The sequence of symptoms and that of the burst of discharges in the insects treated with nereistoxin were quite different from those treated with common insecticides (Fig. 1 and 3).

The increase of the respiratory rate in the neristoxin-poisoned cockroach (Fig. 5) is considered to be due to the nervous excitation, since the compound has no detectable effects on the tissue respiration of the muscle.

It is concluded from the results of the present experiments that nereistoxin acts as a characteristic nervous excitant of which mechanism of action is different from those of common insecticides.

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Reference

1) Sakai, M. : *Jap. J. Appl. Ent. Zool.*, 8, 324 (1964)
2) Hagiwara, H., Numata, M., Konishi, K. and Oka, Y. : *Chem. Pharm. Bull.*, 13, 253 (1965)
3) Sakai, S. : Insect Toxicological Studies on the Joint Action of Insecticides. Tokyo (1960)
4) Yamasaki, T. and Narahashi, T. : *Botyu-Kagaku*, 23, 146 (1958)
5) Nitta, S. : *Tokyo J. Med. Sci.*, 55, 285 (1941)
6) Okaichi, T. and Hashimoto, Y. : *Bull. Jap. Soc. Fish.*, 28, 930 (1962)
7) Sakai, M. : Proceeding of the Annual Meeting of *Jap. Soc. Appl. Ent. Zool.* (March 1965, Tokyo)
8) Sakai, M.: *Botyu-Kagaku*, 31, 61 (1966)

Studies on the Insecticidal Action of Nereistoxin, 4-*N*, *N*-dimethylamino-1, 2-dithiolane. III. Antagonism to Acetylcholine in the Contraction of Rectus Abdominis Muscle of Frog.
Michihiko Sakai (Kyoto Herbal Garden, Research and Development Division, Takeda Chemical Industries Ltd. Ichijōji, Sakyō-ku, Kyoto) Received February 23, 1966. *Botyu-Kagaku*, 31 61. 1966

9. イソメ毒 (Nereistoxin, 4-*N*, *N*-dimethylamino-1, 2-dithiolane) の殺虫作用に関する研究 III. Acetylcholine によるカエル腹直筋収縮に対する拮抗作用

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従来報告されているイソメ毒の脊椎動物における薬理作用から，イソメ毒がコリン作動性の器官の一部，特にアセチルコリン (Ach) 受容体に作用することが予想される.

カエル腹直筋を用いた実験の結果，本薬物はかなりの低濃度で筋の Ach による収縮を抑制するが，筋自体の収縮能は高濃度でも抑制されないことを確めた。イソメ毒のみの施用は筋に収縮反応を起さなかった. 従って，イソメ毒はカエル腹直筋においては Ach 受容体を Ach に対して拮抗的に閉塞するものであって，受容体の deporalizant ではなく，また筋自体の収縮能を抑制するものでもないことが認められる. このことから，おそらく昆虫においてもコリン作動性器官の Ach 受容体がイソメ毒の作用点であることが想像される.

イソメ毒濃度と筋の Ach による収縮を抑制する効果とは一定の関係があったので，これを利用してイソメ毒の topical application で中毒したチャバネゴキブリ体内のイソメ毒を定量したところ，イソメ毒は速やかに表皮を透過して体内に侵入すること，および中毒からの回復は体内のイソメ毒の量の減少に伴なって起ることを認めた.

In the previous report of the author, it was pointed out that the insecticidal action of nereistoxin[1,2] is due to its excitant effect on the nervous discharges[3]. However, the mechanism of its action is still obscure at the present time. To investigate the mechanism in insects, it is consequently necessary to refer to its action in vertebrates. According to Nitta[4], in the experiments with rodents and a frog low dosages of nereistoxin stimulate the pulsation of isolated heart and the contraction of intestine, uterus, bladder and pupil. It has also been demonstrated that the depression of the pulsation rate of the heart evoked by high dosages, and the accelerated secretion of salivary and lachrymal glands by certain dosages are eliminated by the treatment with atropine; and that the relaxation of pupil by a high dosage is avoided by the treatment with eserine.

