Lethal Activity of a Trehalase Inhibitor, Validoxylamine A, and its Influence on the Blood Sugar Level in *Bombyx mori* (Lepidoptera: Bombycidae)¹

Yoshiaki KONO, Satoshi TAKEDA,² Yukihiro KAMEDA,³ Masakazu TAKAHASHI,
Kazuhiro MATSUSHITA,⁴ Masami NISHINA⁴ and Eitaro HORI⁴

Department of Medical Entomology, National Institute of Health, Toyama-1, Shinjuku-ku, Tokyo 162, Japan

²*Department of Insect Physiology and Behavior, National Institute of Sericultural and Entomological
Science, Owashi 1–2, Tsukuba, Ibaraki 305, Japan*

³*Hokuriku University, School of Pharmacy, Kanagawa-machi, Kanazawa 920–11, Japan*

⁴*Department of Medical Zoology, Saitama Medical College, Moroyama, Iruma-gun, Saitama 305–04, Japan*

(Received 2 February, 1993; Accepted 15 April, 1993)

A trehalase inhibitor, validoxylamine A (VAA), gave a lethal effect in *Bombyx mori* larvae. Overall activities were reduced soon after injection of VAA at the 2nd day of the last instar larva. However, the lethal effect was found at the later stages. NMR analysis showed that trehalose was the main blood sugar and its level was strongly enhanced by the VAA injection. It was supposed that utilization of the energy source was prevented due to the trehalase inhibition, and consequently the overall activities were reduced. But, how the trehalase inhibition caused the lethal effect in the later stages remained unclear.

Key words: trehalase inhibitor, validoxylamine A, lethal activity, *Bombyx mori*

INTRODUCTION

Trehalose is a common blood sugar and an energy source in insects, but has not been found in mammals. Trehalase (E.C.3.2.1.28) which hydrolyses the sugar is distributed in most insect tissues and organs for utilizing the sugar. Inhibition of the enzyme is expected to cause disturbance of energy metabolism and critical effects on the insects life. Validoxylamine A (VAA), an amino sugar, has been reported to be a potent and specific inhibitor of trehalases in various organisms (KAMEDA et al., 1987; SALLEH and HONEK, 1990). VAA had an insecticidal activity in *Spodoptera litura* larva (ASANO et al., 1990). In the pupa of silkworm, *Bombyx mori*, VAA induces non-diapause egg production (TAKEDA et al., 1988) and prevents glutinous material production (TAKEDA et al., 1990; YAO et al., 1991) as a result of inhibition of trehalase activity. In the present paper, lethal activity of VAA and NMR analysis of its effect on haemolymph components in the silkworm are reported.

¹ The study was partly supported by a Grant-in-Aid for General Scientific Research (No. 03454057) from the Ministry of Education, Science and Culture.

MATERIALS AND METHODS

Animals. The hybrid race of the silkworm N137 $\times$ C137, was used for the experiments. The eggs were kept under a 18L–6D light regime at 25°C. The larvae were reared on an artificial diet containing about 22% mulberry leaf powder (HORIE et al., 1973) under a 6L–18D light regime at 25°C.

Chemicals. VAA was purified according to the previous paper (KAMEDA et al., 1986). $^{13}\text{C}_1$ -glucose (99 atom %) and D_2O were purchased from Cambridge Isotope Laboratories (England) and CEN Saclay (France), respectively. Other chemicals used were of analytical grade.

Observation of VAA effects on the larvae. Ten microliter ringer solution (0.75% NaCl) of appropriate VAA concentrations was injected into the body cavity of 2nd day 5th instar larva with a microsyringe (10 females and 10 males for each dose). Symptoms of the treated larvae were observed after the injection. Feaces excreted (number or dry weight) and numbers of dead larvae were checked every day, and numbers of cocoons and healthy pupae were also counted.

NMR analysis of the haemolymph. The haemolymph blooded from the cut 1st abdominal leg of several larvae was collected, centrifuged with a small amount of phenylthiourea at 2000 *g* for 10 min at 4°C, and filtrated with a molecule sieve, Molcut™, excluding molecules more than 10,000 molecular weight.

The eluted haemolymph was stored at –20°C until the NMR analysis. The haemolymph samples (450 μl) with 50 μl of 10 mm 3-trimethylsilyl 2,2,3,3-tetradecutropionate (TSP) in D_2O were subjected to ^1H -NMR (400 MHz), ^{13}C -NMR (100 MHz) and ^{31}P -NMR (109 MHz) analysis with a JEOL JNM-EX400 FT-NMR spectrometer.

Assignment of organic chemicals; amino acids, organic acids, choline, phosphoryl choline, trehalose, glycerol, solbitol phosphate, α -glycerophosphate and trimethylamineoxide, was carried out by adding each chemical (2 mg) to the haemolymph before the analysis.

