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FLUORESCENCE MICROSCOPY AND MICROSPECTROFLUORIMETRY OF THE MONOAMINES IN THE BRAIN OF *LOCUSTA MIGRATORIA MIGRATORIOIDES* R. F. (INSECTA, ORTHOPTERA) WITH SPECIAL REGARD TO THE PROTOCEREBRUM

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Among the neurotransmitters of insects the presence of biogenic monoamines was first investigated in the whole body extracts where dopamine, noradrenaline as well as adrenaline were detected (ÖSTLUND, 1953; v. EULER, 1961).

Using the fluorescence histochemical method described by FALCK and HILLARP, numerous papers have been published concerning the localization of the biogenic monoamines in the central nervous system of insects (FRONTALI and NORBERG, 1966; PLOTNIKOVA, 1967; FRONTALI, 1968; KLEMM, 1968; 1971; MANCINI and FRONTALI, 1970; ČECH and KNOZ, 1970; ELOFSSON and KLEMM, 1972; SCHÜRMANN and KLEMM, 1973). KLEMM (1971 a) investigated with that method the distribution of biogenic monoamines even in the stomatogastric nervous system and in the corpora cardiaca of *Schistocerca gregaria*.

Serotonin, noradrenaline and dopamine have been demonstrated in body fragments containing the central nervous system of various insects (GERSCH et al., 1961; FRONTALI and HÄGGENDAL, 1969; HIRIPI and S.-RÓZSA, 1973).

Microspectrofluorimetric measurements have been reported only recently (BJÖRKLUND et al., 1970; KLEMM and BJÖRKLUND 1971; ELOFSSON and KLEMM, 1972; SCHÜRMANN and KLEMM, 1973). Mainly dopamine and less frequently noradrenaline have been detected. Serotonin was also found in the optic lobe of some insects (ELOFSSON and KLEMM, 1972).

The distribution of biogenic monoamines is not uniform in every respect in the central nervous system of insects even in closely related species. In certain parts of the brain there is a specific fluorescence in all species investigated, but in other ones it varies. The data regarding the presence of serotonin are contradictory. In spite of the fact that serotonin had been demonstrated for example in *Periplaneta* extract containing the brain as well as the corpora cardiaca and corpora allata (GERSCH et al., 1961), FRONTALI and NORBERG (1966) failed to find any yellow fluorescence using the fluorescence histochemical method, corresponding to the fluorophore of serotonin. Serotonin was not detected in *Anabolia nervosa* (Trichoptera) either in the extract of the whole head (KLEMM and BJÖRKLUND, 1971) or during the fluorescence histochemical investigation of supra- and suboesophageal ganglia (KLEMM, 1968; 1971).

In the central nervous system of *Locusta migratoria migratorioides* R. F. only some parts of the ventral cord and the tritocerebrum of the brain have been investigated by means of the fluorescence histochemical method (PLOTNIKOVA and GOVYRIN, 1966; PLOTNIKOVA, 1967).

The present work was intended partly at comparing the distribution of nerve elements containing biogenic monoamines in various parts of the brain (proto-, deuto- and tritocerebrum, except the optic lobe), and partly at investigating microspectrofluorimetrically the presence of serotonin the relatively great amounts of which were found during biochemical analyses of locusts (HIRIPI and S.-RÓZSA, 1973).

Material and methods

Adult specimens of *Locusta migratoria migratorioides* R. F. of both sexes were used for investigations. We had to modify in part the fluorescence histochemical method described by FALCK and OWMAN (1965), since the parameters of reaction elaborated on vertebrates failed to give acceptable results. KLEMM (1968) also reported on methodical difficulties. Our modifications concerned the conditions of reaction.

