an unknown factor which protects ChE from inhibitor exists in the thorax and/or abdomen of the resistant house fly. In order to examine the possibility of such an unknown factor in whole body homogenates, sensitivities of ChE of the head and the decapitated body homogenates to propoxur were examined on both strains (Fig. 1). Sensitivities of ChE in the two homogenates to propoxur are almost equal in each strain. Therefore, the possible factor(s) suggested in the house fly is negligible in the whole body homogenate of the green rice leafhopper.

The pre-inhibition technique does not necessarily reflect the conditions in vivo, because in the insect body inhibitors interact with ChE in the presence of ACh as a natural substrate. Therefore simultaneous inhibition experiments were conducted.
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Fig. 1. Inhibition of ChE in head (▲, ●) and decapitated body (△, ○) homogenates of S and N strains by propoxur.

Fig. 2. Inhibition of ChE in whole body homogenates of female (△, ○) and male (▲, ●) adults of S and N strains by propoxur.

with propoxur and carbaryl as inhibitors, though the concentration of ACh tested may not be the natural concentration *in vivo*. The results indicate that $I_{50}$ values of propoxur and carbaryl for ChE in whole body homogenates are $3.4 \times 10^{-5}$ and $3.6 \times 10^{-6}$ M in S strain and $>10^{-2}$ and $2.2 \times 10^{-4}$ M in N strain, respectively. The values obtained here are higher than that obtained by pre-inhibition experiments shown in Table 2. Inter-strain ratio of $I_{50}$ for propoxur and carbaryl are $>290$ and 61 in the case of simultaneous inhibition, and 120 and 43 in pre-inhibition, respectively. From the results of both experiments, it seems that ChE of N strain is not inhibited *in vivo* by the dose of inhibitor which almost perfectly inhibits ChE of S strain.
Sensitivities of ChE in homogenates of males to propoxur were compared with that of females in both S and N strains (Fig. 2). ChE of both sexes showed almost equal sensitivity in each strain, which suggests no inter-sex difference in property of insensitive ChE. Remarkable reduction of sensitivity of the ChE in N strain to all the carbamates tested suggests an alternative configuration of the particular ChE in N strain.

Relationship of anticholinesterase activity to toxicity of carbamate insecticides

From the values shown in Table 2, LD$_{50}$ (log scale) of S and N strains was plotted against $-\log I_{50}$ of the respective strains as shown in Fig. 3. Correlation coefficients ($r$) between $-\log I_{50}$ and log LD$_{50}$ of S and N strains are $-0.73$ and $-0.86$, respectively suggesting good correlation in the two strains. Therefore it can be roughly said that a relatively strong cholinesterase inhibitor exhibits high toxicity, and inversely, a weak cholinesterase inhibitor exhibits low toxicity in both S and N strains.

In order to consider the relationship between anticholinesterase activity and resistance, the ratio of the I$_{50}$ of N strain to that of the S strain was plotted against the ratio of the LD$_{50}$ of N strain to that of the S strain as shown in Fig. 4. High correlation between the two ratios ($r=0.96$) sufficiently explains the resistance spectrum of N strain to carbamate insecticides as anticholinesterase agent. Considering a

![Graph](image_url)

Fig. 3. Relationships between anticholinesterase activity ($-\log I_{50}$) and toxicity (LD$_{50}$) of carbamate insecticides for S and N strains. Cl: carbamate, Cr: carbaryl, H: Hydrol, Me: Meobal, Mi: Mipcin, P: propoxur, T: Tsumacide.
number of steps for inhibitor to reach a target in insect body, such a good correlation apparently indicates that other mechanisms of resistance, if any, is relatively minor. The resistance to carbamate insecticides in N strain of the green rice leafhopper is mostly caused by an insensitive ChE and not by detoxification which is known as the major mechanism of the carbamate-resistance in the house fly (GEORGIOU and METCALF, 1961) and a mosquito (SHRIVASTAVA et al., 1970).

**Anticholinesterase activity and structure of carbamate insecticides**

In a review by METCALF and FUKUTO (1965) a detailed consideration was given to the interaction of substituted phenyl N-methylcarbamates with an active site of ChE of the house fly. Although carbamate insecticides tested in this paper are limited in numbers, some consideration is possible on the relationship between the structure of carbamates and the sensitivity of ChE of the green rice leafhopper in relation to the resistance.

METCALF et al. (1962) showed that anticholinesterase activity and insecticidal activity of N-methylcarbamates for the house fly increased progressively with size and branching of their alkyl and alkoxy substituents until a maximum is attained by isopropyl and sec-butyl. Such a difference in anticholinesterase activity with increasing methylation is fully accountable in terms of the VAN DER WAAL's dispersion forces between substituents and anionic site of ChE (WILSON, 1952; METCALF et al., 1962).

Anticholinesterase activity of Tsumacide (m-CH₃), Mipcin (α-iso-C₃H₇) and propoxur (α-iso-OC₃H₇) for the green rice leafhopper was in the following order: Mipcin>propoxur>Tsumacide in S strain, and Mipcin≠Tsumacide>propoxur in N strain (Table 2). Inter-strain ratio of I₅₀ for Tsumacide, Mipcin and propoxur between N and S strains are 17, 49 and 120, respectively. It is interesting that the bulky alkyl or alkoxy substituted carbamates shows stronger anticholinesterase ac-
tivity than methyl substituted carbamate in S strain just as the case of the house fly, but such a tendency of the activity was not established in N strain. Such a result may suggest that a particular change has occurred at the anionic site of the ChE of N strain affecting the property of the binding forces.

Metcalfe et al. (1962) also showed that, when the affinity of inhibitors to ChE (as $-\log I_{50}$) was plotted against the approximate distance from carbamyl oxygen to the center of the isopropyl substituent group, minimum $I_{50}$ value occurred at 5 Å, which was considered as the distance between an esteratic site which is known to bind carbamyl oxygen, and an anionic site of the surface of ChE. In comparison of propoxur and Mipcin (about 4.7 and 3.7 Å in the distance from carbamyl oxygen to center of isopropyl group, respectively) in the present study, Mipcin shows relatively low $I_{50}$ value as compared with propoxur in both strains, which is different from the case of the house fly.

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REFERENCES


Metcalfe et al. (1962).