These facts suggest that at least a part of the effects of nereistoxin takes place in a part of the cholinergic system in vertebrates. This possibility may be presumed from the fact that in a isolated neuro-muscular preparation of frog treated with nereistoxin the contraction of the muscle itself was not affected, but that the contraction evoked by the stimulation of nerve was suppressed[4]. A possible explanation of the mechanism of these phenomena cited above seems to be nereistoxin blocks the nervous conduction or the neuro-muscular junction.

The present study is mainly concerned with the effects of nereistoxin on the acetylcholine receptor in a skeletal muscle of frog.

The knowledge derived from the present study would be applicable to the investigation of the mechanism of insecticidal action of nereistoxin.

Materials and Methods

Chemicals Pure synthesized nereistoxin hydrogen oxalate[5,6] was used throughout the experiments. Acetylcholine bromide (Ach) was dissolved in distilled water at the concentration of 0.45 M and it was diluted with Ringer solution to a certain concentration.

Experiments with rectus abdominis muscle of frog In order to investigate the action of nereistoxin on a Ach receptor, the contraction evoked by Ach in the rectus abdominis muscle of the frog, *Rana nigromaculatus*, was employed. The contraction of the isolated muscle suspended in the frog Ringer solution was recorded as in the method used for Ach assay.

The Ringer solution used in the experiments contained NaCl, 7.0 g; KCl, 0.14 g; $CaCl_2$, 0.12 g; $NaHCO_3$, 0.2 g; in 1 *l* of distilled water.

Nereistoxin was applied to the muscle either simultaneously with or prior to the addition of Ach to investigate the effect of the test compound on the muscle contraction by Ach.

To study the effect on the contraction of the muscle itself, the muscle immersed in the Ringer solution containing nereistoxin was directly stimulated by electrical impulses (10 V, 1 c/s, duration 20 msec).

When eserinized condition was necessary, eserine salicylate was added to the Ringer solution at the concentration of 0.002%.

Extraction of nereistoxin in the poisoned cockroach The adult German cockroach, *Blattella germanica*, (7~10 days old male) was treated with the acetone solution of nereistoxin at the dosage of 25 μg/g. The solution was topically applied on the ventral side of the abdomen. The treated insect population was divided into groups of 10 individuals. Immediately before homogenization, the number of knocked down insects was recorded and the insects were washed with distilled water to remove nereistoxin that probably might have been remaining on the body surface.

All insects in a single group were homogenized in 2 m*l* of the chilled frog Ringer solution containing 1% of trichloro acetic acid (TCA-Ringer) with a glass homogenizor.

After the homogenate was transferred into a centrifugal tube, the inside and the pestle of homogenizor was washed twice with small portion of TCA-Ringer and the washings and the homogenate were pooled in the tube. The supernatant was decanted after centrifugation at 2000 G for 15 min. The residue was resuspended in 2 m*l* of TCA-Ringer, centrifuged and the supernatants were pooled.

TCA-Ringer has been used by some workers to achieve the stable extraction of Ach from arthropod tissues[7,8,9,10]. To elimanate Ach from the test insect which might interfere with nereistoxin assay, the following procedure was successful.

The supernatant was incubated with 1ml of a cholinesterase preparation for 20 min. at 37°C. The enzyme preparation was the supernatant of

70 homogenized heads of house fly, *Musca domestica*, per 1 m*l* of a Ringer solution containing 0.15 M NaCl and 0.04 M $MgCl_2$.

After the elimination of Ach, the final volume was brought to 10 m*l* with the addition of the frog Ringer solution to make up the "test extract" for a pharmacological assay of nereistoxin on the poisoned cockroaches.

