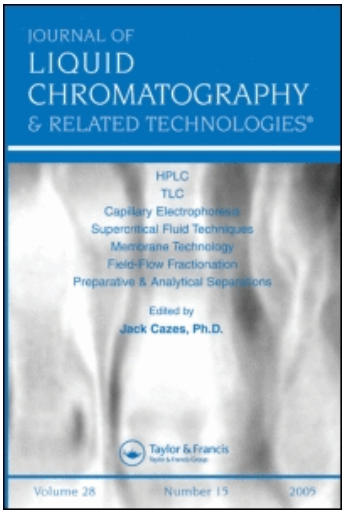

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INFLUENCE OF SAMPLE RECOVERY TECHNIQUES ON DETECTION OF BIOGENIC AMINES IN THE RAT HOOKWORM *NIPPOSTRONGYLUS BRASILIENSIS*

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ABSTRACT

Biogenic amines in *Nematoda* have previously been found by means of high-performance liquid chromatography (HPLC) equipped with various detectors. The methods of sample preparation have differed widely. In this study, we tested the influence of experimental conditions (temperature and duration of exposure to light, centrifugation or individual recovery by pipetting each worm, and saccharose gradient versus physiological saline) on catecholamines and indolamines content in *Nippostrongylus brasiliensis* at various developmental stages (larvae and adults). Amines were determined by liquid chromatography with electrochemical detection.

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To the best of our knowledge, octopamine, tyramin, N-acetylserotonin and N-acetyldopamine were found and determined for the first time in this nematode.

All experimental conditions listed above modify the endogenous amine levels in larval and adult *Nippostrongylus brasiliensis*. This should be taken into account in the design of future research protocols.

INTRODUCTION

Biogenic amines have been found in *Nematoda* by means of various techniques, including histochemistry [1], fluorescence labelling [2-8], gas chromatography [9], radioisotope enzyme assay [1], liquid chromatography (LC) with UV detection [10, 11] or electrochemical detection (ECD) [12-16].

Samples are usually heterogeneous in terms of the body parts analysed (whole worms or various organs), the population (mixed cultures of male and female adults or larvae), and the species (*Caenorhabditis elegans, Rhabditis pseudoelongata, Trichostrongylus colubriformis, Ascaris suum, Ascaris lumbricoides, Ascaridia galli, Nippostrongylus brasiliensis, Setaria cervi, Molinema dessetae, Acanthocheilonema vitae* and *Litomosoides carinii*). This heterogeneity has contributed to large differences between the published results concerning detection and analytical determination of amines. As a result, the published data cannot be directly compared.

Analytical methods are generally well described including the calibration of the detection system used (ECD). Nevertheless, the methods of sample preparation, particularly worm recovery, often are not.

We tested the recovering effect of the larvae and adults of *Nippostrongylus brasiliensis* under various experimental conditions (temperature, light, centrifugation or manual recovery, saccharose gradient or physiological saline) on biogenic amine levels.

Tryptophan (Trp), 5-hydroxytryptophan (5-HTP), serotonin (5-HT), 5-hydroxy-indol acetic acid (5-HIAA), N-acetylserotonin (Na5-HT), noradrenaline (NAd), tyramine (Tyr), octopamine (OA), dihydroxyphenyl acetic acid (DOPAC), dopamine (DA), N-acetyldopamine (NaDA), 3-methoxy-tyramine (3-MT), and homovanillic acid (HVA) were analysed simultaneously by HPLC with electrochemical detection, as described previously [16].

EXPERIMENTAL

Summary of the laboratory cycle

According to Luffau [17], third-stage larvae (L3) were collected from faecal cultures and inoculated into rats by skin puncture (2500 larvae per 250 g female rat). The larvae migrate to the lungs where they develop into fourth-stage larvae within 24 to 32 h. Following subsequent migration from the trachea to the oesophagus and onwards to the intestine, the larvae become adults. The first eggs appear in faeces five to six days after infestation under experimental conditions adopted.

Faecal culture and larval recovery.

