Serotonin and its N-Acetylated and Acidic Derivatives in Insect Brain as Determined by High-Performance Liquid Chromatography with Electrochemical Detection[1]

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ABSTRACT The determination of serotonin (5-HT), N-acetylserotonin (NAS) and 5-hydroxy-3-indoleacetic acid (5-HIAA) in single brains of two acridids (*Paracinema tricolor* and *Oedipoda caerulescens*) was accomplished using a HPLC method combined with amperometric detection. A hydrodynamic voltammetry approach was used to assess the identity of each peak by comparing the voltammograms of standards and those of samples. The analytical method gave satisfactory reproducibility and sensitivity, and detected levels of 5-HT, NAS and 5-HIAA as low as 29, 55 and 10 fmol, respectively. This high sensitivity together with the simplicity of sample processing make the present analytical method suitable for a wide range of studies concerning indoleamine analyses in the insect nervous system. In both acridids, 5-HT showed the largest quantities, while its derivatives occurred in extremely low amounts. The results suggest that N-acetylation of 5-HT is quantitatively preferred to oxidative deamination in both species (NAS levels were 4-fold those of 5-HIAA). The relative importance of each catabolic pathway is discussed as related to physiological and genetic aspects.

KEY WORDS Indoleamines, brain, serotonin, N-acetylation, oxidative deamination, Orthoptera, HPLC, hydrodynamic voltammetry.

The existence of the biogenic amine serotonin (5-hydroxytryptamine, 5-HT) and its role as neurotransmitter and/or neurohormone in insects have been established (reviewed in Evans 1980, and Brown and Nestler 1985). The biosynthesis of serotonin appears to follow the same pathway as in vertebrates. Tryptophan is actively captured by neurons and hydroxylated to 5-hydroxytryptophan, which is then decarboxylated to give serotonin (Livingstone and Tempel 1983). It enters secretory granules where it is stored and transported through neuron cytoplasm to be finally released into the synaptic cleft. There, 5-HT can interact with pre- and postsynaptic receptors, determining a final physiological response, which involves mainly the activation of secondary messengers (Taylor and Newburgh 1978, Baines and Downer 1991). Insect serotoninergic nerve endings have been shown to recapture the released neurotransmitter (Klemm and Schneider 1975).

When 5-HT is released from granules, it is inactivated mainly either by N-acetylation, involving N-acetyltransferase (NAT) activity (Dewhurst et al. 1972, Evans and Fox 1975), or by oxidative deamination involving monoamine

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oxidase (MAO) activity (Richter and Rutschke 1977, Houk and Beck 1978). There is no certainty about which mechanism is used by insects for inactivating 5-HT. In addition, it has been suggested that 5-HT catabolites can be involved as active compounds in certain physiological processes (Evans 1980, Vieira 1991). However, the information about the overall insect 5-HT catabolism is scarce, partly due to the difficulties in quantitatively analyzing 5-HT and its catabolic products.

Our interest was thus (1) to develop a reproducible HPLC with electrochemical detection (ECD) method sensitive enough to carry out a quantitative analysis of indoles in single brains of insects, using two acridids as insect models, and (2) to evaluate the importance of each catabolic pathway in the insect brain under normal conditions.

Materials and Methods

Animals. Adults (males and females) of *Paracinema tricolor* Thunberg and *Oedipoda caerulescens* (L.) (Orthoptera: Acrididae) were obtained in August from a controlled field population in this locality and maintained at 26 ± 1°C under natural daylength. Insects were isolated during the first quarter of the light period.

Tissue preparation. Brains (supraesophageal ganglia) were dissected out from cold anaesthetized animals immediately after decapitation as previously described (Vieira 1991). Heads were pinned to a wax-filled petri dish placed on an ice-cold tray. The head capsule was prised open after an incision was made between the eyes, then the adhering fat body was removed, and the paired cerebral ganglia extracted. A typical dissection was completed within 2 min. Tissues were transferred to 0.5-ml polypropylene microtubes containing 120 µl 0.4 N perchloric acid (PCA) with 0.1% sodium metabisulphite and 0.01% EDTA. Samples were homogenized using an ultrasonic cell disruptor (Kontes) and centrifuged (40000 g) at 4°C for 15 min. An aliquot (10 µl) of the supernatant was injected directly onto the chromatographic column for indoleamine analysis. The remaining tissue pellets were resuspended in 100 µl of 1 N NaOH and the protein content was measured (Bradford 1976).