Pharmacological assay of nereistoxin in the test extraxt As will be described later, the rate of suppressive effect of nereistoxin on the muscle contraction evoked by Ach was proportional to the nereistoxin concentration (Fig. 3). Therefore the amount of nereistoxin in the extract of the poisoned cockroach could be assayed pharmacologically with the rectus abdominis muslce of the frog.

The contraction evoked by 7×10^{-7}M Ach was recorded at first, then the muscle was thoroughly washed with the Ringer solution until the muscle returned to be relaxed. Then the Ringer solution was replaced with the test solution (extract). After the incubation for 10 min, the muscle was washed with the Ringer solution three times and the contraction by 7×10^{-7}M Ach was recorded.

Because of the presence of sensitizing substances in the extract[7,8,11,12,13,14], 7×10^{-7}M Ach was prepared with the extract of the normal cockroaches. To make up a standard test solution of nereistoxin, the homogenate of 10 cockroaches (ca. 0.5g) to which 12.5μg of the compound was added was samely extracted as in the preparation of the test extract.

The assay was conducted under the eserinized condition.

The rate of suppressive effect of nereistoxin on the muscle contraction was calculated as follows:

$$100-\frac{\text{Contraction height after the treatment with test solution}}{\text{Normal contraction height}}\times100$$

=Suppression Rate (%)

The amount of recovered nereistoxin in the extract was estimated from the graph in which the suppression rates were plotted versus the known concentrations of nereistoxin (Fig. 3b).

Temperature The kymography and the test with German cockroach were conducted in the room at the temperature of 25±1°C.

Results

Effects of nereistoxin on the contraction of frog muscle evoked by Ach The contraction of uneserinized muscle evoked by a high concentration (1.2×10^{-5}M) Ach was not affected by nereistoxin which was applied together with or prior to the application of Ach (Fig. 1 a, b). The record in Fig. 1 b shows that a single application of nereistoxin did not evoke any response of the muscle. When the concentration of Ach was low (7×10^{-7}M), the contraction was considerably affected. Fig. 1 c shows the shortening of the muscle did not reach to the normal level by the application of nereistoxin. The sensitivity of the muscle to Ach was suppressed even after the washing of the muscle with the Ringer solution. Nevertheless, the suppression was eliminated and the sensitivity to Ach was greatly increased by eserinization of the muscle (Fig. 1 c).

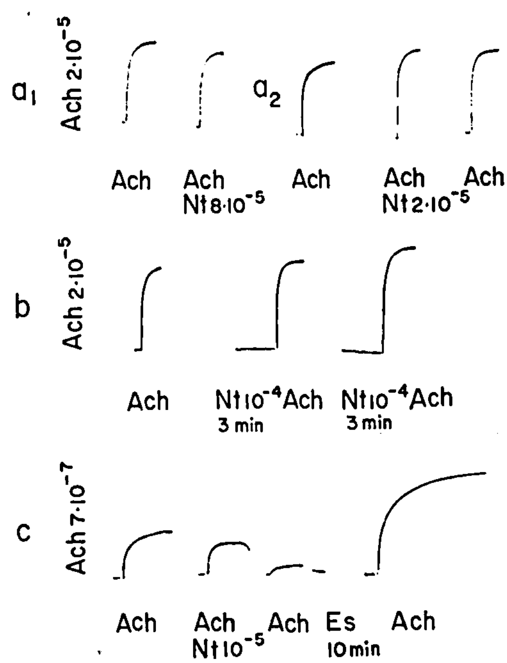

Fig. 1. Effects on nereistoxin on contraction of rectus abdominis muscle of frog evoked by acetylcholine under uneserinized condition. Ach:acetylcholine, Nt:nereistoxin, Es:eserine.

In the muscle sensitized to Ach by eserinization, the contraction was affected by nereistoxin of a lower concentration than in the uneserinized preparation. As shown in Fig. 2a, nereistoxin applied together with Ach depressed the shortening of muscle and then relaxed the muscle from contraction. However, the sensitivity of the muscle to Ach recovered gradually after replacing nereistoxin with the fresh Ringer solution.

