Immunocytochemical demonstration of octopamine-immunoreactive cells in the nervous system of *Locusta migratoria* and *Schistocerca gregaria*

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Summary. The distribution of octopamine in the metathoracic ganglion, brain and corpus cardiacum of *Locusta migratoria* and *Schistocerca gregaria* was investigated by means of immunocytochemistry with an antiserum against octopamine. The dorsal unpaired median (DUM) cells of the metathoracic ganglion were found to be strongly octopamine-immunoreactive. In the rostroventral part of the protocerebrum a group of seven immunopositive cells was demonstrated. Stained nerve fibres of these cells run into three directions: circumoesophageal connectives, midbrain, and optic lobes. As far as the protocerebrum is concerned, immunoreactive fibres were found in the central body, the protocerebral bridge, and in other neuropile areas. In the optic lobe a dense plexus of immunopositive fibres was found in the lobula and in the medulla. In the brain one other immunopositive cell was demonstrated, situated at the lateral border of the tritocerebrum. Octopamine could not be shown to occur either in the globuli cells of the mushroom bodies or in the dorsolateral part of the protocerebrum, where the perikarya of the secretomotor neurones are located that innervate the glandular cells of the corpus cardiacum. In the nervi corporis cardiaci II, which contain the axons of the neurones that extend into the glandular part of the corpus cardiacum, and in the corpus cardiacum proper no specific octopamine immunoreactivity could be found.

Key words: Octopamine – Immunocytochemistry – Nervous system – *Locusta migratoria* – *Schistocerca gregaria*

on the release of adipokinetic hormone from the glandular cells (Orchard and Loughton 1981; Orchard et al. 1983; Orchard et al. 1986; Pannabecker and Orchard 1986). A neuromodulator role of OA has been demonstrated in relation to neuromuscular transmission in insect skeletal muscle (Evans 1982). Under the dorsal surface of thoracic ganglia of several insects, a group of unpaired nerve cells, called DUM (dorsal unpaired median) neurones (Hoyle et al. 1974), is present including the so-called DUMETi-neurone, which is involved in the innervation of the tibia extensor muscle. Evidence for the octopaminergic nature of these neurones is derived from biochemical (Hoyle 1975) and pharmacological studies (Evans and O'Shea 1978; Evans 1981). Evidence is also accumulating for a neurohormonal role of OA. In *Schistocerca gregaria*, the concentration of OA in the haemolymph increases dramatically during the first 10 min of flight (Goosey and Candy 1980) and in response to stress (Orchard et al. 1981). Under these conditions circulating OA may act as a sympathetic or stress hormone increasing the availability of carbohydrate and lipid and stimulating the oxidation of these substrates (Goldsworthy and Wheeler 1986).

To date, OA was found to be present in the central nervous system of insects only by use of biochemical methods (for review, see David and Coulon 1985). In the present study the distribution of OA in metathoracic ganglion, brain and corpus cardiacum of *Locusta migratoria* and *Schistocerca gregaria* was investigated by means of immunocytochemistry with an antiserum raised against OA (Mons and Geffard 1987).

Materials and methods

Investigations were performed on adult male specimens of *Locusta migratoria*, 11 days after imaginal ecdysis. The insects were reared under crowded conditions, at 30° C, and at a relative humidity of 40%; they were fed on a diet of reed grass supplemented with rolled oats. After decapitation of the animals the brains, corpora cardiaca and metathoracic ganglia were dissected and fixed (a) in 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 1% sodium metabisulfite (SMB), final pH 7.4 or 4.0, for 1.5 h, at 0° C; or (b) in a mixture of glutaraldehyde 25 ml, picric acid 75 ml, and acetic acid 1 ml (GPA), containing 1% SMB, final pH 1.8. For comparison some brains and metathoracic

In the insects, octopamine (OA) has been proposed to act as a neurotransmitter, neuromodulator, or neurohormone (David and Coulon 1985; Evans 1985). Strong evidence for a neurotransmitter function has come from studies on the role of OA in light production in fire flies (Robertson and Carlson 1976; Carlson and Jalenak 1986). Also in the glandular part of the corpus cardiacum of locusts OA may have a neurotransmitter function. This evidence is derived mainly from pharmacological studies on the influence of OA on the cyclic AMP level in the corpus cardiacum and

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ganglia of adult *Schistocerca gregaria* (obtained from the Pharmacological Laboratory, University of Amsterdam, Dr. T. Piek) were used. After dehydration in increasing steps of alcohol with 1% SMB added to the aqueous component, the tissue was embedded in paraffin. Five- μ m serial frontal and horizontal sections (from the metathoracic ganglion also sagittal sections) were made and mounted on gelatin-coated slides.