Indoleamine analysis. Concentrations of 5-HT, N-acetylserotonin (NAS) and 5-hydroxy-3-indoleacetic acid (5-HIAA) were determined by HPLC with electrochemical detection. The mobile phase was prepared from a stock buffer (110 ml of 0.1 M sodium acetate and 890 ml of 0.1 M acetic acid, ph 3.8), 0.10 mM Na_2-EDTA and 10% methanol (v/v). The mobile phase was filtered through a 0.22-µm filter, degassed under vacuum, and finally pumped by a Kontron 420 solvent delivery system (Kontron, Zürich, Switzerland) at a flow rate of 1.0 ml/min. Samples were applied to the column by means of a Rheodyne injection valve with a 20-µl loop. Indoles were separated on an Spherisorb 5-µm ODS I column (150 × 4.0 mm) (Tracer Analítica, Barcelona, Spain). The electrochemical detector was a BAS LC-4B equipped with a TL-5 glassy carbon electrode (West Lafayette, IN, USA) set at +0.60 V (versus Ag/AgCl). Chromatograms were recorded using a Hewlett Packard HP-3390 A integrator. Amine concentrations were calculated from peak area measurements compared to external standards run the same day. Stock solutions (500 µM) of amine

standards were made up in 0.1 M PCA, 0.1 mM Na_2-EDTA, 0.2 mM sodium metabisulphite, and stored at -80°C. Working standard solutions were prepared each day to the desired dilution with mobile phase. Recovery estimates of 5-HT, NAS and 5-HIAA added to brain homogenates were higher than 98%, whereby no correction was used in determination of indole concentrations.

Hydrodynamic voltammetry. In order to obtain hydrodynamic voltammograms, step-by-step reductions of the applied electrode potential were made from 0.8 to 0.3 V. Aliquots (20 µl), taken from the standard mixture (400 nM) and from samples of each species (10 homogenized pooled brains per sample), were chromatographed at each potential. Characteristic current-voltage curves were generated by plotting peak currents versus applied potential.

Chemicals. 5-HT creatinine sulphate, N-acetyl-5-hydroxytryptamine, 5-hydroxy-3-indoleacetic acid, and the other standards were obtained from Sigma (St. Louis, MO, USA). Citric acid, Na_2-EDTA and acetic acid from Scharlau (Barcelona, Spain). Methanol (gradient grade), sodium acetate, sodium metabisulphite and perchloric acid were from Merck (Darmstadt, Germany). HPLC-quality water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

Results

Quality of the method. The HPLC elution profiles obtained with the present analytical method after injection of a standard mixture and authentic insect brain samples are shown in Fig. 1. The retention times for 5-HT, 5-HIAA and NAS were about 7, 9, and 17 min, respectively. The system provided a good baseline separation between indoles and the other standards assayed, and no interfering peaks were observed in the chromatograms. The identification of each peak was accomplished by comparing both the retention times and hydrodynamic voltammograms with those of the standards. Fig. 2 shows that the characteristic electrochemical behaviour of each standard 5-HT, NAS and 5-HIAA is parallel to that of the indole components separated in real samples. All the current-voltage curves perfectly fitted to the same sigmoid curve (data not shown), verifying that the electrochemical behaviour of the indole compounds can be considered as normal.

A linear relationship between increasing amounts of the indole standards and detector response was confirmed with calibration curves of indoles covering 0.1-5 pmol total injected onto the column. The line of best fit was determined by linear regression analysis (5-HT: $y = 0.675 + 71.37x$, $r = 0.999$; NAS: $y = 3.52 + 31.95x$, $r = 0.999$; 5-HIAA: $y = 0.149 + 04.76x$, $r = 0.999$). Reproducibility of the method was examined intra- and inter-assay (for two consecutive days), with coefficients of variation below 6% for brain 5-HT, NAS and 5-HIAA concentrations exceeding 0.1 pmol. The detection limits obtained for standard 5-HT, NAS and 5-HIAA were as low as 0.029, 0.055 and 0.010 pmol per injection (assuming a signal-to-noise ratio of 2), respectively.

Quantitative analysis of 5-HT, NAS and 5-HIAA in orthopteran brain. Table 1 shows the content of serotonin and its derivatives in the brains of the acridids *O. caerulescens* and *P. tricolor*. As observed in both acridids, serotonin showed the highest amounts, representing about 85% in *P. tricolor* and 95% in