As in the uneserinized preparation, the contraction of eserinized muscle was suppressed by the exposure to nereistoxin prior to the application of Ach (Fig. 2). The incubation of the muscle

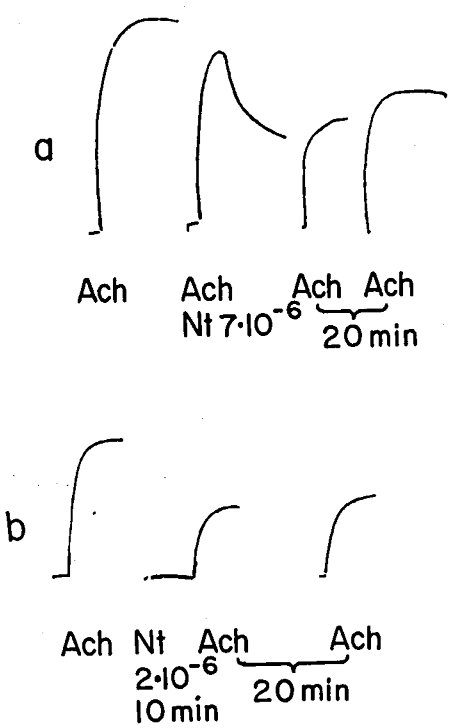

Fig. 2. Effects on nereistoxin on contraction of rectus abdominis muscle of frog evoked by acetylcholine under eserinized condition. Ach:acetylcholine, Nt:nereistoxin. Acetylcholine concentration was 7×10^{-7}M.

in 2×10^{-6}M nereistoxin for 10 min. reduced the shortening evoked by 7×10^{-7}M Ach approximately by half of the normal shortening (Fig. 2b). Also in this experiment, the reduced sensitivity of the muscle to Ach recovered after the immersion in the fresh Ringer.

To evaluate the effect of concentration of nereistoxin on the contraction of the muscle by Ach, the muscle was exposed to various concentrations

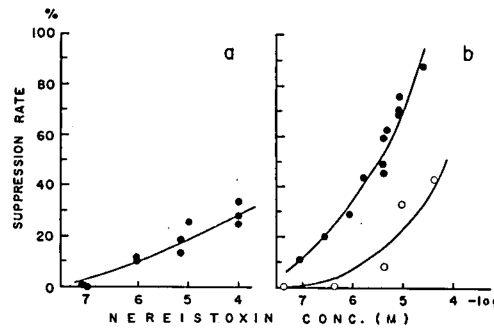

Fig. 3. Suppression effect of nereistoxin on the contraction of rectus abdominis muscle of frog evoked by acetylcholine. a: nereistoxin was applied simultaneously with acetylcholine. b: nereistoxin was applied 5 (open circle) and 10 (solid circle) min. prior to the application of acetylcholine. Acetylcholine was 7×10^{-7}M throughout.

of nereistoxin, and the shortening evoked by 7×10^{-7}M Ach was recorded. The suppression rate plotted against the nereistoxin concentration is shown in Fig. 3. The data show that nereistoxin applied prior to the application of Ach evoked stronger suppression to the contraction than when applied simultaneously with Ach. For instance, 10^{-5}M nereistoxin reduced the shortening only about 20% in the simultaneous application, but it reduced 30% and 70% when applied 5 and 10 min. prior to Ach, respectively. It was revealed in Fig. 3b that in the prior application the immersion of longer period evoked stronger depression to the contraction.

Effect of nereistoxin on the contraction of frog muscle evoked by direct stimulation. The contraction of the muscle due to the direct electrical stimulation was not significantly affected by 10^{-5} M nereistoxin. However, in the same preparation, the contraction evoked by 3.5×10^{-7} M Ach was considerably reduced still after the muscle was washed with the fresh Ringer solution (Fig. 4).