In order to observe the effect of VAA, 10 $\mu\text{g}/10 \mu\text{l}$ /larva VAA solution was injected to the 2nd day last instar larva with or without additional injection of 400 $\mu\text{g}/\text{larva}$ $^{13}\text{C}_1$ -glucose.

For determining the haemolymph trehalose concentration, a calibration curve of trehalose concentration (*Y*) against the ratio (*X*) of signal strength of trehalose (methyl signal at C-6) at 3.46 ppm to signal strength of TSP at 0 ppm in ^1H -NMR chart was drawn (Fig. 1). The concentrations could be calculated according to a linear relationship between *Y* and *X*: $Y = 3.83X$ (mg/ml).

RESULTS

Effect of VAA on the larva

When dosages of 1–100 $\mu\text{g}/\text{larva}$ of VAA were injected to the 2nd day last instar larvae, relaxation of the segmental muscle of the larva was observed several hours after the injection, and the overall activity of the larva decreased. These decreased activities lasted through larval life at doses higher than 5 $\mu\text{g}/\text{larva}$. The amount of feaces excreted (Table 1) was reduced from the day of the injection, and the reduction was dose dependent and was accompanied by a decrease of food up-take in the treated

Fig. 1. Calibration line of trehalose concentration. The concentration (Y) can be calculated from the ratio (X) of trehalose peak height to TSP peak height in NMR chart, according to a formula $Y=3.83X$.

Table 1. Effect of validoxylamine A on the amount of faeces in the 5th instar silkworm

Dose injected (μg)	No. of faeces ^a (/larva)	Dry weight of faeces (mg/larva)				
		2	3	4	5	6 ^b
	1	days after injection				
None	25.0	184	303	352	379	—
1	19.2	120	161	184	257	—
5	19.3	124	135	164	243	—
10	18.3	116	123	125	146	—
50	17.4	108	103	91	141	—
100	11.0	71	71	91	—	—

^a Counted 16 h after injection.

^b Not counted.

larvae. The lethal effect of VAA appeared at the later stages of the instar, especially wondering and spinning stages or later (Table 2). With 1 $\mu\text{g/larva}$ injection of VAA, 20% of the treated larvae were dead in larval stage, 35% of the larvae failed to spin the cocoon and only 20% of the larvae succeeded in normal pupation. The lethal effect increased as the dosage of injection became higher, and consequently, no normal pupa were obtained at doses higher than 10 $\mu\text{g/larva}$ and no cocoons at doses higher than 50 $\mu\text{g/larva}$.

Characteristic symptoms observed in the later stages of the treated larvae were the extrusion of the midgut and hindgut from the anus and the failure of larval-pupal molt.

Haemolymph components and effect of starvation on them

Figure 2a shows ^1H -NMR spectrum of the haemolymph of the normal 2nd day

Table 2. Effect of validoxylamine A on the development of the 5th instar silkworm

Dose injected (μ g)	No. treated	No. of larvae dead						No. of cocoons obtained	No. of healthy pupae
		1	2	3	4	5	6		
None	20	0	0	0	0	0	0 (8) ^a	20	19
1	20	0	0	0	0	4	0 (1)	9 (4) ^b	4
5	20	0	0	0	1	7	2 (2)	6 (3)	1
10	20	0	0	1	0	8	4 (1)	2 (0)	0
50	20	0	0	0	1	11	4	0	0
100	20	0	0	2	4	12	1	0	0

Validoxylamine A dissolved in 0.75% NaCl solution was injected into the body cavity of 1 day 5th instar larvae (10 females and 10 males).

^a Numerals in the parenthesis indicate numbers of mounting larvae.

^b Numerals in the parenthesis indicate numbers of coarse cocoons.

Table 3. Trehalose concentration in the haemolymph of the 5th instar silkworm

Age of sampling	Treatment	Conc. (mg/ml)
2nd day	None	3.6
3rd day	Starvation for 1 day	3.2
3rd day	Treatment of VAA for 1 day	7.4
5th day	None	4.7
5th day	Starvation for 3 days	0.8
5th day	Treatment with VAA for 3 days	5.7

last instar larvae. Most of the signals were assigned by adding the standard chemicals: leucine (0.98d, 1.75d), valine (1.04d), lactate (1.33d), alanine (1.48d), lysine (1.90s), arginine (1.92t), acetate (1.93s), glutamine (2.15q), pyruvate (2.38s), succinate (2.42s), citrate (2.5–2.7q), choline (3.20s), phosphoryl choline (3.23s), trimethylamineoxide (3.27s), trehalose (3.4t, 3.6t, 3.8q), and glycine (3.56s), where numerals and letters in the parenthesis indicate position of the signals (ppm) and the number of peaks; singlet(s), doublet(d), triplet(t) and quartet(q), respectively. Concentrations of most components in the haemolymph of untreated larvae were maintained at constant levels until the 5th day of the last instar.