Immunoreactivity was visualized by the peroxidase-antiperoxidase (PAP) technique of Sternberger (1979). The antiserum against OA was obtained by immunizing rabbits with OA conjugated with a protein carrier by glutaraldehyde. Before immunization the conjugate was reduced by borohydride. The antiserum obtained had a very high affinity for the protein carrier-OA conjugate. The crossreactivity with tyramine was greater than 1:100 (affinity of the OA antiserum was 100 times greater for OA than for tyramine), and with dopamine and noradrenaline 1:120 and 1:90, respectively (Mons and Geffard 1987).

After rehydration the sections were washed successively, for 5 min in tap water, for 2×5 min in 0.05 M Tris-HCl buffer, pH 7.6, containing 1% SMB (Tris-HCl-SMB buffer), for 5 min in a 0.13 M sodiumborohydride (NaBH_4 , Fluka) solution in Tris-HCl buffer to saturate double bonds (McRae-Degueurce and Geffard 1986), and for 4×15 min in Tris-HCl-SMB buffer, followed by preincubation in normal goat serum diluted (1:25) in Tris-HCl-SMB buffer for 1 h, at 4°C . The sections were then incubated for 20 h, at 4°C , in the primary antiserum diluted 1:2000 to 1:5000 with Tris-HCl-SMB buffer, washed in Tris-HCl buffer for 2×10 min, and exposed to goat anti-rabbit IgG diluted 1:25 in Tris-HCl buffer, for 3 h, at 4°C . After washing for 2×5 min in Tris-HCl buffer, the sections were treated with rabbit PAP-complex (1:200) for 1 h, at 4°C , washed in Tris-HCl buffer (2×5 min), and then treated with 0.5 mg 3,3-diaminobenzidine (DAB)/ml Tris-HCl buffer containing 0.01% H_2O_2 for 7 min, at 20°C . Following two 5 min washes in Tris-HCl buffer and another wash in distilled water the sections were dehydrated and mounted with DPX.

To establish the specificity of the immunoreaction, in the tissue the primary antiserum was omitted or sections were immunostained with the primary antiserum that earlier had been subjected to a preadsorption treatment with a) OA (DL-OA, Sigma 0-0250) in a concentration of 10^{-7} , 10^{-5} or 10^{-3} M, with b) bovine serum albumin (BSA), or with c) one of the following glutaraldehyde-coupled conjugates: BSA-OA, BSA-p-tyramine (Fluka 93810), BSA-dopamine (Sigma H-8502), and BSA-noradrenaline (Fluka 74470). The conjugates were prepared according to Geffard et al. (1984). For preadsorption the OA antiserum, diluted at working dilution in Tris-HCl-SMB buffer, was incubated with the preadsorption component on a rocking table for 60 min at 37°C followed by 20 h at 4°C . Control antiserum without preadsorption component was treated similarly.

Results

With glutaraldehyde at pH 7.6 as fixative the histological structure of the tissue, especially of the corpus cardiacum, was mostly not well preserved. With glutaraldehyde at pH 4.0 instead of 7.6, a better structural preservation was obtained. The lower pH may cause a better penetration of the fixative. However, sectioning of the glutaraldehyde-fixed tissue was difficult in both cases. GPA gave an excel-

Table 1. Results of control reactions with preadsorbed antiserum. The BSA-amine conjugates were prepared according to Geffard et al. (1984). Given that the molar ratio of amine to BSA is about 40:1 (Geffard et al. 1984) and that the molecular weight of BSA is about 68000, it can be calculated that 2.5 μg BSA-amine is approximately equivalent to an amine concentration of 1.3×10^{-6} M. +++ Regular staining (result with OA antiserum without preadsorption); ++ moderate staining; + weak staining; — no staining