Faeces from infected rats were collected between six and eight days after infestation. The droppings were macerated in water and mixed with an approximately equal volume of granular animal charcoal to form a paste. The mixture was spread on the center of a moist filter paper which was then placed on a wet sponge in a Petri dish and incubated at 25°C for 6-8 days [15,17]. Third-stage larvae were harvested by filling the Petri dish with water at 25°C, spontaneous sedimentation of the larvae recovered, then rinsed in distilled water.

They were divided into three groups and incubated in distilled water at 4°C, 25°C or 37°C for 4 hours. After incubation and spontaneous sedimentation, the supernatant was discarded and the worms were sponged with filter paper and stored at -20°C until analysis.

Harvest and preparation of adult worms

One week after infestation, rats were killed by ether asphyxiation and the small intestine was removed. The intestine was filled with 0.15 M NaCl at 37°C and opened longitudinally. The mucosa was excised and placed in a gauze bag, which was then placed in a beaker containing 0.15 M NaCl at 37°C. Under these conditions, adult worms settle to the bottom of the beaker, and a mixed population of male and female worms can be harvested after one hour of spontaneous sedimentation [17].

Four preparation methods were compared using nematodes from the same batch (See Table I) :

All samples were then sponged, weighed and stored at -20°C. Worm number and weight varied to different samples.

Sample preparation

Samples were placed in test tubes and homogenized (Ultraturrax, PolyLabo, Paris, France ; 30 sec.) in 0.4 N $HClO_4$ solution containing antioxidants (0.1% cysteine, 0.1% sodium metabisulphite and 0.1% sodium edetate) (1 mg of sample in 50 µl). Proteins were precipitated with perchloric acid and removed by centrifugation (Dupont Superspeed, Saint-Quentin en Yvelines, France ; 4°C, 20 min at 2 000 *g*) ; clear supernatants were used for the analysis.

Table I: Schema of the preparation of Nematodes samples before analysis

A pool of Nematodes is obtained by sedimentation and treated differently

Lot A : (about 75 % of adults)				**Lot B : about 25 % of adults**
Lot A_1 : Males and Females rinsed in NaCl 0.15 M	Lot $A_{1.1}$ analysed immediately	**Lot A_2** individually sexed Females were taken to test effects of light and temperature after exposure	Lot $A_{2.1}$ =1 hour	in sucrose (35 % in water) then centrifugation
	Lot $A_{1.2}$ analysed after light-temperature exposure during 3 hours		Lot $A_{2.2}$ =3 hours	
			Lot $A_{2.3}$ =5 hours	

Apparatus

Separation and determination of biogenic amines.

Samples were analysed by liquid chromatography with electrochemical detection according to Barreteau *et al* [16]. The chromatographic system consisted of a Beckman 112 pump (USA, Palo Alto, California) (constant flow rate of 1 ml/min); a Rheodyne injection valve (Touzart, Vitry, France) with a 20 µl loop ; a reverse-phase column (Ultrasphere ODS, 5 µm, 150 x 4 mm, Beckman) and a Metrohm 641 VA electrochemical detector (Roucaire, Vélizy, France) equipped with a glassy carbon electrode (set to a 0.85 V potential versus a KCl/AgCl reference electrode) [15].

A mixture of phosphate buffer [0.1 M KH_2PO_4, heptane sulphonic acid (5 mM)] and methanol (90/10, v/v) was used as the mobile phase ; pH was adjusted to 3.8 by using 3 M KOH.

Total elution was reached within 25 min. The system allowed the simultaneous detection of NAd, 5-HTP, OA, NaDA, DOPAC, DA, 5-HIAA, Na5-HT, HVA, Tyr, Trp, 3-MT and 5-HT (in the order of elution).

The perchloric acid medium formed a relatively large solvent front, hindering the detection of compounds eluted before Noradrenaline (*i.e.* during the first three minutes of each chromatographic analysis).

Chemicals

All reagents were of analytical grade ; methanol was from Merck, while Trp, 5-HTP, 5-HT, 5-HIAA, Na5-HT, NAd, Tyr, OA, DOPAC, DA, 3-MT, HVA and NaDA were from Sigma Co.