The brains without the optic lobes were quenched in isopentane cooled by liquid nitrogen and freeze-dried in an apparatus type HVG 1 (Ilmeneau, GDR). The cooling agent was dry ice-aceton mixture and the end vacuum reached 10^{-5} Torr. The formaldehyde treatment was carried out with paraformaldehyde of 80% relative humidity for 30 min at 80° C (during experiments durations from 15 min. till 3 hours this time proved to be optimal). After treatment the material was kept in a closed vessel over phosphorus pentoxide for 1 hour at room temperature in darkness. Embedding in paraffin lasted 1 hour at 60° C in vacuo. The specificity of fluorescence reaction was tested on a material, heated (30 min at 80° C) without formaldehyde gas. Serial sections of about 10 microns were either uncovered or covered with Entellan (Merck) + 10% xylene, however, the majority of micrographs were taken from uncovered slides. Zeiss NU2 microscope was used as a fluorescence microscope with BG12 excitation and OG1 ocular filters. The light source was HBO 200 mercury-vapour lamp.

Microspectrofluorimetric analysis was carried out from the protocerebral lobe containing fluorescence fibres and from the central body. Leitz-MPV cytophotometer attached to Leitz Ortholux II microscope was used with a photomultiplier type RCA 1P-21. Suitable interference filters were applied for taking the excitation and emission spectra. The correction of curves and the further details are described elsewhere (ZS.-NAGY and DEÁK, 1973). For chemical identification of fluorophores, the deparaffinized sections were treated with HCl-vapour according to the method of BJÖRKLUND et al. (1968).

Results

We found only green fluorescence in the parts of the locust-brain (proto-, deuto- and tritocerebrum, except the optic lobe). According to the controls, the fluorescence was the result of formaldehyde treatment, i.e. specific to the

biogenic monoamines. The most intensive fluorescence reaction occurred in the central body and in some parts of the corpora pedunculata. The characteristic features of parts of the brain termed according to BULLOCK and HORRIDGE (1965) is now described in detail.

I. *Protocerebrum*

1. Corpora pedunculata: The perikaryon layer and the calyx neuropile contained no fluorescing elements. The parts of the pedunculus adjacent to the calyx displayed intensely fluorescing varicose fibres of rather short diameter, running nearly parallel to the long axis of the pedunculus *(Fig. 1)*. In oblique or longitudinal sections of the pedunculus, a narrow fluorescence-free stripe could be observed running centrally over whole pedunculus.

The fluorescence reaction was more intense in the alpha and beta lobes than that of the pedunculus. In the sections of given thickness single fibres were not separated *(Fig. 2)*. The fluorescence showed some layering perpendicular to the longitudinal axis in both lobes but mainly in the beta lobe.

2. Central body: It showed very intense fluorescence in the centre of the brain consisting of two parts. Fluorescence was more intense in the dorsal part showing a septate shape like a fan *(Fig. 3)*. The ventral part was of paler fluorescence and of more homogeneous in appearance. The pictures give the impression that the fluorescing fibre bundles of the dorsal part pass over into the ventral one at some places *(Fig. 3)*.

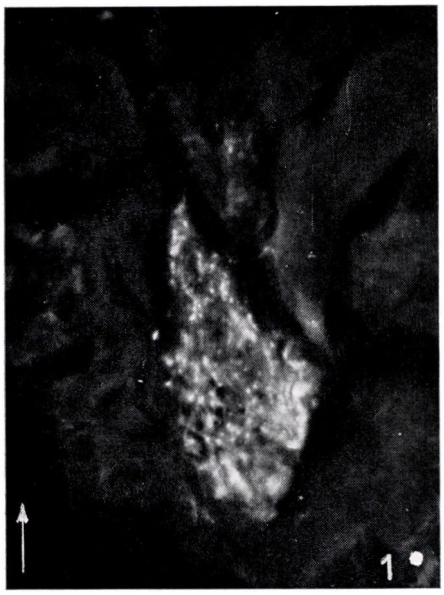

Fig. 1. Beginning of the pedunculus of mushroom body. Frontal section, ×470. (Arrow indicates the dorsal direction in each micrograph.)

