Biogenic Amine Levels in the Haemolymph of the Cabbage Armyworm Larvae *(Mamestra brassicae)* Following Injection of Octopaminergic Insecticides

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Changes in haemolymph biogenic amine levels of the cabbage armyworm, *Mamestra brassicae,* last instar larvae following injection of octopaminergic insecticides were investigated by using HPLC-ECD. When octopaminergic insecticides, chlordimeform or 2-(4-chloro-*o*-toluidino)-2-oxazoline (AC-6), are injected, 4-tyrosine (TYR-4) is increased 10 min after injection. Clonidine injection also caused an increase of TYR-4. Based on these results, chemically induced insect stress is discussed.

An increase in haemolymph octopamine levels appears to be a general response to chemically induced stress [1]. Octopamine has been measured by radioenzymic assay in Lepidopteran haemolymph from adults and larvae of *Spodoptera littoralis* [2] and *Manduca sexta* [2, 3], but analysis of this compound by HPLC-ECD has not been extensively surveyed in any species of Lepidopterous insects. Thus very little is known about the levels of the biogenic amine in Lepidoptera, despite their importance as the main target for the formamidine pesticides [see 4; 5–7]. It is known that when Locusta is subjected to insecticide stress, the concentration of octopamine in the haemolymph increases [1].

The present study using HPLC-ECD focuses on changes in haemolymph biogenic amine levels in the cabbage armyworm, *Mamestra brassicae,* following injection of octopaminergic insecticides.

Materials and Methods

Insects

Larvae of the cabbage armyworm, *Mamestra brassicae,* were reared on an artificial diet (Silkmate: Nihonnosan Kogyo Co.) at 25 °C under a long-day photoperiod. Pre-wandering stage larvae (day 4) were used in all experiments.

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Chemicals

Chlordimeform HCl (more than 98% pure) was from Nihon Noyaku Co., Ltd. (Tokyo, Japan). Clonidine HCl was purchased from Sigma Chemical Company. 2-(4-Chloro-*o*-toluidino)-2-oxazoline (AC-6) (purity technical grade) was from American Cyanamid Research Center. The structures of the compounds used in this experiment are shown in Fig. 1.

Preparation of chemicals

Chlordimeform HCl and clonidine HCl were prepared at 1000 ppm in a 0.9% NaCl solution; 2-(4-chloro-*o*-toluidino)-2-oxazoline was also prepared at 1000 ppm in acetone. These doses of each test compound are not toxic to the insect.

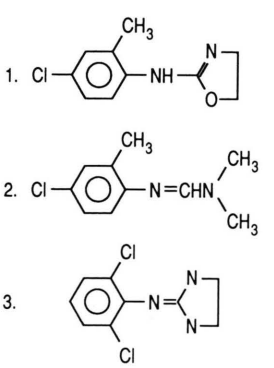

Fig. 1. Chemical structure of octopaminergic insecticides. 1. AC-6, 2. chlordimeform, 3. clonidine.

Injection

A microsyringe was used to inject 2 µl of each compound into the abdominal haemocoel through the first abdominal proleg. Controls were injected with 2 µl of 0.9% NaCl solution or acetone in the same manner. Haemorrhage from an injection hole injured with injector needle was soon ceased, because of melanization occurring in the wounded epidermis to seal off the wound. Since physical stress due to injection is known to elevate haemolymph octopamine levels in insects [8, 1], a sample of blood was also collected from non-treatment (no injection) animals.

Sampling of haemolymph

10 Min after injection, haemolymph was rapidly collected from a pin hole made in the first pair of abdominal prolegs in 50 µl microcaps lined with a silicone film. The haemolymph (50 µl) was mixed with 150 µl 0.1 N HCl solution and frozen. Immediately before use, the haemolymph was thawed and then centrifuged at 10,000 × *g* for 10 min to remove cell debris and precipitated protein. The supernatant (100 µl) was then mixed with 0.4 N perchloric acid (PCA) (50 µl), and 80 µl solution was injected.

High performance liquid chromatography (HPLC)

A neurochem HPLC neurochemical analyzer (ESA, Inc., Mass., U.S.A.) was used. This equipment consists of a gradient HPLC system and 16 high sensitivity coulometric electrochemical detectors coupled with a compatible computer. The concept and inherent advantage of multi-electrode HPLC system have been described elsewhere [9, 10]. The neurochemical analyzer was set to run a mixed linear and step gradient with a reverse phase C 18 column allowing separation of 23 compounds shown with their retention times and dominant electrode in Table I. Mobile phase A consisted of 0.1 M sodium phosphate and 10 mg/ml of sodium dodecyl sulfate at a pH of 3.35. Mobile phase B consisted of 50% methanol/water and 50 mg/l sodium dodecyl sulfate at a pH 3.45. The 16 serial electrodes were set in an incremental 60 mV array from 0 mV to 900 mV. The column and electrodes were maintained at a temperature of 34 °C throughout the run. Data from each electrode were collected on the computer and stored to the

Table I. List of standards used.