Amount of nereistoxin in the poisoned cockroach The consistent relationship between the concentration of nereistoxin and the suppression rate of the frog muscle contraction (Fig. 3) was believed to be applicable to the assay of small amounts of nereistoxin. A longer period of the exposure of the muscle to nereistoxin prior to

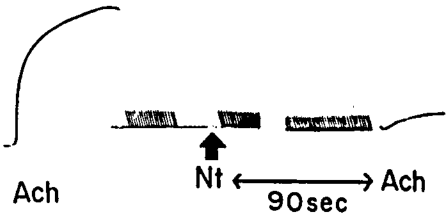

Fig. 4. Effects of nereistoxin on contraction of rectus abdominis muscle of ofg evoked by direct electrical stimulations. Ach:acetylcholine (3.5×10^{-7}M), Nt:nereistoxin (10^{-5}M). Under eserinized condition.

the application of Ach of a low concentration resulted in the higher sensitivity to nereistoxin. Therefore, in this experiment the muscle was immersed for 10 min. in the test solution (extract) prior to the application of 7×10^{-7}M Ach. From the observed suppression rate, the amounts of nereistoxin in the extracts were estimated from the graph shown in Fig. 3b.

The aim of this experiment was undertaken to know whether the revival from the poisoning[2] was due to the decrease of nereistoxin in the insect body.

The results are shown in Table 1. Immediately after the treatment, nereistoxin was scarcely detected. The maximum amount which was equal to the total amount of topically applied dosage was found in the completely knocked down insects after 25∼30 min. from the treatment. However, it was shown that after 55∼60 min., the amount of nereistoxin decreased to 12μg/g, although the knock-down rate was 100%. Along with the revival from the poisoning, the amount of nereistoxin decreased rapidly.

Discussion

In the experinents with the frog rectus abdominis muscle, the following results were obtained; 1) nereistoxin of a concentration which positively suppressed the muscle contraction evoked by a low concentration of Ach did not affect the contraction by a high concentration of Ach (Fig. 1); 2) the contraction which was suppressed by nereistoxin under the uneserinized condition recovered considerably after the increase of actual concentration of Ach acting on the receptor site by eserinization; *viz.* the action of nereistoxin was antagonized by eserine (Fig. 1); and 3) the contraction evoked by the direct stimulation of the muscle was not significantly affected by nereistoxin of which concentration was sufficient to suppress the contraction by Ach (Fig. 4).

These results demonstrate that the action of nereistoxin suppressing the muscle contraction is the blockade of Ach receptor and that it does not affect the contractile power of the muscle fiber itself. The block by nereistoxin is presumed not to be due to deporalization, since the blocking action is antagonized by eserine and the single

Table 1. Amount of nereistoxin in topically treated* German cockroaches assayed pharmacologically by frog muscle contraction.

Sample	Knock-down rate (%)	Suppression rate (%)	Nereisotxin found
Extract of untreated insects	0	2	0
Nereistoxin 6×10^{-6}M in the extract of normal insects	—	63	7×10^{-6}M
Nereistoxin 12.5μg added to the homogenate of normal insects (0.5g) and extracted.	—	60	25μg/g**
Extract of treated insects			
Immediately after the treatment	0	10	0.4 μg/g**
25∼30 min	100	60	25 μg/g**
55∼60 min	100	43	12 μg/g**
3 hr	60	11	0.4 μg/g**
16 hr	10	9	0.3 μg/g**

* 25μg/g of nereistoxin hydrogen oxalate
** Amount of nereistoxin calculated as the weight per 1 g of insect body.

application of nereistoxin only does not stimulate the frog muscle to contract as a deporalizant, for instance, decamethonium does[15].

However, the action of nereistoxin to some visceral muscles of vertebrates[4] and to the nervous activity of insects[3] is considered to be stimulative, not being depressive as on the frog muscle.