Statistical analysis

When sufficient repetitions were possible, analysis of variance (Fisher's test, ANOVA using Stat View software on a Macintosh computer) was used to

compare the different groups. However, a batch of worms obtained from one rat must be divided in several series to compare the effect of different sample recovery, and samples were often pooled to obtain quantities allowing assays; sometimes, the number of assays was insufficient and, in such a case, no statistical analysis was performed.

RESULTS

Two important facts are found. First, we have succeeded to detect some catechol and indol compounds for the first time in nematodes : octopamine (OA), tyramine (Tyr), N-acetylserotonin (Na5-HT) and N-acetyldopamine (NaDA) were found in larvae and adults.

Second, sample recovery including adaptative responses to environmental conditions greatly influenced the concentration of the indol and catechol compounds.

1- Effect of temperature on amine content of free third-stage larvae (fig.1)

Tryptophan was detected in all samples. Its concentration in larvae incubated at 4°C (15.4 ± 3 µg/g) was higher than in those incubated at either 25°C (5.6 ± 1.1 µg/g) or 37°C (6.7 ± 1.7 µg/g) ($p < 0.01$).

5-HTP was detected in all but two of the 11 samples (one at 4°C and one at 25°C) but accurate quantitative analysis was difficult because 5-HTP was generally eluted at the tail of the very broad solvent peak. When quantification was possible, 5-HTP concentrations were in the range of 170 to 195 ng/g.

Serotonin was detected (44 ng/g) in only one sample at 37°C.

5-HIAA was determined in all samples ; its concentration decreased with increasing incubation temperature (4°C : 353 ± 136 ng/g ; 25°C : 307 ± 246 ng/g ; 37°C : 87.5 ± 32 ng/g) ($p < 0.05$).

