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Effects of dietary precursors to biogenic amines on the behavioural response from groups of caged worker honey bees (*Apis mellifera*) to the alarm pheromone component isopentyl acetate

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Abstract. The sound or 'buzzing response' from groups of fifteen worker honey bees, *Apis mellifera* L., to the presentation of isopentyl acetate, an alarm pheromone component, was recorded through a microphone connected via a digital–analogue converter into a computer. The effects of ingested biogenic amine precursors 5-hydroxytryptophan, L-DOPA and tryptophan were tested on three variables measurable from the sound traces: onset of the buzzing response after presentation of the pheromone stimulus, the duration of the buzzing event, and the maximal sound frequency produced during the buzzing event. Bees fed high doses of 5-hydroxytrytophan were found to react significantly more slowly and with a significantly lower maximum frequency than bees that were only fed sucrose controls. Bees fed DOPA were no different from controls for any of the variables measured, and bees fed tryptophan were so hyperactive that reliable responses to the presentation of alarm pheromone could not be made. Finally, the brains of worker bees fed these different precursors were examined for content of various neurochemicals. Bees fed 5-hydroxytryptophan were found to have dose-dependent elevations of both 5-hydroxytryptophan and serotonin within their brains. Bees fed tryptophan were found to have dose-dependent elevations of tryptophan and kynurenine in the brain but no change in brain serotonin. Bees fed DOPA had dose-dependent increases in DOPA and dopamine levels within their brains. These results suggest that the hyperactive condition results from kynurenine metabolism, and the reduced response to alarm pheromone is related to serotonin metabolism.

Key words. Alarm pheromones, biogenic amines, honey bees, 5-hydroxytryptophan, kynurenine, L-DOPA, tryptophan.

Introduction

Colony defence behaviour in honey bees (*Apis mellifera* L.) can be quantified in both field and laboratory tests (Stort, 1974; Collins & Rothenbuhler, 1978; Collins & Kubasek, 1982). Various genetic parameters related to the overall colony reaction and the response to alarm pheromone components have been outlined using field and laboratory bioassays. These tests measured the speed, intensity and duration of the colony behavioural response to a mechanical disturbance after the brief presentation of test chemicals (Boch *et al*., 1962; Shearer & Boch, 1965; Blum *et al*., 1978; Collins *et al*., 1980; Collins & Blum, 1983; Collins, 1989). Distinctions between Africanized and European honey bee defence behaviour have been determined using these techniques (Collins *et al*., 1982; Collins & Rinderer, 1988). Another laboratory test for honey bee responses to alarm pheromones measured metabolic oxygen consumption by groups of bees subsequent to release of a test chemical (Moritz *et al*., 1985; Southwick & Moritz, 1985). The common element of all of these tests is that responses to alarm

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pheromones are made only on caged groups or whole colonies of bees.

It has been difficult to relate the physiology of individual bees to overall colony defence. Our particular focus is on the relationship between biogenic amine neuromodulators within the honey bee brain and specific behavioural response parameters to an olfactory stimulus, such as the primary alarm pheromone component, isopentyl acetate. Recent work with vertebrates and invertebrates indicates that a variety of behaviours are mediated by the actions of biogenic amines within the central nervous system (Kravitz, 1988; Bicker & Menzel, 1989). Indeed, the examination of olfactory learning in bees has shown that memory retrieval and storage (complex nervous system functions) are mediated by biogenic amines (Menzel *et al*., 1990). Brain biogenic amine levels have been shown to change with worker honey bee reproductive status (Harris & Woodring, 1995), after exposure to carbon dioxide narcosis (Harris *et al*., 1996), season (Harris & Woodring, 1992) and the amount of dietary pollen (Harris *et al*., 1996). Recent work by Burrell & Smith (1994, 1995) showed possible modulatory roles by octopamine on the muscular and motor-neural components of the honey bee sting extension motor program within the ventral nerve cord. A recent preliminary report showed that European honey bees had significantly higher levels of the neurochemical beta-alanine in the brain than do Africanized honey bees (Last *et al*., 1994). This latter report presents the possibility that differences in behaviour between Africanized and European honey bees may be related to chemicals within the CNS.