Octopamine antiserum at working dilution preadsorbed with:	Staining intensity
Octopamine 10^{-7} M	+
10^{-5} M	±
10^{-3} M	—
BSA-octopamine	
2.5 $\mu\text{g}/\text{ml}$ antiserum	+
25 $\mu\text{g}/\text{ml}$ antiserum	—
250 $\mu\text{g}/\text{ml}$ antiserum	—
BSA-dopamine	
2.5 $\mu\text{g}/\text{ml}$ antiserum	+++
25 $\mu\text{g}/\text{ml}$ antiserum	+++
250 $\mu\text{g}/\text{ml}$ antiserum	++
BSA-tyramine	
2.5 $\mu\text{g}/\text{ml}$ antiserum	+++
25 $\mu\text{g}/\text{ml}$ antiserum	++
250 $\mu\text{g}/\text{ml}$ antiserum	+
BSA-noradrenaline	
2.5 $\mu\text{g}/\text{ml}$ antiserum	+++
25 $\mu\text{g}/\text{ml}$ antiserum	++
250 $\mu\text{g}/\text{ml}$ antiserum	+
BSA	
250 $\mu\text{g}/\text{ml}$ antiserum	+++

lent histological structure, but in this case some cells and nerve fibres showed a non-specific immunostaining. This non-specific staining appeared as a positive reaction after omission of the first antiserum and can therefore be considered to be of methodological origin (Sternberger 1979). The non-specific reaction was exclusively restricted to some cells in the rostral part of the pars intercerebralis, to the nervi corporis cardiaci I, and to the corpus cardiacum (neurohaemal and glandular portion). Except for this non-specific staining after GPA fixation, no other differences between the effects of the two fixatives on the number and position of immunopositive cell bodies and nerve fibres were observed.

The immunopositive structures described below exhibited a specific immunoreaction; they were positive both after glutaraldehyde and after GPA fixation, and negative after omission of the first antiserum and after preadsorption tests with OA and BSA-OA (Table 1). This table also shows that the OA antiserum displays only a slight crossreactivity with the other amines used in the preadsorption tests. Some of the results of these tests are represented also in Fig. 3a–j. The results described below are based on immunostaining of about 30 animals of the species *Locusta migratoria* and of 4 animals of the species *Schistocerca gregaria*. No differences were observed between the two species.

Fig. 1a–e. Sections through the metathoracic ganglion of *Schistocerca gregaria* (a–c) and *Locusta migratoria* (d, e). Horizontal sections (a–c) with the rostral side of the ganglion at the top show the immunoreactive DUM-cells and the bifurcating bundle containing axons of these cells. Note stained fibres towards the frontally directed connectives (*thick arrows*) and towards nerve 1 (*thin arrow*). **Fig. 1d** is a frontal section through the rostral part of the metathoracic ganglion showing two DUM-cells at the dorsal aspect. **Fig. 1e** (frontal side of the ganglion is on the right) is a sagittal section displaying stained somata belonging to the DUM-cells (*thick arrow*) and five other dorsally situated, unpaired immunopositive cells (*thin arrows*). In the ventral part a perikaryon belonging to a pair of cells can be seen. Slightly oblique section as indicated by the absence of the unpaired immunopositive cells at the ventral aspect. Fig. 1a–c $\times 90$; Fig. 1d, e $\times 60$

In the frontal half of the metathoracic ganglion a group of 15–20 immunopositive neurones was found (Figs. 1a–e; 2a) located in the midline under the dorsal surface. Number and position of these cells correspond well with the data of Evans (1980, 1982) concerning the dorsal unpaired median (DUM) cells. Nerve fibres of the immunopositive perikarya form a compact bundle, which initially follows a caudal course, then bends sharply in ventral direction and subsequently in frontal direction. In a position ventral to the immunopositive perikarya from which the axons originate, the bundle shows a bifurcation (Figs. 1b, c; 2a). The two branches can be followed laterally up to the site where they leave the ganglion via the nervi 3, 4, and 5 (Burrows 1982). Thin fibres can be seen bifurcating from the laterally running branches and bending forward towards the nerve 1 (Burrows 1982) (Figs. 1c; 2a) and towards the frontally directed connectives (Figs. 1b, c; 2a). Caudal to the compact group of 15–20 immunoreactive neurones, a further five positive cell bodies can be detected at the dorsal side (Figs. 1e; 2a) and four other positive neurones at the ventral side of the metathoracic ganglion (Fig. 2a). In addition to these unpaired cell bodies a few small-sized, paired im-

muno-positive perikarya occur; they are situated in the ventral half of the ganglion (Fig. 2a). Fine immunopositive fibres from unknown origin form a plexus in the neuropile area of the ganglion (Fig. 1d, e).