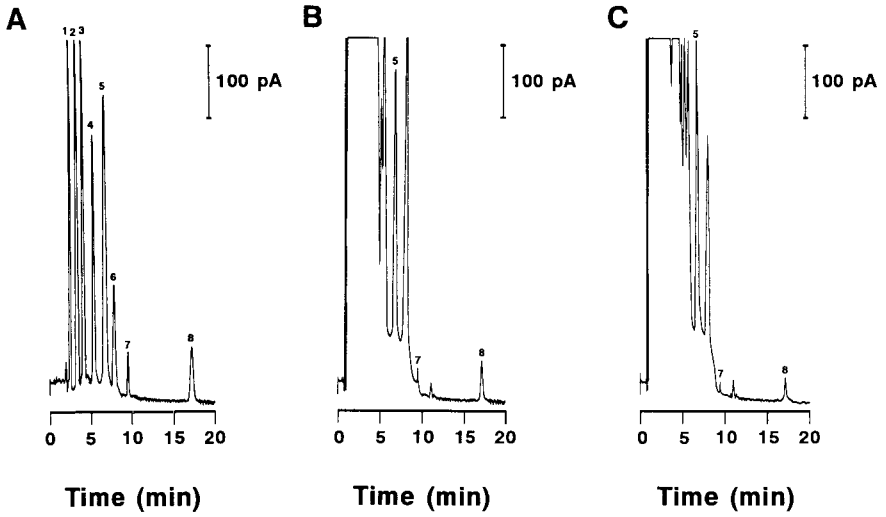

Fig. 1. Determination of brain indoleamines (5-HT, NAS and 5-HIAA) in *P. tricolor* and *O. caerulescens* by HPLC with amperometric detection. The elution profiles represent (A) a working standard solution containing 1 pmol of noradrenaline (1), dopamine (2), 5-hydroxytryptophan (3), 3,4-dihydroxyphenylacetic acid (4), serotonin (5) and N-acetylodopamine (6), and 0.1 pmol of 5-hydroxy-3-indoleacetic acid (7) and 0.2 pmol of N-acetylserotonin (8), (B) a brain extract from a male of *P. tricolor,* and (C) a brain extract from a male of *O. caerulescens.*

O. caerulescens of the total electroactive serotoninergic metabolism. With respect to the catabolic products, brain levels of NAS in both species were 4-fold those of 5-HIAA, which only represented about 1% in *O. caerulescens* and 2-3% in *P. tricolor* of all indoles analyzed considered as a whole. In addition, male-female differences were noted in *P. tricolor* with respect to brain NAS and 5-HIAA content, while brain 5-HT content differed between sexes in *O. caerulescens.*

Discussion

Insect neuroendocrinology has gained increased interest since these invertebrates can be used as models to investigate basic biological phenomena applicable to higher organisms including mammals (Scharrer 1987, Vieira 1991). Serotonin is known to act as a neurotransmitter and/or a neurohormone in the insect nervous system (Evans 1980). Such a monoamine has been directly related to circadian rhythms (Muszynska-Pytel and Cymborowski 1978a,b, Pandey and Habibulla 1982), determination of behaviour (Kostowski and Tarchalska 1972), learning performance (Vaysse et al. 1988) and development (Vieira et al. 1991a). In addition, 5-HT and other neurotransmitter systems

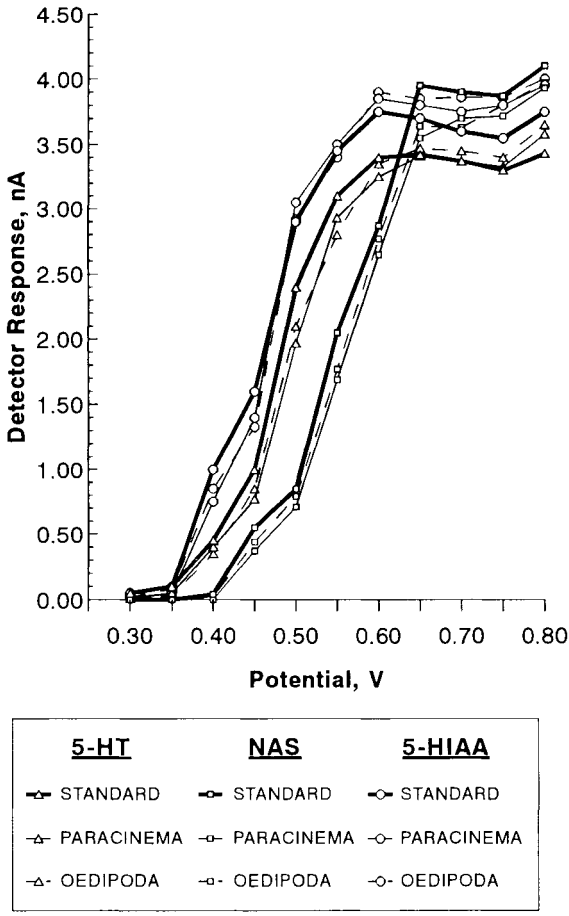

Fig. 2. Hydrodynamic voltammograms for 5-HT, NAS and 5-HIAA standards and for the same indole compounds present in brain samples of *Paracinema tricolor* and *Oedipoda caerulescens*.

have received special attention as targets for those compounds showing insecticide action (Wierenga and Hollingworth 1987, Sattelle and Yamamoto 1988, Sloley and Orikasa 1988). For these purposes the measurement of 5-HT and related substances from the insect CNS provides an interesting way to study the functionality of the serotoninergic system. Most of the studies on 5-HT and its metabolites demand highly sensitive analytical methods due to very low amounts occurring in the insect CNS. In connection with this, HPLC techniques have received preferential attention because of the simplicity of sample preparation and great versatility of application. Likewise, one fruitful approach has been to combine reversed-phase HPLC with ECD (Nässel and Laxmyr 1983, Nagao and Tanimura 1989) which allows a high sensitivity.