The purpose of the current study is to test the potential effects of ingested biogenic amine precursors on behavioural responses to the presentation of isopentyl acetate. In particular, the laboratory bioassay used in this study involved the quantitative measurement of variables related to the previously described 'buzzing response' produced by small groups of bees within screened cages when exposed to isopentyl acetate in paraffin oil (Collins & Rothenbuhler, 1978; Collins & Blum, 1983). The buzzing responses were monitored using a small microphone located within the screened cages. The variables measured were: the time delay or onset of the buzzing response after presentation of the isopentyl acetate chemical stimulus, the duration of the buzzing response, and the maximal frequency of the response. The effects of various doses of ingested biogenic amine precursors (L-DOPA, tryptophan and 5-hydroxytryptophan) on the buzzing response to isopentyl acetate were investigated. Finally, the brains of individual bees that were fed the same doses of the various precursors used in the behavioural study were analysed to indicate possible correlations between the behavioural studies and the relative levels of biogenic amines and amine precursors within the CNS of individual bees.

Materials and Methods

Behavioural responses to isopentyl acetate

The behavioural experiments were conducted during May–July 1995, at Louisiana State University. Forager honey bees were collected with forceps without use of an anaesthetic as they returned to their observation colony. Fifteen bees were placed into each clear plastic cylinder (8 cm diameter, 15 cm length) which were screened on both ends with a plastic mesh. Bees were collected between 08.00 and 10.00 hours on each day that tests were to be performed.

Each group of bees was held without food for the first 30–40 min after capture, then each group was fed 2 m sucrose solution *ad libitum* over the next 5–6 h. The syrup was pipetted into a small vial cap placed on the bottom screen of each cage through a small access hole drilled in the side of each cage. Each refill was approximately 700 µl and cages received two or three refills throughout the entire experiment. Each cage of bees was clamped to a ringstand so that the bottom screen was accessible for the presentation of test solutions soaked on a cotton swab. A small tie-clip type of microphone was anchored to the inside top screen of each cage and the analogue signals from the microphones were conducted to separate channels on a MacLab 4S analogue–digital converter. The raw waveform data generated from the MacLab were converted to frequency data using the MacLab Chart program. Prior to each test session, all microphones were calibrated using a frequency generator and a speaker at a set distance. Baseline noise from resting bees was found to be around 12 Hz. Maximal responses to any disturbance from these groups of bees was found to be between 200 and 350 Hz; therefore the microphones were calibrated at frequency signals of 200 and 350 Hz of the sound generator to ensure that all microphones responded the same. The temperature of the test room was 28–30°C and the overhead lights remained on throughout the experiment. Groups of bees were placed at least 2 m apart to avoid possible influences of one group of bees on a neighbouring group.

To test the effectiveness of isopentyl acetate at eliciting buzzing responses from caged bees, twenty cages of bees were assembled and fed sucrose as described above. After a 5-h acclimation period, groups were exposed to a 30-s presentation of either a swab soaked in paraffin oil or one soaked in paraffin oil containing 5% isopentyl acetate (ten groups per test chemical). Five hours was selected as the time interval to test the response to the pheromone, because preliminary tests indicated that maximal levels of ingested precursors were appearing in the brain within 5 h. The swabs were placed close to the centre of the bottom screen of each cage without touching the mesh. Each group was tested only once. Response measurements were made relative to a mark placed on the data trace with the simultaneous presentation of the test swab. The variables measured were the onset (in seconds) of the response, the duration (s) of the response, and the maximum frequency of the response, reported here as the maximum value (Hz) of the frequency data trace. If a group of bees did not respond, the group was assigned an onset value of 30 s, and duration and maximal response values of 0.