When the larvae were starved for 1 day from the 2nd day, concentrations of amino acids, lactate, succinate decreased markedly, whereas concentration of citrate increased (Fig. 2b). Starvation for 3 days from the 2nd day brought on more prominent changes in the concentrations of components (Fig. 2c). Concentrations of amino acid, succinate, citrate and trehalose decreased, while those of lactate, acetate, pyruvate and trimethylamine increased.

As for the concentration of trehalose (Table 3), the value of the normal 2nd day larvae (3.6 mg/ml) declined to 3.2 mg/ml and 0.8 mg/ml by the starvation of 1 day and 3 days, respectively.

Effect of VAA on the haemolymph components

When the larvae were injected with 10 μ g/larva of VAA and kept without food

Fig. 2. ^1H -NMR spectrum of the haemolymph of (a), 2nd day last instar larva without treatment. (b), 3rd day last instar larva previously starved for 1 day; (c), 5th day last instar larva previously starved for 3 days; and (d), 3rd day last instar larvae which were injected with $10\ \mu\text{g/larva}$ VAA at the 2nd day and starved after injection. 1: leucine, 2: valine, 3: lactate, 4: alanine, 5: leucine, 6: lysine, 7: arginine, 8: acetate, 9: glutamine, 10: pyruvate, 11: succinate, 12: citrate, 13: phosphoryl choline, 14: trimethylamineoxide, 15: trehalose, 16: glycine.

Fig. 3. ^1H -NMR (a) and ^{13}C -NMR (b) spectra of the haemolymph of 5th day last instar larvae which were injected with $10\ \mu\text{g}/\text{larva}$ VAA at the 2nd day and with $400\ \mu\text{g}/\text{larva}$ ^{13}C -glucose at the 4th day. 15: trehalose C_6 signal, G- $\text{C}_{1,3}$: glycerol C_1 and C_3 signal, T- C_1 -T- C_6 : trehalose C_1 - C_6 signals respectively. (The same abbreviations are used in Fig. 4.)

for 24 h, trehalose concentration increased to $7.4\ \text{mg}/\text{ml}$ (Fig. 2d), 2.3 times higher than that of the control (Fig. 2b, Table 3), but other components were not affected. Figures 3 a and b show the results of ^1H - and ^{13}C -NMR analysis of the haemolymph from the 5th day larvae which had been injected with VAA ($10\ \mu\text{g}/\text{larva}$) on the 2nd day and with $^{13}\text{C}_1$ -glucose ($400\ \mu\text{g}/\text{larva}$) on the 4th day. The ^1H -NMR analysis (Fig. 3 a) shows that the trehalose concentration increased to $5.7\ \text{mg}/\text{ml}$, and other components (amino acids, organic acids) decreased but not so strongly as in the starved larvae (Fig. 2 c). Difference of peak height ((T- C_1)-(T- C_3)) on ^{13}C -NMR chart (Fig. 3 b) indicates the amount of trehalose converted from $^{13}\text{C}_1$ -glucose. It is obvious from the figure that no $^{13}\text{C}_1$ -glucose (signals appear at 94.8 and 98.6 ppm) remained in the haemolymph and a large amount of trehalose was produced. This result also shows glycerol production from $^{13}\text{C}_1$ -glucose (appearance of peak G- $\text{C}_{1,3}$).

Figures 4 a and b show the results of ^{13}C -NMR analysis of the haemolymph of the larvae injected with $400\ \mu\text{g}/\text{larva}$ $^{13}\text{C}_1$ -glucose 2 h before sampling of the haemolymph with and without pretreatment of VAA ($10\ \mu\text{g}/\text{larva}$). It appears that $^{13}\text{C}_1$ -glucose injected was rapidly converted to $^{13}\text{C}_1$ -trehalose and that VAA treatment enhanced the accumulation of the trehalose and suppressed the conversion to glycerol. These results indicate that some glycerol was produced through the pathway of trehalose breakdown.

Figure 5 shows the effect of VAA on the phosphorus components in the haemolymph. Inorganic phosphate, phosphoryl choline, α -glycerophosphate and sorbitol 6-phosphate were detected by the ^{31}P -NMR analysis. Three days after VAA injection, ratio of sorbitol 6-phosphate to phosphorylcholine decreased.