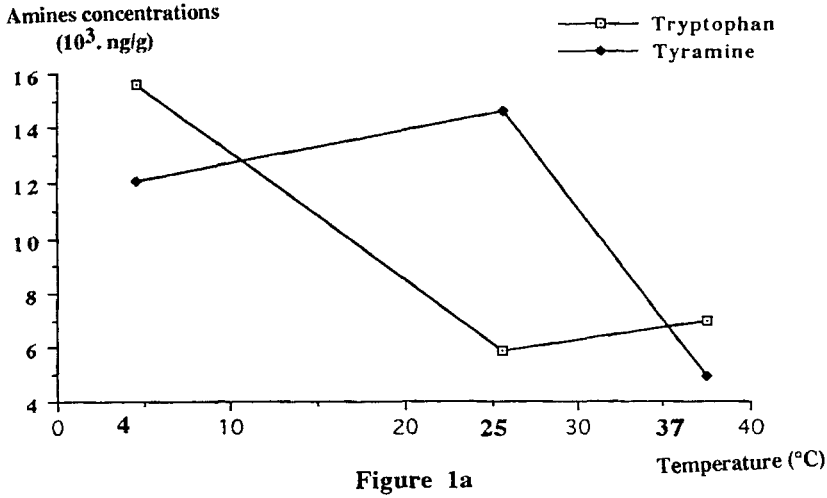

Figure 1a

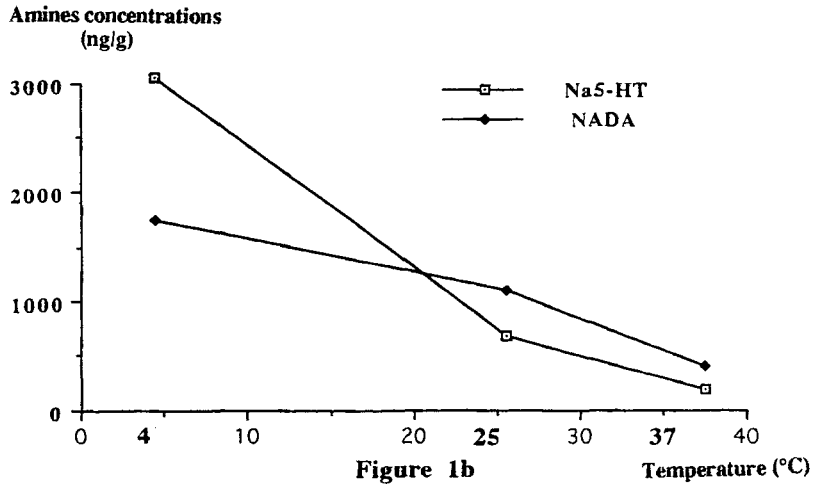

Figure 1b

Figure 1 : Effect of temperature on amine content of free third-stage larvae. Results are given only for some biogenic amines, for the others see text. Figure 1a : Tryptophan and Tyramine ; figure 1b : 5-HIAA, DA, DOPAC and 5-HT ; figure 1c : N-acetyl derivatives of 5-HT and DA.

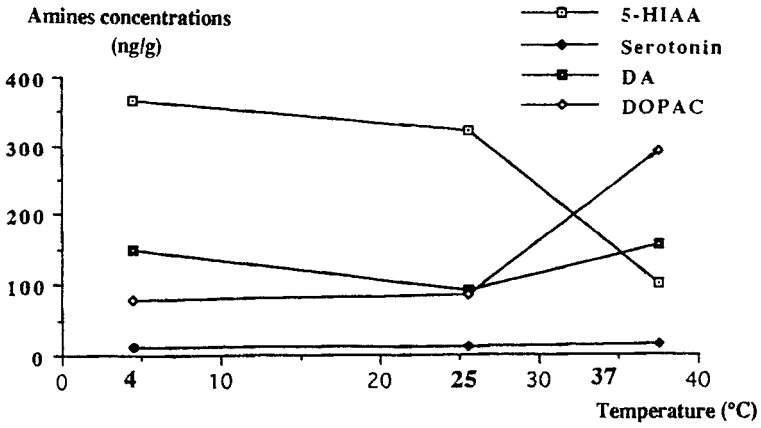

Figure 1c

Levels of Na5-HT were high at 4°C (2977 ng/g) in the three samples, but was either drastically lower or absent in samples incubated at 25°C (614 ng/g in one of four samples, mean : 153.5 ng/g) and 37°C (110 ng/g, 2 of 4 samples). Na5-HT was detected in all the samples, but levels were below the limit of accurate detection (2 ng/g).

Catecholamine concentrations also varied depending on incubation temperature.

Tyramine was detected in most samples but the quantitative determination was not always possible. It was determined in one of four samples at 4°C (35 µg/g), three of four at 25°C (mean : 14 ± 8.8 µg/g), and two of four at 37°C (9.1 µg/g, 9.34 µg/g). Thus, a tendency for concentration decrease with increasing temperature was observed.

Octopamine was detected in all samples at 4°C and 25°C, but in only 2 of 4 samples at 37°C. Accurate quantification was difficult, because octopamine eluted at the tail of the very broad solvent peak, OA was however determined at

4°C (32 µg/g, 1 out of 3 samples), 25°C (6.7 µg/g, 2 out of 4 samples), and 37°C (2 µg/g, 3 out of 4 samples).

Noradrenaline was present in all samples, often at higher concentrations than 3 µg/g. However, accurate determination was not always possible since Noradrenaline also eluted at the tail of the very broad solvent peak.

Dopamine was detected in 2 samples out of 3 at 4°C (137 ± 107 ng/g), 2 out of 4 at 25°C (78.8 ± 69 ng/g) and 3 out of 4 at 37°C (142 ± 61 ng/g).

DOPAC concentrations increased from 67.7 ± 48 ng/g at 4°C to 277 ± 125 ng/g at 37°C ($p < 0.05$).

Concentrations of NaDA decreased with increasing temperature (1659 ± 715 ng/g at 4°C ; 1026 ± 129 ng/g at 25°C ; and 338 ± 119 ng/g at 37°C ; $p < 0.05$).

2- " Temperature-illumination " effects in adults (Table II).