Compound	Abbreviation	Oxidation Potential First [mV]	Second [mV]	Retention time [min]
Vanillylmandelic acid	VMA	300	600	3.38
Norepinephrine	NE	180	–	4.89
Dopa (L-)	LD	150	–	5.45
Methoxy-Hydroxyphenyl glycol (3-, 4-)	MHPG	450	–	6.16
Octopamine	OCT	620	–	7.70
Epinephrine	E	180	–	8.29
Tyrosine (4)	TYR	650	–	8.70
Dihydroxyphenylacetic acid (3-, 4-)	DOPAC	150	–	9.90
Normetanephrine	NMN	480	–	10.70
Methyldopa (3-O)	MD	450	–	11.38
Hydroxytryptophan (5)	5HTP	300	650	11.52
Hydroxyindoleacetic acid (5)	5HIAA	180	750	11.77
Dopamine	DA	150	–	11.92
Acetylserotonin (N)	NACET5HT	180	700	12.45
Epinine	EPIN	120	–	12.64
Vanillic acid	VA	480	–	12.94
Homovanillic acid	HVA	450	–	13.60
Tyramine (4)	TYRA	620	–	14.35
Serotonin	5HT	180	700	15.42
Methylserotonin (N)	NMET	300	700	15.94
Methoxytyramine (3)	3MT	450	–	16.10
Melatonin	MEL	600	–	18.28
Tryptophan	TRP	600	–	18.95

hard disc for post run analysis. Each compound would typically be detected on three electrodes, with an average ratio in peak height between the electrodes of 1:6:1. However, the exact ratio was specific for each compound and could be used to establish the compound purity of unknown peaks in the sample eluting from the column at the same time as a known standard. Special compound recognition algorithms are used with the Neurochem analyzer which could match standards with unknowns using both retention time and ratio across the electrodes on which the compound was detected. Final concentration data were calculated based on comparing the peak height on the dominant electrode of a known standard with that of dominant unknown peaks in the sample.

Results

Basal levels of haemolymph biogenic amines

As shown in Table II and Fig. 2A, B, six biogenic amines were detected in the untreated groups. Among them, 4-tyrosine (TYR-4) was high in concentration, *ca.* 32081 ng/ml. This TYR-4 level did not change by injection of 0.9% NaCl solution or acetone solution as compared with that of the untreated groups (Fig. 2B), but other biogenic amine levels such as L-DOPA, DA and HVA increased with 0.9% NaCl or acetone solution treatment. Larvae injected with acetone solution (2 µl) responded rapidly to increased excitation, but show sedation after excitation.

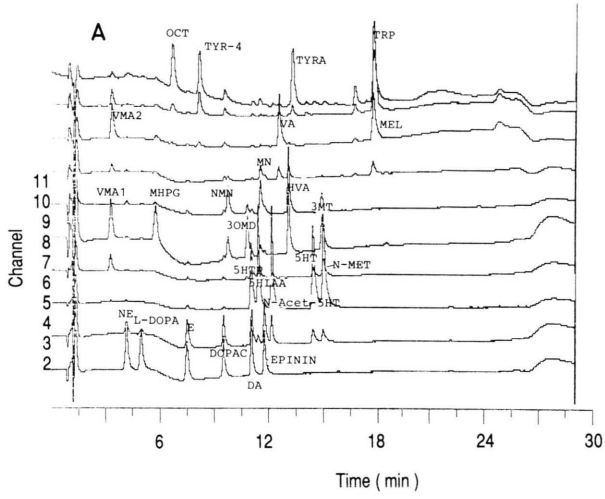

Table II. Concentrations of biogenic amines in the hemolymph of *Mamestra brassicae*.