Fig. 4. ^{13}C -NMR spectra of the haemolymph of 2nd day last instar larvae which were injected with, (a) $400\text{ }\mu\text{g/larva}$ ^{13}C -glucose 2 h before the sampling, and (b) $10\text{ }\mu\text{g/larva}$ VAA and $400\text{ }\mu\text{g/larva}$ ^{13}C -glucose 4 h and 2 h before the sampling, respectively.

Fig. 5. ^{31}P -NMR spectra of the haemolymph of the 3rd day (a), the 3rd day with $10\text{ }\mu\text{g/larva}$ VAA injection at 2nd day (b) and 5th day last instar larvae with $10\text{ }\mu\text{g/larva}$ VAA injection at the 2nd day (c). 17: inorganic phosphate, 18: phosphoryl choline, 19: α -glycerophosphate, 20: sorbitol 6-phosphate.

DISCUSSION

VAA, an amino sugar, shows potent inhibitory activity against trehalases in fungi, bacteria, insects and mammals (ASANO et al., 1987; KAMEDA et al., 1987; SHIGEMOTO et al., 1989; SALLEH and HONEK, 1990). Its inhibitory activity is very selective to trehalase because no significant inhibitory activity was detected against other sac-

charide hydrolytic enzymes such as cellulase, pectinase, chitinase, amylase, α -glucosidase and β -glucosidase (ASANO et al., 1987). In insects, *in vitro* I-50 was reported to be 4.8×10^{-8} M against the trehalase of *Spodoptera litura* larva (KAMEDA et al., 1987). From *in vivo* experiments, it has been reported that 1 μ g/pupa VAA injection inhibited 78% of trehalase activity in the colleterial gland arborescent region of the silkworm (YAO et al., 1991) and that 10 μ g/pupa VAA injection induced a total inhibition against ovary trehalase of the silkworm (TAKEDA et al., 1988). Taking these results into account, 10 μ g/larva VAA injection seems to give the strong inhibition against the trehalases in tissues and organs of the silkworm larva. The present result that VAA injection induced the accumulation of trehalose without changes of other components (Fig. 2 b, d) supports the specific and strong inhibition of the trehalase. The results of experiment using $^{13}\text{C}_1$ -glucose, on the other hand, show that the trehalose is the main blood sugar and active synthesis of trehalose occurs in the larva. Body relaxation and reduction of activity by VAA treatment are highly understood to be caused by prevention of an energy source supplied to the muscle. However, the mechanism of trehalase inhibition, the primary effect of VAA, resulting in abnormalities in the later stages is to be left for further studies.

REFERENCES

- ASANO, N., T. YAMAGUCHI, Y. KAMEDA and K. MATSUI (1987) Effect of validamycins on glycohydrolases of *Rhizoctonia solani*. *J. Antibiot.* **40**: 526–532.
- ASANO, N., M. TAKEUCHI, Y. KAMEDA, K. MATSUI and Y. KONO (1990) Trehalase inhibitors, validoxylamine A and related compounds as insecticides. *J. Antibiot.* **43**: 722–726.
- HORIE, Y., T. INOKUCHI, K. WATANABE, A. NAKASONE and K. YANAGAWA (1973) A few experiments on the improvement of the silkworm artificial diet. *Bull. Seric. Exp. Stn.* **96**: 7–20.
- KAMEDA, Y., N. ASANO, T. YAMAGUCHI and K. MATSUI (1987) Validoxylamines as trehalase inhibitors. *J. Antibiot.* **40**: 563–565.
- KAMEDA, Y., N. ASANO, T. YAMAGUCHI, K. MATSUI, S. HORII and H. FUKASE (1986) Validamycin G and Validoxylamine G, new members of the validamycins. *J. Antibiot.* **39**: 1491–1494.
- SALLEH, H. M. and J. F. HONEK (1990) Time-dependent inhibition of porcine kidney trehalase by aminosugars. *FEBS Lett.* **262**: 359–362.
- SHIGEMOTO, R., T. OKUNO and K. MATSUURA (1989) Effect of validamycin A on the activity of trehalase of *Rhizoctonia solani* and several sclerotial fungi. *Ann. Phytopathol. Soc. Jpn.* **55**: 238–241.
- TAKEDA, S., Y. KONO and Y. KAMEDA (1988) Induction of non-diapause eggs in *Bombyx mori* by a trehalase inhibitor. *Ent. exp. appl.* **46**: 291–294.
- TAKEDA, S., Y. KONO and Y. KAMEDA (1990) Non-glutinous egg production by using a trehalase inhibitor, validoxylamine A, in *Bombyx mori*. *J. Seric. Sci. Jpn.* **59**: 360–365.
- YAO, X., H. FUGO and S. TAKEDA (1991) Effect of validoxylamine A on the trehalase activity in the colleterial glands of the silkworm, *Bombyx mori*. *J. Seric. Sci. Jpn.* **60**: 296–301.