In the rostroventral part of the protocerebrum, at the border with the tritocerebrum, a paired group of immunoreactive cells is present (Figs. 2c; 3a–j; 4a, b). These neurones form part of the so-called ventral cell-body group (Williams 1975), a paired cluster of cell bodies in which seven large and an undetermined number of smaller cells are present. Of these cells four large and three small neurones on each side are immunopositive. Reconstruction (as far as immunopositivity allows) of the fibres of the positive neurones showed immunoreactive branches of these fibres extending in three directions. One branch (Fig. 2c, branch 1) ascends in the centrodorsal direction. From the present study, it cannot be conclusively stated whether the immunopositive fibres observed in neuropile areas such as the mushroom body (Fig. 5a), protocerebral bridge (Fig. 5b), and central body (Fig. 5c), arise from this branch. The second branch (Fig. 2c, branch 2) descends in the tritocerebrum and circumoesophageal connectives. This branch is rather thin and,

Fig. 2. Diagrammatic representation of the metathoracic ganglion (modified after Hoyle (1978)) (a) and the brain of *Locusta migratoria* (b, c). In **Fig. 2a** the immunopositive neurones are indicated by closed circles. **Fig. 2b.** Brain with its major neuropile areas seen from the caudal aspect. **Fig. 2c** represents the horizontal brain sections (A–F) indicated in **Fig. 2b**. Cell body *VCBG* represents one of the seven immunoreactive perikarya in the ventral cell group. The branches of the immunopositive nerve fibres of this cell are labelled: 1 ascending to the midbrain; 2 descending to the circumoesophageal connectives; 3 ascending to the optic lobe with a side branch (3^b) crossing over. Note *T* a single immunopositive cell in the tritocerebrum. Immunopositive fibres and varicosities in the major neuropile areas are represented by dots. **Abbreviations:** *AC* anterior connectives; *AL* antennal lobe; *C* calyx of the mushroom body; *CB* central body; *DUM* dorsal unpaired median cells; *LA* lamina; *LO* lobula; *M* medulla; *N* 1,3,4,5 nerve trunks; *P* pedunculus of the mushroom body with α and β lobe; *PB* protocerebral bridge; *PC* paired cells; *T* immunopositive cell in tritocerebrum; *VCBG* immunopositive cell in ventral cell body group; *VUM* ventral unpaired median cells

Fig. 3a-j. Frontal sections through the ventral part of the protocerebrum showing immunopositive perikarya in the ventral cell-body group. **a, d, f** Normal immunoreaction in sections from three animals. **g** Result after omission of the first antiserum. **b, c, e, h, i, j** Effects of preadsorption of the first antiserum with, respectively, BSA, 250 µg (**Fig. 3b**), 10^{-5} M OA (**Fig. 3c**), BSA-OA, 25 µg (**Fig. 3e**), BSA-tyramine, 25 µg (**Fig. 3h**), BSA-noradrenaline, 25 µg (**Fig. 3i**), and BSA-dopamine, 25 µg (**Fig. 3j**). **Fig. 3a-j.** $\times 145$. Compare with Table 1

therefore, difficult to trace. The third branch (**Fig. 2c**, branch 3; **Fig. 4a-f**) initially runs in a caudal direction and then ascends along the caudal margin of the protocerebrum to the optic lobe. On its course to the optic lobe this bundle gives off a side branch (**Fig. 2c**, branch 3b; **Fig. 4d-e**), which crosses over to the contralateral side. In the optic lobe the immunoreactive fibres arborise in lobula and medulla (**Fig. 5e**). In the lobula a fine plexus of positive fibres and varicosities can be seen. In some layers of the medulla positive fibres are present, especially in those adjacent to the lamina. In the latter layers the arrangement of the positive fibre elements is highly ordered.