Ingested amine precursors and the behavioural response to isopentyl acetate

The effects of ingested amine precursors tryptophan, 5-hydroxytryptophan and DOPA on the behavioural response to

the presentation of isopentyl acetate was tested during June–July 1995. These precursors were chosen based on the results of the behavioural tests conducted in May–July 1995 and on earlier determinations of brain levels of amine. A procedure identical to the above was used for all experiments, except that the control groups of fifteen bees were fed 700 µl of 2 M sucrose and each test group was fed 700 µl of 2 M sucrose containing one of four doses (1, 2, 4 or 8 mM) of one of the above three precursors (ten groups of bees tested for each dose and drug combination; 120 total groups were tested). All feeding started exactly 5 h before performing the isopentyl actetate bioassay. After the test solutions were totally consumed, each group of bees was fed 2 M sucrose to keep them alive throughout the test. Because of the logistics involved in testing many groups of bees, tests were conducted over a 2-week period. Hence, on any test day, equal numbers of controls (groups only fed 2 M sucrose) and of drug-fed groups were tested. For simplicity, equal doses of all three drugs were tested on the same days. The data were analysed using an analysis of variance with a model including major terms for the drug treatment and dose of drug. Also included was the interaction term for these two sources of variation.

Ingested amine precursors and brain amine content

The effects of the ingested amine precursors tryptophan, 5-hydroxytryptophan and DOPA on amine levels within the brains of individual bees were tested during late summer 1995. Individual foragers were captured from the observation colony and placed singly into a screened cage identical to those used for the isopentyl acetate tests. Each bee was starved for 30–40 min before being fed 45 µl of test solution. The treatment groups were: controls = groups fed only 2 M sucrose; all other groups received one drug (tryptophan, 5-hydroxytrypophan or DOPA) and dose (0.5, 1, 2, 4 or 8 mM) dissolved in 2 M sucrose. The brains from the test bees were removed for amine analysis 5 h after the experiment began. An HPLC with electrochemical detection was used to quantify levels of brain neurochemicals (Harris & Woodring, 1992). For simplicity, controls and all doses of a single drug were tested on one day. A separate statistical analysis was carried out for each precursor. An analysis of variance tested the effects of dose of ingested precursor on resulting levels of octopamine, serotonin, dopamine, tryptophan, kynurenine and 5-hydroxtryptophan.

Results

Effects of 5% isopentyl acetate versus paraffin oil controls on buzzing responses of bees

There were significant differences between those groups of bees exposed to 5% isopentyl acetate in paraffin oil and those exposed to only paraffin oil for all three variables measured (Fig. 1). Paraffin controls required an average (mean ± SE) onset of 25.37 ± 3.09 s to react, whereas groups treated with isopentyl acetate reacted four times more quickly

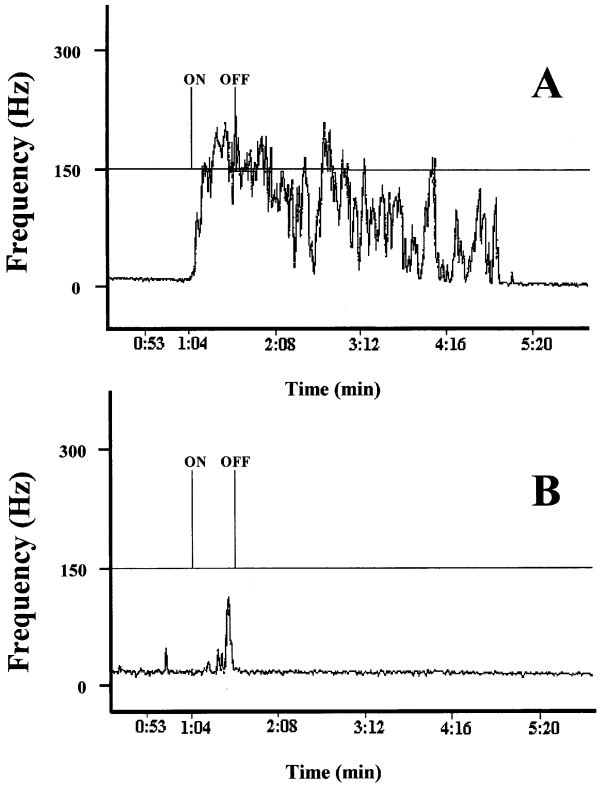

Fig. 1. A comparison of the buzzing responses produced by groups of fifteen bees exposed to 5% isopentyl acetate in paraffin oil (trace A) versus paraffin oil only (trace B). Each recording began about 1 min prior to the presentation of a cotton swab soaked in test solution at the screened bottom of the cage holding each group of bees. A test swab was held beneath the bees for 30 s. **ON** indicates presentation of the swab, and **OFF** indicates the removal of the swab. The response produced by 5% isopentyl acetate in paraffin oil was significantly faster (almost immediate), lasted significantly longer (1–5 min) and produced a higher maximal frequency (> 200 Hz). The maximal frequency was derived from raw oscillation data and converted into a plot of frequency against time. A higher maximal frequency reflects a higher pitch of the buzzing sound. The minimal response to paraffin oil alone represents the minimal disturbance (probably visual) of presenting the cotton swab.