Treatment	ng/ml hemolymph L-DOPA	DA	EPININE	HVA	NE	TYRA	TYR-4
non-treatment	631.098 ± 393.80 (3)	98.575 ± 15.20 (3)	20.012 ± 1.41 (3)	5.641 ± 0.72 (3)	N.D. (3)	123.642 ± 30.55 (3)	32081.248 ± 1196.87 (3)
NaCl	3623.954 ± 758.12 (4)	257.403 ± 226.28 (4)	14.266 ± 3.52 (4)	36.915 ± 23.88 (4)	507.291 ± 274.27 (4)	165.988 ± 19.73 (4)	28994.487 ± 4673.63 (4)
Acetone	4231.083 ± 861.56 (3)	120.507 ± 26.84 (3)	5.251 ± 1.81 (3)	18.167 ± 0.80 (3)	461.371 ± 214.43 (3)	154.365 ± 5.18 (3)	25972.667 ± 1254.21 (3)
Clonidine	3414.077 ± 1041.83 (5)	182.367 ± 128.35 (5)	21.710 ± 10.32 (5)	42.241 ± 31.01 (5)	437.084 ± 308.04 (5)	130.909 ± 32.29 (5)	45236.499 ± 9730.19 (5)
CDM	6883.528 ± 5703.31 (4)	439.581 ± 214.04 (4)	29.208 ± 8.00 (4)	53.150 ± 34.31 (4)	267.315 ± 230.46 (4)	131.334 ± 18.35 (4)	45560.767 ± 7638.49 (4)
AC-6	3137.770 ± 1238.26 (4)	170.375 ± 8.40 (4)	14.705 ± 5.77 (4)	40.020 ± 10.99 (4)	451.654 ± 189.38 (4)	139.233 ± 9.09 (4)	53600.135 ± 9833.41 (4)

L-DOPA, DA, EPININE, HVA, NE, TYRA, and TYR-4; see abbreviation of Table I.
N.D.; not detected.
NaCl, 0.9% NaCl solution, CDM; chlordimeform, AC-6; 2-(4-chloro-*o*-toluidino)-2-oxazoline.
Values indicate mean ± S.D. for the number of determinations shown in parentheses.