Of the three branches mentioned only the branch to the optic lobe (branch 3) forms together with the corresponding branches of the other immunopositive cells a compact bundle of fibres (**Fig. 4c-f**). Especially in oblique, frontally cut sections (**Fig. 4d-f**) this bundle was shown to con-

sist of seven individual fibres, indicating that each immunopositive cell sends a fibre into this bundle. Since the centrodorsally directed branches (branch 1 in **Fig. 2**) of the individual immunopositive neurones are not united in a compact bundle it is difficult to determine whether each of the immunopositive perikarya possesses such a branch. The same applies to branch 2.

In the brain only one other pair of cells shows immunopositivity. This strongly positive cell is situated at the lateral edge of the tritocerebrum (**Figs. 2c; 4d; 5d**); a fibrous extension of this neurone appears to branch in the neuropile of the tritocerebrum (**Fig. 5d**).

No immunopositivity could be observed either in the globuli cells (Kenyon cells) of the mushroom bodies (**Fig. 5a**) or in the lateral part of the protocerebrum, where the cell bodies of the secretomotor neurones are located that innervate the glandular cells of the corpus cardiacum

Fig. 4a-f. Horizontal (a-c) and oblique frontal sections (d-f) through the brain of *Locusta migratoria*, showing the immunopositive fibre tract to the optic lobe. The immunoreactive fibres are indicated by *thin arrows*. In sections 4d, e also the side branches (branch 3b in Fig. 2c) crossing over to the contralateral side (*thick arrows*) can be seen. In **Fig. 4d** note the single tritocerebral immunopositive cell (T). Fig. 4a-f $\times 90$

(Rademakers 1977); the same holds true for the nervi corpori cardiaci II, which contain the nerve fibres of these cells extending into the glandular part of the corpus cardiacum. In the corpus cardiacum no specific anti-OA immunopositivity could be observed; after glutaraldehyde fixation the corpus cardiacum was immunonegative (Fig. 5f). After GPA fixation, in the corpus cardiacum a non-specific positive reaction could be observed; the distribution and intensity of the immunocytochemical reaction product were the same in sections treated with anti-OA-serum and in sections subjected to the immunocytochemical procedure omitting the anti-OA-serum (Fig. 5g, h).

Discussion

A large amount of data indicates the presence of octopamine (OA) in the nervous system of insects (for review see David and Coulon 1985). The presence of OA in the DUM-cells is probably best established (Evans 1980, 1985). In DUM-cells, OA acts as a modulator of neuromuscular transmission (Evans 1982), but seems to be also a source of circulating OA during the onset of flight (Goosey and Candy 1982). In the present study the octopaminergic nature of the DUM-cells is confirmed by their immunopositive reaction with the anti-OA-serum. The number and posi-

Fig. 5a-h. Sections through brain, optic lobe and corpus cardiacum of *Locusta migratoria*. **Fig. 5a-c.** Immunoreactive fibres in mushroom body; note calyx (C), pedunculus (P) and globuli cells (Kenyon cells) (K) (5a), protocerebral bridge (PB) (5b), and central body (CB) (5c). **Fig. 5c** also shows immunopositive cell bodies of the ventral cell-body group (VCBG). **Fig. 5d.** Single tritocerebral immunopositive cell (T) emitting an axon to the neuropile of the tritocerebrum (*thick arrows*). This section also displays the fibres from the VCBG-cells to the optic lobe (*thin arrows*). **Fig. 5e** represents a section through the optic lobe with lobula (LO) and medulla (M). The incoming fibres from the VCBG-cells are indicated by *thin arrows*. **Fig. 5f-h.** Sections of the corpus cardiacum fixed in glutaraldehyde (Fig. 5f) or in GPA (Fig. 5g, h). **Fig. 5f, g.** Sections of the corpus cardiacum after normal immunocytochemical reaction; **Fig. 5h** section consecutive to section 5g, immunostained after omission of the first antiserum. CCS storage part of the corpus cardiacum; CCG glandular part of the corpus cardiacum; A lumen of the aorta. Fig. 5a-e $\times 90$; Fig. 5f-h $\times 145$

tion of the compact group of 15–20 immunoreactive cells located under the dorsal surface in the midline of the frontal half of the metathoracic ganglion of *Locusta migratoria* and *Schistocerca gregaria* match very well the data of Evans (1982) on the DUM-cells. Reconstruction from horizontal and sagittal sections shows a bifurcating bundle of coarse immunopositive fibres originating from the positive DUM-cell bodies. After bifurcation both branches retain the size of the original undivided bundle, which strongly suggests that the individual fibres bifurcate, as already described for one of these neurones, the DUMETi neurone, by Evans (1982). The two branches can be followed up to the site where they leave the ganglion. In the present study other immunopositive cells were observed both at the dorsal and the ventral side of the metathoracic ganglion. No further information on the course of their fibres was obtained.