(8.10 ± 1.20 s) (Student's t; $P < 0.001$; unequal variances). Groups exposed to isopentyl acetate buzzed above baseline levels (12 Hz) for an average of 39.53 ± 9.00 s (mean ± SE), whereas controls responded only for 2.03 ± 1.38 s (Student's t; $P < 0.003$; unequal variances). The maximal frequency in groups exposed to isopentyl acetate was 233 ± 17.83 Hz (mean ± SE), whereas controls had an average maximum of 20 ± 13.66 Hz (Student's t; $P < 0.001$; equal variances).

Effects of trytophan, 5-hydroxytryptophan and DOPA on the response to isopentyl acetate

All doses of tryptophan produced a hyperactive condition in the groups of bees that did not permit measurement of the three

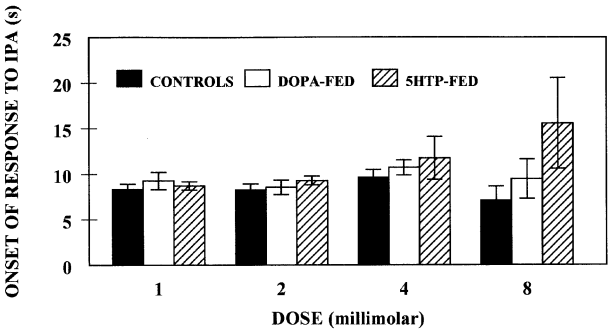

Fig. 2. Effects of ingested L-DOPA and 5-hydroxytryptophan on the onset of the buzzing response elicited from small groups of fifteen worker bees by exposure to 5% isopentylacetate in paraffin oil. The timing of the buzzing response began with the 30-s presentation of an applicator swab soaked in the 5% isopentyl acetate at the bottom screen of each cylinder (see Fig. 1). There were no significant differences in the onset of the buzzing responses between controls (black bars), DOPA-fed bees (open bars) and 5-hydroxytryptophan-fed bees (striped bars) for any of the doses tested.

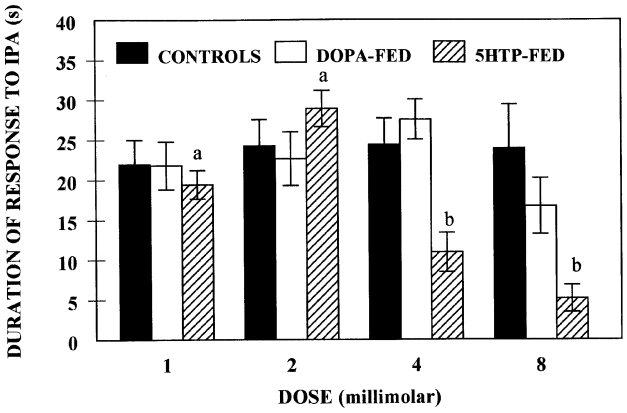

Fig. 3. Effects of ingested L-DOPA and 5-hydroxytryptophan on the duration of the buzzing response elicited from groups of fifteen worker bees by exposure to 5% isopentylacetate in paraffin oil. Only high doses of 5-hydroxytryptophan (striped bars) significantly reduced the duration of the buzzing response to 5% isopentyl acetate; bars with the same letter are not significantly different as determined by the Tukey mean separation test ($\alpha = 0.05$). Controls (black bars) and DOPA-fed bees (open bars) were not significantly different at all doses tested. Each bar respresents the mean (± SE) duration for ten groups of bees.

behavioural parameters (onset, duration and maximum). The baseline noise level for control bees never exceeded 12 Hz. For all doses (0.5, 2, 4 and 8 mM) of tryptophan the baseline noise level exceeded 20–30 Hz and sporadically exceeded 200 Hz throughout the test. The irregular and unpredictable buzzing from the tryptophan-fed bees made statistical evaluation of an isopentyl acetate response impossible.