We observed marked differences inside and between groups (*i.e.* lot $A_{1.1}$ and lot $A_{1.2}$). Except for DOPAC, we found a general decrease in amine levels. However, statistical analysis showed that, OA, DOPAC, NaDA and tryptophan were unaffected by "temperature-illumination", whereas 5-HTP, dopamine, 5-HIAA and Na5-HT levels decreased.

3- Effect of temperature-illumination exposure time in female worms (Figure 2)

The analysis concerned the lots $A_{2.1}$, $A_{2.2}$ and $A_{2.3}$ (males and females in mixture).

Serotonin and tyramine were not detected, in any sample.

Tryptophan and 5-HIAA levels remained constant (21-26 µg/g and 120-148 µ/g, respectively).

There was a trend towards an increase in 5-HTP levels when the time of illumination increased from 1 to 5 hours (11 vs 32 ng/g, $p < 0.05$). Finally, Na5-

Table II : "Temperature-illumination" effects on amine contents in adults.

Content of biogenic amines (ng/g)	Physiological saline Illumination time 0 min	Physiological saline Illumination time 3 hours
NAd	74.26 ± 14	55.96 ± 10.59
5-HTP	62.05 ± 4.6*	20.33 ± 14.83 *
OA	11.22 ± 4.78 10^3	8.69 ± 4.27 10^3
DOPAC	38.53 ± 4.64	76.78 ± 42.15
NaDA	86.98 ± 12.57	40.99 ± 19.69
DA	38.59 ± 11.9 **	0.83 ± 0.18 **
5-HIAA	169.01 ± 6.78*	116.39 ± 27.5*
N-Ac-5-HT	285.56 ± 231.8**	2.3 ± 0.13**
Trp	18.35 ± 3,02.10^3	13.2 ± 3.44.10^3
Tyr	not detected	not detected
5-HT	not detected	not detected

* p = 0.05 ; ** p = 0.02. Each value reports mean ± S.E.M. for 3 samples.

HT was detected in all samples, but below the quantitative determination limit in one sample at 1, 3 and 5 hours. Mean levels decreased as the time of illumination increased.

There was a significant increase ($p < 0.05$) in noradrenaline (66 vs 222 ng/g), NaDA (140 vs 307 ng/g) and dopamine (0.92 vs 97 ng/g) levels when the time of illumination increased. In contrast, DOPAC levels decreased from 46 ng/g to about 29 ng/g after a 5-hour illumination period ($p < 0.05$). Finally, octopamine levels were not affected by illumination (13-17 µg/g).

4- Effect of centrifugation and sucrose gradient recovery on biogenic amines (Figure 3).

As a consequence of the large variability, no significant differences were observed between sucrose gradient (lot B) and saline (lot A) recovery groups (with the exception of tryptophan : 17.3 ± 1.64 vs 23.9 ± 2.57 µ/g, p = 0.04).