The different actions of drugs rendering a muscl inexcitable, *viz.* block by deporalization, block by competition and dual block are explained on the basis of the rate of drug-receptor combination[16]. Hence, a different type of blocking action is not only specific to the nature of a drug, but also it is observed in the muscle of different species and sometimes even in the different muscles of a same species[17]. Decamethonium and hexamethonium are the drugs that cause block by deporalization in some muscles, but dual block in others[18,19,20,21,22]. Therefore, the existence of these opposite actions in a single compound, nereistoxin, would not be contradictory.

If nereistoxin blocks Ach receptor only, it is interesting and worthwhile to demonstrate what the site of action is in insects. Vertebrate skeletal muscles are cholinergic, but many evidences have been presented to show insect peripheral muscles are not cholinergic[12,23,24,25,26]. Accordingly, a conceivable mechanism of action of nereistoxin in insects is the block of Ach receptors in the central nervous system which is regarded to be cholinergic[23,27].

The pharmacological assay using the frog rectus abdominis muscle was applicable for detecting the approximate amount of nereistoxin. This assay was sensitive enough to assay nereistoxin of concentration in the order of 10^{-7}M (ca. 0.02 μg/m*l*). The sensitivity was much higher than that of the bioassay with azuki bean weevil, *Callosobruchus chinensis*, to which the LD-50 of this compound was approximately 7μg/Petri dish :that is, 1 m*l* of 3×10^{-5}M solution per a dish is required to kill 50% of the weevils[2].

If it is assumed that the cockroach did not convert nereistoxin to substances which still suppress the muscle contraction, the results shown in Table 1 explain that intoxication and the revival from the poisoning were in accordance with the amount of nereistoxin existed internally. These results show that the speed of nereistoxin to penetrate the insect cuticle is fairly rapid and that the revival from the poisoning is due to detoxication or excretion of the toxic substance.

Summary

The effect of nereistoxin on the contraction of isolated rectus abdominis muscle of frog was investigated.

The application of nereistoxin simultaneously with and preliminarily to the application of Ach suppressed the muscle contraction that might be evoked by Ach. A single application of nereistoxin did not stimulate the muscle to contract. The contraction evoked by direct stimulation of the muscle was not affected by nereistoxin.

From these results, it was concluded that the action of nereistoxin suppressing the muscle contraction is due to the blockade of the Ach-receptors. Therefore, a conceivable mechanism of insecticidal action of this compound is the block of Ach-receptors.

The proportional relationship between the concentration of nereistoxin and the strength of the suppressive effect to the muscle contraction was applicable to assay small amounts of nereistoxin. This procedure was applied for detecting the amount of this compound in the body of German cockroach. The result showed that the compound penetrated through the cuticle rapidly, and the revival from the poisoning was in accordance with the decrease of nereistoxin in the body.

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References

1) Okaichi, T. and Hashimoto, Y. :*Bull. Jap. Soc. Sci. Fish.* 28, 930 (1962)
2) Sakai, M. :*Jap. Jour. Appl. Ent. Zool.* 8, 324 (1964)
3) Sakai, M. :*Botyu-Kagaku.* 31, 53 (1966)