Neither DOPA nor 5-hydroxytryptophan significantly affected the onset of buzzing responses versus untreated controls

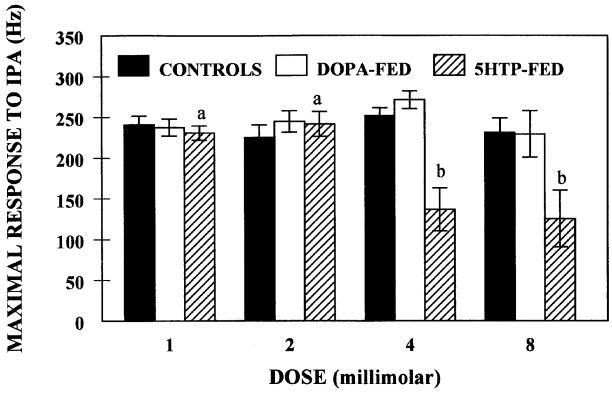

Fig. 4. Effects of ingested L-DOPA and 5-hydroxytryptophan on the maximal response during the buzzing response elicited from groups of fifteen worker bees by exposure to 5% isopentylacetate in paraffin oil. The maximal response (Hz) was correlated to the sound intensity produced by the buzzing bees. Only high doses of 5-hydroxytryptophan (striped bars) significantly reduced the maximal response to 5% isopentyl acetate; bars with the same letter are not significantly different, as determined by the Tukey mean separation ($\alpha = 0.05$). Controls (black bars) and DOPA-fed bees (open bars) were not significantly different at all doses tested. Each bar represents the mean (± SE) maximal response for ten groups of bees.

for any dose tested (Fig. 2). None of the model terms were significant sources of variation: main drug treatment term ($P > 0.08$), dose term ($P > 0.33$) and the treatment–dose interaction term ($P > 0.35$). Although there were no significant differences in onset between drug treatments, those groups of bees fed the highest dose of 5-hydroxytryptophan required twice as much time to respond as did controls (Fig. 2). Perhaps higher doses of 5-hydroxytryptophan would have an effect on the onset of the isopentyl acetate response.

Only 5-hydroxytryphtophan had an effect on the duration of the isopentyl acetate response, and the highest doses (4 and 8 mM) were most effective at reducing the total duration of the isopentyl acetate response (Fig. 3). Groups fed DOPA were not significantly different from controls, regardless of the dose. All model terms were significant sources of variation: drug treatment term ($P < 0.002$), dose term ($P < 0.003$) and the drug treatment–dose interaction term ($P < 0.002$).

Only high doses of 5-hydroxytryptophan significantly reduced the maximal response produced by groups of bees exposed to isopentyl acetate (Fig. 4). L-DOPA had no significant effect. All model terms were significant sources of variation: drug treatment term ($P < 0.001$), dose term ($P < 0.03$) and the drug treatment–dose interaction term ($P < 0.001$).

Effects of ingested amine precursors and brain amine levels in individual bees

Levels of all three precursors (L-DOPA, 5-hydroxytryptophan and tryptophan) were found to increase in the brains of bees with an increase in the dose ingested by bees (Figs 5–7).

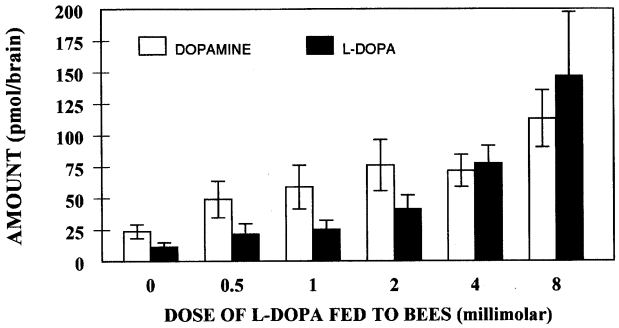

Fig. 5. Effects of various doses of ingested L-DOPA on L-DOPA (black bars) and dopamine (open bars) levels in the brains of worker honey bees. The brains were sampled for biogenic amine content 5 h after the experiment began (see Methods). Both brain L-DOPA and dopamine levels increased significantly ($\alpha = 0.05$) with increasing dose of ingested L-DOPA. Octopamine and serotonin levels were also measured from these brains, but their levels were not affected by ingested DOPA.