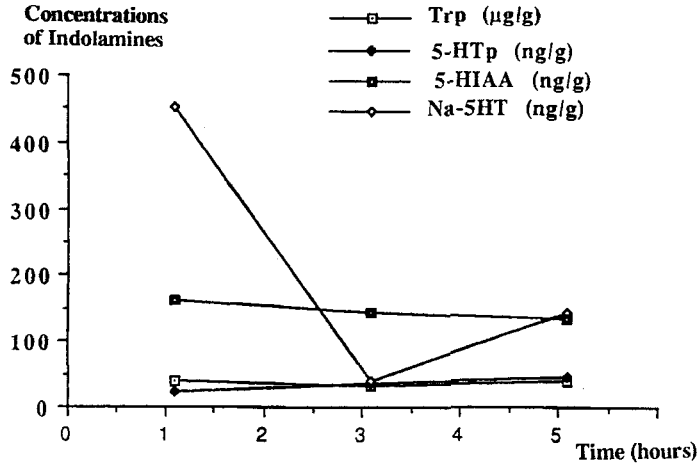

Figure 2a: indol compounds

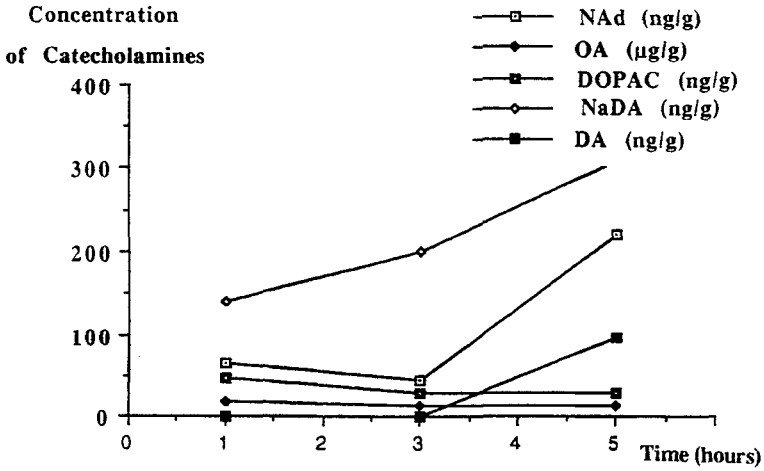

Figure 2b : catechol compounds

Figure 2 : Effect of the time of temperature-illumination and stress on biogenic amine content in females.

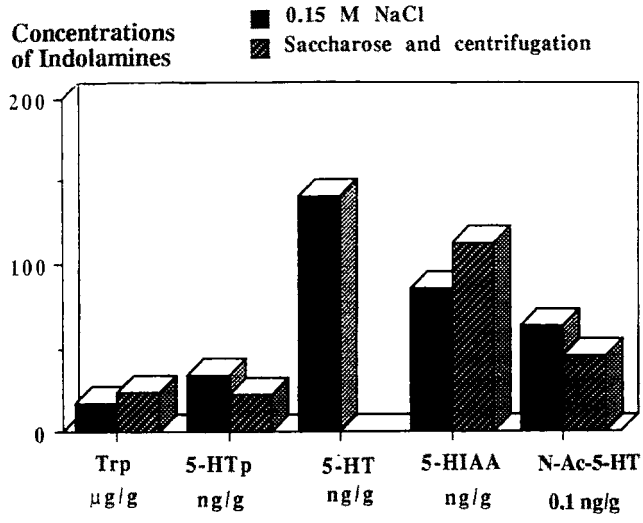

Figure 3a : indol compounds

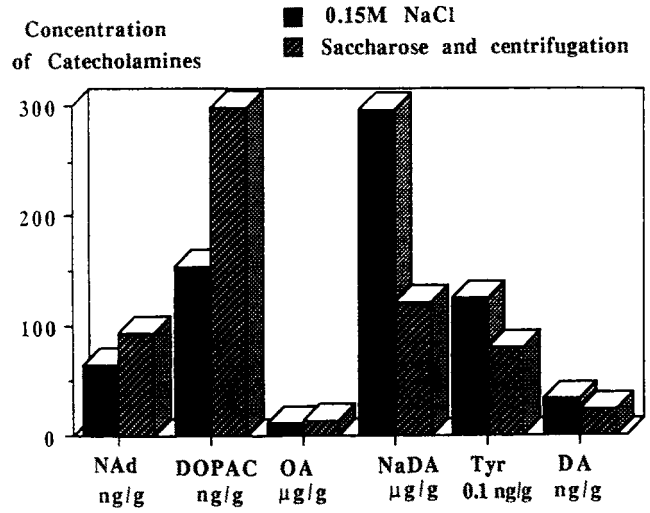

Figure 3b : catechol compounds

Figure 3 : Effect of worm recovery by a sucrose gradient and centrifugation on biogenic amines.

Tryptophan, 5-HTP, 5-HIAA and Na5-HT were detected in all samples (Fig. 3a). Serotonin was only determined in one saline-treated sample (142 ng/g). In saccharose-treated worms, 5-HTP and Na5-HT levels slightly decreased (21.24 ± 6.73 vs 34.07 ± 7.4 ng/g; 452.9 ± 127.4 vs 640.1 ± 160 ng/g respectively), 5-HIAA rose slightly (112.7 ± 26.6 vs 86.5 ± 25.5 ng/g) when compared to saline recovery group.