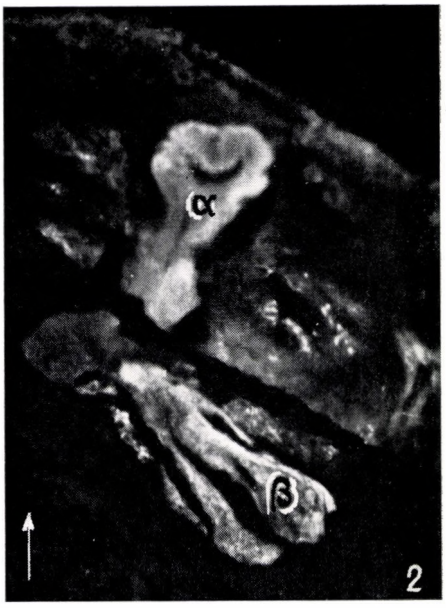

Fig. 2. The alpha *(α)* and the beta *(β)* lobes of mushroom body. Frontal section, ×190

3. Other protocerebral parts: The definite structures mentioned above are surrounded by the protocerebral neuropile. Scattered varicose, green fluorescing fibres were found even in this neuropile. These fibres formed groups in some parts *(Fig. 5)*, while there were only few of them elsewhere *(Fig. 4)*. Especially numerous fluorescing fibres were seen around the alpha lobe and in the vicinity of the frontal part of the central body, as well as in the fibre bundles running toward the optic lobe *(Fig. 5)*.

In the protocerebrum green fluorescing perikarya were also visible. They were solitary or grouped in the pars intercerebralis *(Fig. 6* and *7)*. Further investigations will reveal the topography and connections of those cells.

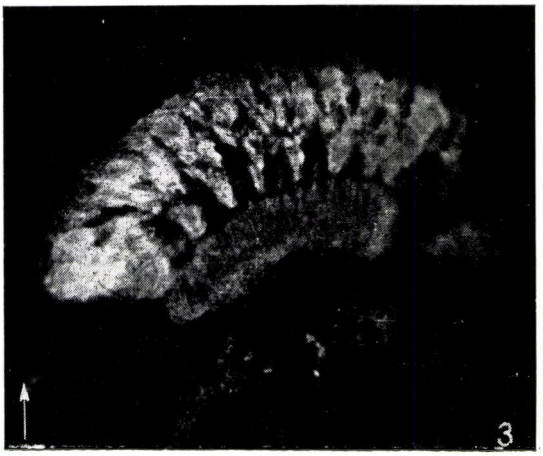

Fig. 3. Central body. The dorsal and ventral portions are well separated. Frontal section, ×190

Fig. 4. Scattered varicose fibres in the protocerebral lobe. Frontal section, ×470.

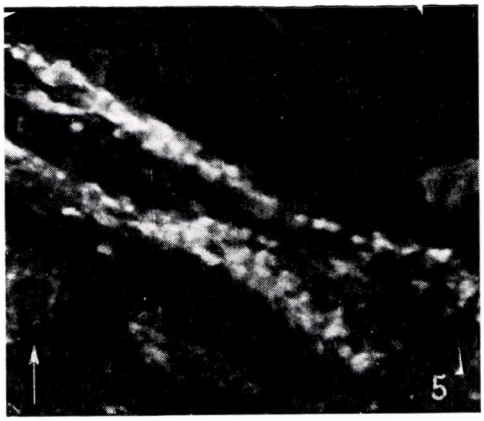

Fig. 5. Detail from the protocerebral lobe. The fluorescing varicose fibres are concentrated here and run toward the optice lobe. Frontal section, × 250.

Fig. 6. Solitary fluorescing perikaryon in the pars intercerebralis. Frontal section, ×470

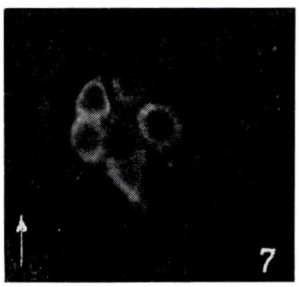

Fig. 7. Grouped fluorescing perikarya in the pars intercerebralis. Frontal section, ×470

II. *Deutocerebrum*

We failed to observe any specific fluorescence in this part of the brain. It should be noted that the structural preservation of this region was always worse than that of the other parts, therefore the absence of specific fluorescence should be not intergreted as an absence of biogenic monoamines.