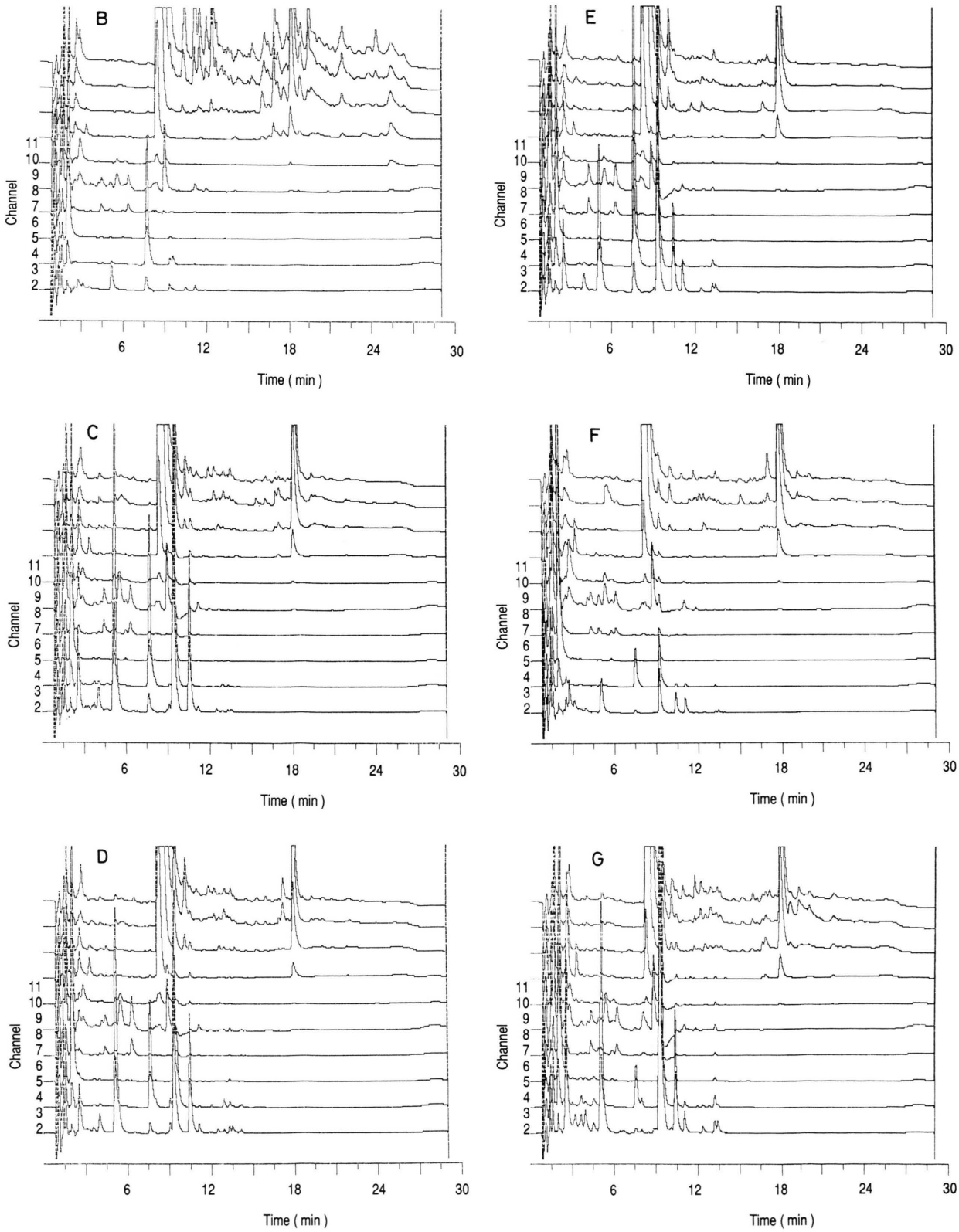

Fig. 2. HPLC-ECD chromatograms. A, peak identification of standards; B, hemolymph of the non-treatment; C, hemolymph following acetone solution injection; D, hemolymph following AC-6 injection; E, hemolymph following 0.9% NaCl solution injection; F, hemolymph following chlordimeform injection; G, hemolymph following clonidine injection.

Biogenic amine levels following treatment of octopaminergic insecticides

When either 2 μg chlordimeform, clonidine or AC-6 were injected, TYR-4 increased about twice as compared with that of control (0.9% NaCl or the acetone solution treatment) (Table II and Fig. 2C–G). With clonidine treatment, the other 6 biogenic amines did not change drastically as compared with that of their control. Except for NE and TYRA, all biogenic amine levels increased with chlordimeform treatment. With injection of AC-6, only HVA increased more than the controls (non-treatment or acetone). L-DOPA increased in some treatments, especially following chlordimeform injection, more than that of its control (NaCl) (Table II).

Discussion

This is the first report on biogenic amine analysis by HPLC-ECD in Lepidoptera haemolymph as far as we know. However, in Lepidoptera there were some recent reports on biogenic amines measured by radioenzymic assay [1, 2, 8]. According to Davenport and Wright [2], octopamine levels in the haemolymph of 6th instar *Spodoptera littoralis* are about 20 pg/μl (radioenzymic assay). In the present experiments, the octopamine level with acetone treatment on the same noctuid moth, *Mamestra brassicae,* is 4.23 pg/μl haemolymph (HPLC-ECD).

All chemicals, chlordimeform, AC-6 and clonidine act to increase levels of TYR-4. It is known that clonidine, α-adrenoreceptor agonist, had the potent stimulation of light organ adenylate cyclase [11], and also increased cardioactivity in *Manduca sexta* [12]. Clonidine and chlordimeform stimulate the elevation of C-AMP [11] and are known as an octopamine agonist [11]. In *Mamestra brassicae,* C-AMP levels in the haemolymph with a treatment of chlordimeform (2 μg) were increased 4-fold as compared with that of 0.9% NaCl solution injection (Shimizu, unpublished data). Octopamine levels with chlordimeform treatment were 67.124 pg/μl of haemolymph in the present experiments, these levels were high as compared with that of acetone treatment.

AC-6 was a potent octopaminergic agonist (measured by the elevation of C-AMP) and produced the characteristic poisoning symptomology seen with the formamidines [13]. The structure of AC-6 is similar to the chlordimeform phenyl substitution pattern. Previously we have noted that three compounds with 2-methyl substitution on phenyl group evoked continuous bursts of mandibular movements (CBMM) [14]. Lund *et al.* [15] reported that changes in the ring substituents markedly affected activity, producing tremors and feeding inhibition, and that a compound showing this was a 2,4-dichloro substituted compound. In a comparative study of AC-6 and chlordimeform on insect behavior, chlordimeform shows CBMM, but clonidine and AC-6 were not shown CBMM (Shimizu, unpublished observation).

4-Tyrosine (TYR-4) is a precursor for two biosynthetic pathways, tyramine-octopamine and L-dopa-dopamine. TYR-4 is increased, thereby insects might be physiologically ready to resist soon for chemical stress. Additionally, L-dopa was increased with chlordimeform treatment. From these results, we speculated the following pathway for chemical stress.

tyrosine (increase) → L-dopa (increase) → dopamine (no increase)
→ tyramine → octopamine

A similar increase of TYR-4 (60%) and L-dopa (150%) was obtained in the Corpus cardiacum of the American cockroach following AC-6 (2 μg) injection [16].

Already, Davenport and Evans [1] reported that chlordimeform caused significant increase in the haemolymph levels of octopamine 30 min after application on *Locusts*. Therefore, TYR-4 may be a metabolite of tyramine-octopamine [see 17, 18]. Experimentally Chang *et al.* [19] found increases of both tyramine and octopamine levels in the American cockroach after treatment with chlordimeform or deltamethrine.

Now we are investigating changes of biogenic amines in the ganglion following octopaminergic insecticide treatment for comparison with results contained herein.

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