Tyramine, noradrenaline, DOPAC, dopamine, octopamine and NaDA were detected in all samples (Fig. 3b). In saccharose-treated worms, level decreases were registered for tyramine (800 ± 70 vs 1250 ± 600 ng/g), NaDA (121.25 ± 19.53 vs 295.7 ± 115 ng/g) and dopamine (24 ± 8.7 vs 33.3 ± 8.5 ng/g) while levels of octopamine (13.17 ± 1.62 vs 10.62 ± 1.69 µg/g), noradrenaline (93 ± 25.9 vs 65.5 ± 16.3 ng/g) and DOPAC (297.8 ± 69.45 vs 153.3 ± 42.2 ng/g) were enhanced, when compared to saline recovery group.

DISCUSSION AND CONCLUSION

A simultaneous determination of octopamine, tyramine, Na5-HT and NaDA was performed for the first time in *Nippostrongylus brasiliensis* by means of LC-ECD. In chromatographic separation followed with the electrochemical detection, behavior of these compounds was identical with standards.

Noradrenaline was detected in all samples, but both its high concentration and its elution in the tail of the solvent peak often precluded a quantitative analysis. For the same reason, concentrations of 5-HTP and octopamine were accurately not determined in some samples .

The presence of serotonin in nematodes has been reported by several authors [10, 11, 13-16], however this compound was hardly detectable in most of

our samples (even in presence of serotonin derivatives). This inconsistency with previous reports [15, 16] may be accounted for by the detection limit of our method, since in the present study we used 1 mg of worms per 50 µl of buffer, whereas Barreteau [15] used 1 mg per 10 µl.

3-MT and HVA were never detected, confirming the previous reports.

In addition to the detection of various indol and catechol compounds in nematodes, our study clearly shows that endogenous levels of bioamines depend not only on analysis method but mainly on worm recovery conditions.

Cooling of larvae increased the concentration of all the biogenic amines determined, with the DOPAC and tyramine exception, which remained constant. The stress induced by modifying temperature conditions could be evidenced by an acceleration of arylalkylamines, indolamines pathways, contrarily to dopaminergic pathways.

We observed the differences in biogenic amine contents at 25°C (typical temperature of free larval cultures) and 37°C (host temperature). Concentration of serotonin derivatives (tyramine, octopamine and NaDA) decreased with increasing temperature, while those of dopamine and DOPAC increased. A general decrease in N-acetyl derivatives was observed when temperature increased.

In mixed male and female adult worms, exposure to light and high temperatures reduced the biogenic amine content. If it is likely that enzyme activities are affected by temperature changes, especially above 37°C, these data are difficult to interpret, because they are a result of the combined influence of light and temperature. In most cases, the registered effect was negative (DOPAC was the only increasing biogenic amine which increased). However increasing times of illumination resulted in females by a significant increase in NaDA and

noradrenaline levels and a decrease in Na5-HT levels, other amines being unaffected.

To estimate the effects of handling (*i.e.* manipulation of worms under a microscope lamp for sexing), two different populations were used (handled females and a mixture of both sexes) and the results therefore cannot be directly compared. Indeed, it has been recently demonstrated that, biogenic amine contents in males and females are minimal after 7 days post-infestation [18]. Despite this restriction, manipulation of the worms seemed to induce modifications, with levels varying in the opposite way due to temperature-illumination exposure variations.

Centrifugation and osmotic pressure stress in sucrose solution induced variations of the level of most biogenic amines in worms.

Temperature, light and handling affect the physiology of living organisms, as indicated in this study by changes in biogenic amine contents. Recovery and buffer washing, followed by spontaneous sedimentation or centrifugation methods resulted also in adaptative responses, but to a lesser extent. We could not determine where, precisely, modifications took place, because this was a whole worm assay.

Our findings suggest that sample recovery techniques should be taken into account when measuring biogenic amines, and that experimental culture and recovery conditions should be defined clearly. Combined with information on the physiological state of the population (stage, sex, reproductive period, etc..) and the recovery of samples, LC-ECD is likely to be fruitful for simultaneous studies of biogenic amines in nematodes.

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