III. *Tritocerebrum*

The neuropile contained uniformly distributed green fluorescin gfibres. In some places the varicose fibres were concentrated according to the description of PLOTNIKOVA and GOVYRIN (1966) concerning this area of the brain.

Microspectrofluorimetry:

1. Excitation spectra: The excitation spectra were registered in 3 quadrangular regions of $20 \times 20\ \mu^2$ size of both the central body and the fluorescing fibres of the neuropile of the protocerebral lobe. The averages of these measurements are shown in Figs 8 and 9. The excitation maximum is at 425 nm corresponding to that of the fluorophore originating from the catecholamines.

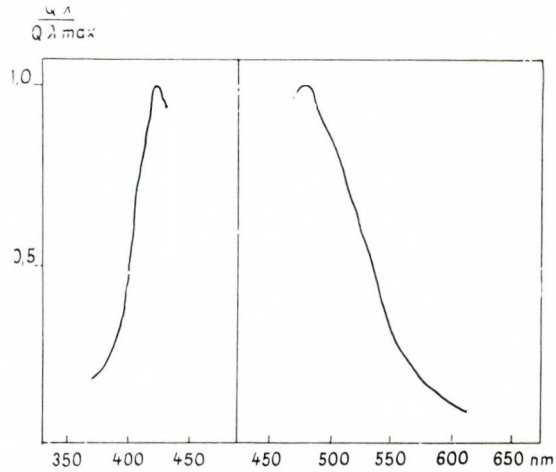

Fig. 8. Excitation (left) and emission (right) spectra of the fluorophore in the protocerebral neuropile. The spectra are expressed as relative quanta versus wavelength Corrected curves

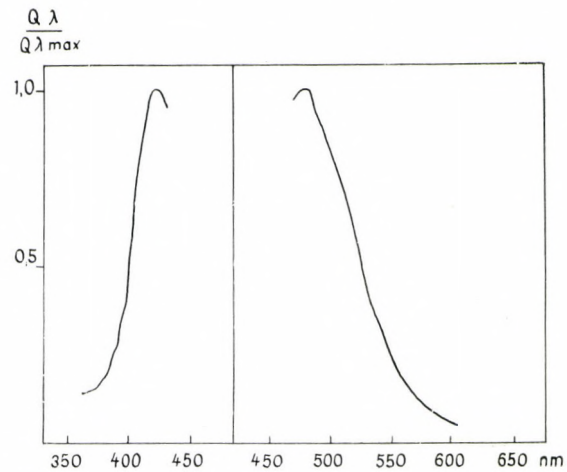

Fig. 9. Excitation (left) and emission (right) spectra of the fluorophore of central body The spectra are expressed as relative quanta versus wavelength. Corrected curves

2. Emission spectra: The emission spectra were recorded at an excitation wave-length of 410 nm. Under given methodical conditions (Zs.-Nagy and Deák, 1973) the recording of the emission spectrum was possible from 470 nm. Areas of 10×40 μ^2 containing at least 2—3 varicose fibres were measured in order to achieve the necessary intensities. The corrected curves are shown in *Figs 8* and *9*. The emission maximum is at 482 nm and the curve is characteristic of recordings obtained from vertebrate nerve elements containing only catecholamines (Björklund et al., 1968). There are no other maxima or plateau on the curves indicating the simultaneous presence of catecholamines and serotonin (Möllmann et al., 1972) in the parts of the brain under investigation.

Results of exposure to HCl-vapour: The excitation spectrum was altered already after very short treatments of 20—30 seconds in so far as the maximum shifted to shorter wave-lengths of about 390 nm. Extending the time of treatment to 2 minutes the maximum shifted further to 380 nm. Further increase of the exposure time to 4—5 min induced no change of the maximum. After exposures longer than 4 min strong background fluorescence appeared, therefore, the measurements became impossible. These results show that the fluorophore present in the neurons originates mainly from dopamine (BJÖRKLUND et al., 1968).