4) Nitta, S. : *Tokyo Jour. Med. Sci.* 55, 285 (1941)
5) Hagiwara, H., Numata, M., Konishi, K. and Oka, Y. : The 6th symposium on the Chemistry and Natural Products. Sapporo, July. 1962; Abstracts. p. 88 (1962)
6) Hagiwara, H., Numata, M., Konishi, K. and Oka, Y. : *Chem. Pharm. Bull.* 13, 253 (1965)
7) Lewis, S. E. and Smallman, B. N. : *J. Physiol.* 134, 241 (1956)
8) Fowler, K. S. and Lewis, S. E. : *ibid.* 142, 165 (1958)
9) Mehrotra, K. S. : *J. ins. Physiol.* 6, 180 (1961)
10) Colhoun, E. H. and Gielberg, J. : *Ann. Ent. Soc. Amer.* 54, 140 (1961)
11) Feldberg, W. : *J. Physiol.* 130, 367 (1945)
12) Colhoun, E. H. : *J. ins. Physiol.* 2, 108 (1958)
13) Colhoun, E. H. : *ibid.* 2, 117 (1958)
14) Mehrotra, K. N. : *ibid.* 5, 129 (1961)
15) Paton, W. D. M. and Zaimis, E. J. : *Brit. J. Pharm.* 4, 381 (1949)
16) Paton, W. D. M. : *Proc. Roy. Soc. B. 154*, 21 (1961)
17) Jewell, P. A. and Zaimis, E. J. : *J. Physiol.* 124, 417 (1954)
18) Zaimis, E. J. : *ibid.* 112, 176 (1951)
19) Zaimis, E. J. : *ibid.* 122, 238 (1953)
20) Burns, B. D. and Paton, W. D. M. : *ibid.* 115, 34 (1951)
21) Paton, W. D. M. and Zaimis, E. J. : *Pharmacol. Rev.* 4, 219 (1952)
22) Bovet, D. and Bovet-Nitti, F. : *Sci. Med. Ital.* 3, 484 (1955)
23) Colhoun, E. H. : Advances in Insect Physiology, Vol. 1, Academic Press Inc., London and New York. 1 (1963)
24) Wigglesworth, V. B. : *Quart. J. Micr. Sci.* 99, 441 (1958)
25) Colhoun, E. H. : *Can. J. Biochem, Physiol.* 37, 1127 (1959)
26) O'Conner, A. K., O'Brien, R. D. and Salpeter, M. M. : *J. ins. Physiol.* 11, 1351 (1965)
27) Yamasaki, T. and Narahashi, T. : *ibid.* 4, 7 (1960)

Determination of Malathion Residues on and in Rice Plant and Chinese Cabbage. Masao Yamauchi. (Agricultural Chemicals Inspection Station, Ministry of Agriculture and Forestry, Kodairashi, Tokyo) Received January 12, 1966. *Botyu-Kagaku* 31, 67. 1966. (with English Summary 77)

10. 稲および白菜におけるマラソン残留量の定量

山内正雄（農林省農薬検査所，東京都 小平市）

M. V. Norris らの植物体におけるマラソン残留量の定量法に，アルカリ分解試薬として，前報において用いた水酸化カリウムエタノール溶液を適用し，操作簡便な定量法を作り，稲および白菜におけるマラソンの残留量を定量した．ただし抽出およびクリーナップの方法は，ほぼ H. W. Conroy の方法に従ったが，抽出溶媒にはメタノールを用い，抽出液中に定量妨害物質が含まれている場合には，A. N. Bates らの方法にならい塩酸処理アルミナによるカラムクロマトグラフィーを適用した．白菜については，圃場および孵卵器に保温した場合における全残留量を定量し，温度によるマラソンの消失速度の変化を調べ，稲については，全残留量と表面残留量を定量し，その差より吸収浸透量を求め，Gunther-Blinn の解析法および同様な理論に基づく残留量の対数の対数と経過日数とより求めた回帰方程式により，稲および白菜におけるマラソンの消失機構を論議した．

1. 緒　　言

農作物の害虫防除に殺虫剤を使用する場合，直接害虫に散布する場合と，浸透性殺虫剤の如く植物体に吸収浸透せしめて防除する場合とあるが，前者の場合にあっても作物体に付着した農薬と害虫とを接触せしめて殺虫効果をあげ，また付着，吸収した残留量により長期間にわたって防除効果を期待するものである．このように，浸透性，非浸透性を問わず，いづれの場合においても作物体を介して防除を行なうものであるから，作物体に付着，吸収，浸透した農薬の動向をできるだけ明確に究明することは，より効果的，経済的防除法ならびに残留毒性に対する正しい知識を得る点から極めて重要なことである．このような観点から殺虫剤の植物体における残留量の定量法，消失機構などについての研究を試み，今回はマラソンの残留量の定量