Table 1. Mean amounts of indoles in the brain of *P. tricolor* and *O. caerulescens*.

Species	5-HT	NAS	5-HIAA
P. tricolor			
females	21.79 ± 2.76(6)	2.51 ± 0.21(5)**	0.54 ± 0.03(5)***
males	24.15 ± 2.72(7)	3.39 ± 0.06(5)	0.87 ± 0.04(5)
O. caerulescens			
females	36.79 ± 3.43(6)*	1.90 ± 0.10(8)	0.48 ± 0.08(8)
males	50.34 ± 3.59(5)	1.99 ± 0.10(5)	0.49 ± 0.03(5)

Indole concentrations are expressed in pmol/mg protein. Values represent the mean ± S.E.M. of (n) determinations. Statistical male-female differences were evaluated using one-way ANOVA. *$P < 0.05$, **$P < 0.005$, and ***$P < 0.0005$.

Sensitivity and selectivity are key factors for the development of any HPLC method. Both factors can be efficiently studied by a hydrodynamic voltammetry approach. In hydrodynamic voltammetry, the applied electrode potential is held constant at several potentials while peak currents are measured for one or more compounds separated chromatographically from each other. The bidimensional information provided by this approach allowed us (1) to select the optimum potential required for the compounds of interest in order to attain a good signal-to-noise ratio, and (2) to assess the identity of each peak by comparing the characteristic electrochemical behaviours of the indole standards and those of the samples. Although Kissinger et al. (1979) considered hydrodynamic voltammetry as a valuable tool for determining peak purity in chromatography, few works using this methodology have been published (Nagao and Tanimura 1989).

The present analytical method proved to be reproducible and sensitive enough to determine 5-HT and its catabolic products, NAS and 5-HIAA, in single brains of both acridids studied. Likewise, the simultaneous detection of both 5-HT derivatives allowed us to begin to study the activity of the serotoninergic system in insect brain (Vieira et al. 1991b). Moreover, not only monoamines but also their metabolites simultaneously can be candidates that control physiological processes (Evans 1980). In this way, recent findings have directly involved 5-HT, NAS and 5-HIAA in protein synthesis induction during vitellogenesis (Goudey-Perrière et al. 1991) and in sex-related reproductive and/or metabolic differences (Vieira et al. 1991b).

The determination of NAS and 5-HIAA in this paper confirms the coexistence of both N-acetylation and oxidative deamination pathways, although the first catabolic route is quantitatively preferred in both species studied (Vieira et al. 1991b). Characterization of both pathways were made also clear in *Periplaneta americana* (L.) (Barreteau et al. 1991), *Blaberus craniifer* Burmeister (Barreteau et al. 1991, Goudey-Perrière et al. 1991), and in mites (Kadir and Knowles 1989) and crustaceans (Ehrenström and Berglind 1988) as well. Likewise, the presence of 5-HIAA in very low levels agrees with recent

reports indicating the existence of this 5-HT metabolite in the nervous system of crickets (Nagao and Tanimura 1988, Nagao and Tanimura 1989), cockroaches (Scott et al. 1985) and damselflies (Vieira et al. 1991a). This suggests that the very low MAO activity found in insects (Dewhurst et al. 1972) is enough to generate 5-HIAA or else this catabolite can be produced via another pathway (Scott et al. 1985).

All of the above data contrasts with the idea supported by some authors (Omar et al. 1982, Downer and Martin 1987, Sloley and Downer 1988) that N-acetylation would be the only 5-HT catabolic pathway occurring in insects. At any rate, it seems evident that the broad phylum of Arthropoda has exploited any of the 5-HT catabolic pathways, either inactivating this indoleamine mainly by N-acetylation, or by oxidative deamination or else combining both. The continuing evolution occurring among present-day insects and their ability to adapt to and colonize successfully all type of habitats are facts that fit with the existence of the above mentioned differences in monoaminergic metabolism. It is known that insects can respond to small environmental changes selecting favourable characters which results, for example, in an important physiological change. This has proved to be occurring in several mutants of *Drosophila,* which present an altered monoaminergic metabolism together with certain physiological and behavior alterations (Restifo and White 1990). Therefore, further studies are needed in order to fully understand the physiological role of 5-HT metabolism, which could help us to confirm (1) the possible advantages of one catabolic pathway as opposed to others, and (2) which evolutionary strategies have been adopted by the diverse species with regard to this neurotransmitter system.

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