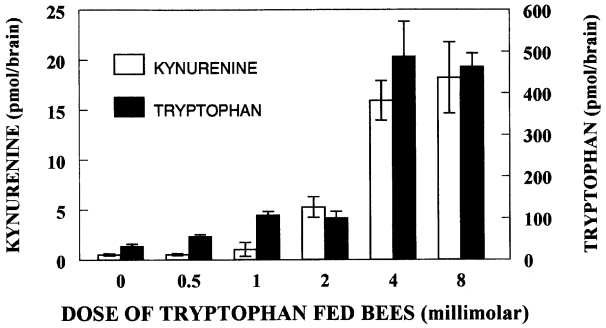

Fig. 7. Effects of various doses of ingested tryptophan on tryptophan (black bars) and kynurenine (open bars) levels in the brains of worker honey bees. The brains were sampled for biogenic amine content 5 h after the experiment began (see Methods). Both brain tryptophan and kynurenine levels increased significantly ($\alpha = 0.05$) with increasing dose of ingested tryptophan. Octopamine, dopamine and serotonin levels were also measured from these brains, but their levels were not affected by ingested tryptophan.

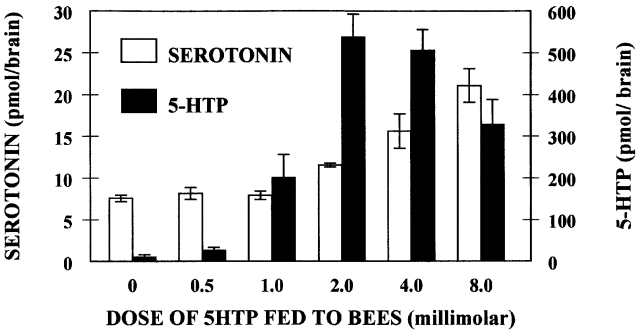

Fig. 6. Effects of various doses of ingested 5-hydroxytryptophan on 5-hydroxytryptophan (black bars) and serotonin (open bars) levels in the brains of worker honey bees. The brains were sampled for biogenic amine content 5 h after the experiment began (see Methods). Both brain 5-hydroxytryptophan and serotonin levels increased significantly ($\alpha = 0.05$) with increasing dose of ingested 5-hydroxytryptophan. Octopamine, dopamine and tryptophan levels were also measured from these brains, but ingested 5-hydroxytryptophan did not affect their levels.

This result would be expected if all three could cross the insect 'blood–brain barrier' to be absorbed by cells within the brain, and our results indicate that indeed these materials do cross the blood–brain barrier. Also, the levels of at least one metabolite were found to increase with increasing dose of ingested precursor for all three precursors. Dopamine ($P < 0.001$) and L-DOPA ($P < 0.001$) levels in the brain increased significantly with increasing dose of ingested L-DOPA (Fig. 5). Octopamine ($P > 0.45$) and serotonin ($P > 0.17$) did not increase with dose of ingested L-DOPA. For example, bees fed only sucrose ($n = 19$) had 9.21 ± 1.87 pmol/brain octopamine and 7.97 ± 0.26 pmol/brain serotonin, whereas bees fed 8.0 mM L-DOPA ($n = 7$) averaged 12.65 ± 4.63 pmol/brain octopamine and 8.87 ± 1.10 pmol/brain serotonin (mean $\pm$ SE).

Brain levels of serotonin ($P < 0.001$) and 5-hydroxytryptophan ($P < 0.001$) were elevated significantly with increasing dose of ingested 5-hydroxytryptophan (Fig. 6). Levels of octopamine ($P > 0.06$), dopamine ($P > 0.16$) and tryptophan ($P > 0.40$) were not significantly different between controls and 5-hydroxytryptophan-fed bees. For example, bees fed only 2 M sucrose ($n = 10$) averaged (mean $\pm$ SE) 5.54 ± 0.56 pmol/brain octopamine, 11.04 ± 1.45 pmols/brain dopamine and 94.66 ± 15.31 pmol/brain tryptophan. Bees fed 8.0 mM 5-hydroxytryptophan ($n = 8$) had 3.55 ± 0.79 pmol/brain octopamine, 9.67 ± 1.14 pmol/brain dopamine and 98.97 ± 6.95 pmol/brain tryptophan.