Octopamine-immunoreactive neurones in the ventral cell group of the protocerebrum have never been described before. Judged from the morphology of their processes it is unlikely that the OA-immunoreactive neurones correspond to the ventral giant neurones or the tritocerebral commissure giant cell described by Williams (1975) and Bacon and Tyrer (1978) in *Schistocerca gregaria*.

The presence of OA in the optic lobe has been suggested earlier by biochemical methods (for review, see David and Lafon-Cazal 1979). The location of OA as demonstrated immunocytochemically in the present study corresponds very well with the site where Klemm (1976) supposed OA to be present. After incubation with highly fluorogenic amine substrates he observed a selective accumulation of these substances in the outer stratum of the medulla, suggesting that nerve processes in this region possess an amine uptake mechanism. On the basis of this evidence Klemm (1976) proposed the presence of a non-fluorogenic amine such as octopamine in this region of the medulla. The present study demonstrates this to be exactly the site where a dense plexus of immunopositive fibres can be seen.

The octopaminergic nature of the globuli cells of the mushroom bodies could not be confirmed. These cells or at least some of them were suggested to be octopaminergic by Dymond and Evans (1979), Evans (1980), and Mercer et al. (1983). In favour of this hypothesis is that all globuli cells, non-fluorescent with the Falck-Hillarp technique (Klemm 1976; Mercer et al. 1983), take up the dye neutral red (Mercer et al. 1983); this type of uptake has been claimed to be a property of aminergic cells (Stuart et al. 1974). Furthermore, by radioenzymatic assay, OA was described to be present in considerable quantities in micro-dissected samples of globuli cell bodies (Dymond and Evans 1979; Mercer et al. 1983). However, the globuli cell bodies do not take up highly fluorogenic compounds used to demonstrate non-fluorogenic amine-containing neurones (Klemm 1976). Also, the present findings are not indicative of the octopaminergic nature of (at least some of) the globuli cells. Therefore, the origin of the immunopositive fibres in the mushroom bodies remains unknown. The same applies to the immunopositive fibre elements in the central body and the protocerebral bridge.

OA is also supposed to be present in the brain-corpus cardiacum axis. The secretomotor neurones situated in the dorsolateral part of the protocerebrum, the axons of which innervate the glandular cells of the corpus cardiacum via the nervi corporis cardiaci II (Mason 1973; Rademakers 1977), have been proposed to be octopaminergic (Orchard

and Loughton 1981; Orchard et al. 1983). In the present study, however, no specific OA-immunoreaction was found either in the corpus cardiacum or in the perikarya of the secretomotor cells and in their axons innervating the glandular lobe of the corpus cardiacum. In light of the biochemical evidence for the presence of OA in the corpus cardiacum (David and Lafon-Cazal 1979; Orchard et al. 1986) and the pharmacological evidence for the involvement of OA in the release of adipokinetic hormones from the glandular cells (Orchard et al. 1983; Pannabecker and Orchard 1986), the absence of OA-immunoreactivity in the brain-corpus cardiacum axis is rather peculiar. However, since (i) in two other portions of the nervous system of the same animals and (ii) with the same first OA-antiserum, the supposed octopaminergic nature of certain cells and neuropile areas could be confirmed (as shown by biochemical and histochemical methods), the octopaminergic nature of the innervation of the glandular part of the corpus cardiacum must be reconsidered. The effects of OA and of agonists or antagonists of OA (i) on cyclic AMP level in the glandular part of the corpus cardiacum and (ii) on release of adipokinetic hormones from the glandular cells can possibly be caused by OA from other sources than endings of the nervi corporis cardiaci II. It is possible that OA, present in the haemolymph, penetrates the corpus cardiacum and brings about the above-described effects. In a preliminary study it was shown that horseradish peroxidase readily penetrates the corpus cardiacum from the haemolymph (Diederer et al. 1985).

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