Brain levels of kynurenine ($P < 0.001$) and tryptophan ($P < 0.001$) increased with increasing dose of ingested TRP (Fig. 7). Brain levels of octopamine ($P > 0.08$), dopamine ($P > 0.31$) and serotonin ($P > 0.24$) were not affected significantly by ingested tryptophan. Bees fed 2 M sucrose ($n = 6$) averaged (mean $\pm$ SE) 1.89 ± 0.44 pmol/brain octopamine, 6.07 ± 1.12 pmol/brain dopamine and 2.91 ± 0.48 pmol/brain serotonin. Bees fed 8.0 mM tryptophan in sucrose ($n = 8$) averaged 2.70 ± 0.68 pmol/brain octopamine, 8.96 ± 1.40 pmol/brain dopamine and 2.27 ± 0.18 pmol/brain serotonin.

Discussion

It has been known for a long time that biogenic amines can modulate, through both excitatory and inhibitory effect, the dorsal unpaired median (DUM) motor neurones in the thorax and thereby potentiate specific muscular responses (Evans, 1980). Serotonin increased the frequency of spontaneous spikes in the DUM motor neurones in *Periplaneta americana* (Washio & Tanaka, 1992). The increased maximal frequency

induced by ingestion of serotonin and tryptophan in this study indicates some kind of modulation or potentiation of the thoracic muscles, which causes the increased frequency of the buzzing response.

Of the three ingested amine precursors (tryptophan, 5-hydroxytryptophan, and L-DOPA), 5-hydroxytryptophan was most effective at reducing the total duration and maximal response of the buzzing event produced by a 30-s exposure to 5% isopentyl acetate. The reductions in these two variables were dose-dependent. Also, it was shown that both 5-dydroxytryptophan and serotonin levels in the brains of bees increased in a dose-dependent manner with increasing dose of ingested 5-hydroxytryptophan. These experiments cannot show conclusively that reductions in the behavioural variables were related to either 5-hydroxytryptophan or serotonin, but the evidence suggests that some factor related to serotonin metabolism within the honey bee CNS may be responsible for reduced reactivity to the presentation of isopentyl acetate. It has been shown that serotonin often reduces the responsiveness of neural components (Erber *et al*., 1991, 1993), but more work is needed to locate and identify the exact mode of action.

Ingested tryptophan was shown to produce a hyperactive state in groups of bees, and it appears that this behaviour may be related to metabolic routes involved in the conversion of tryptophan into kynurenine and not to serotonin. Serotonin levels in the brains of bees did not increase within the first 5 h after ingestion of any dose of tryptophan. It seems likely that the hyperactive state in bees is more related to the increased kynurenine than to the increased brain tryptophan levels. This conclusion is supported by other studies which have indicated that tryptophan in the CNS tends to depress neural activity and that kynurenine stimulates CNS activity (Lopatina & Dolotovskaya, 1984; Lopatina *et al*., 1985).

Although ingested L-DOPA was found to elevate dopamine levels in the brain, it had no obvious effect on the buzzing response bees made in response to isopentyl acetate. This result suggests that dopamine may not be involved in neuromodulation of the olfactory response to isopentyl acetate. However, more than 5 h response time or higher doses may be needed for dopaminergic neural elements to respond to increased L-DOPA levels in the haemolymph and/or brain.

It was shown that a simple but effective procedure for evaluating a response to the honey bee alarm pheromone (isopentyl acetate) can be used to test the effects of ingested biogenic amine precursors on behavioural responses. The results of this study suggest that hyperactivity in bees that were fed tryptophan probably results from molecules derived from kynurenine metabolism. The reduced response to the alarm pheromone in bees that were fed 5-hydroxytryptophan probably arose from molecules derived from serotonin metabolism. One shortcoming of this current study is that the buzzing responses of small caged groups of bees to the presentation of isopentyl acetate were not correlated to the defence response of colonies in the field. However, Collins & Rothenbuhler (1978) showed significant correlation of caged responses to isopentyl acetate and field temperament of inbreed lines.

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