Using the fluorescence microscope with BG12 excitation filter, we failed to observe any changes after HCl-treatment. This is in accordance with the facts mentioned above.

Discussion

Our results partly agree, partly disagree with the data obtained on other Orthoptera and Trichoptera species. Apart from the pedunculus of mushroom body specific fluorescence had been detected in every part of the brain of other species where we observed too (FRONTALI and NORBERG, 1966; FRONTALI, 1968; PLOTNIKOVA and GOVYRIN, 1966; PLOTNIKOVA, 1967; KLEMM, 1968; 1971; MANCINI and FRONTALI, 1970; KLEMM and BJÖRKLUND, 1971). As regards the pedunculus, the literary data are contradictory. Specific fluorescence could not been detected in the pedunculus of *Periplaneta americana* (Orthoptera) nor in that of several Trichoptera species (FRONTALI and NORBERG, 1966; KLEMM, 1968; 1971), whereas in the brain of *Acheta domesticus* (also Orthoptera) the pedunculus proved to be rich in fluorescing fibres (SCHÜRMANN and KLEMM, 1973). Our observations agree with those of the latter authors who are on the opinion that the globuli cells of the mushroom body are of catecholaminergic character in spite of the fact that catecholamines are found only in the nerve fibres and not in the soma. Those authors explained the exclusive axonal occurrence of catecholamines with the absence of synapses around the somas, hence the catecholamines required for the synaptic functions are produced and stored only in the axons, while the soma contains catecholamines only below the threshold of demonstrable amount. The question is further complicated by the fact that the fibres of the globuli cells are also present in the calyx glomeruli (VOWLES, 1955) where fluorescence characteristic of catecholamines was not found. Since the globuli cells are considered to be unipolar, it has to be supposed that the branches of the same nerve can chemically be differentiated in so far as one of them entering the calyx neuropile does not contain detectable amount of catecholamines, whereas the other one reaching other parts of the mushroom body does. The chemical differentiation may be related to the postsynaptic, dendritic character of the intrinsic fibres present in the calyx neuropile (ZS.-NAGY and B.-MUSKÓ, 1972) while in the pedunculus and in both lobes the intrinsic fibres are presynaptic, axonic in character.

A further question is what kind of mediation realizes the transmission of impulses in species where the pedunculus contains no catecholamines? since synaptic structures were encountered in the pedunculus of all species investig-

ated so far electron microscopically (SCHÜRMANN, 1970; ZS.-NAGY and B.-MUSKÓ, 1972; SCHÜRMANN and KLEMM, 1973).

The microspectrofluorimetric analyses show that the fluorophore originates only from catecholamines in the regions investigated. In cells where catecholamines occur together with serotonin, e.g. in the glomus caroticum of rabbit, the emission curve recorded by microspectrofluorimetry after FALCK—HILLARP method is characteristically of a "two-humped" appearance (MÖLLMANN et al., 1972). It has been shown that this curve represents mathematical summation of emissions of catecholamines and serotonin. On the basis of our results we have to deny the assumption that the fluorophores of catecholamine and serotonin would be present together in the fluorescing regions only, the green fluorescence of the former covers the yellow one of the latter. Such simultaneous presence has been assumed among others by SCHÜRMANN and KLEMM (1973) in the central body of the *Acheta* brain. The questions of localization of relatively great amount of serotonin revealed by biochemical analyses of homogenates of locust ganglions (HIRIPI and S.-RÓZSA, 1973) remains yet to be answered. Since the latter authors analysed homogenates containing the whole cerebral ganglion including even the optic lobes as well as the suboesophageal ganglia, it is possible that serotonin is bound to the parts have not been investigated by us. In other species some neuropile regions of the optic lobe have shown yellow fluorescence characteristic of serotonin (ELOFSSON and KLEMM, 1972). It cannot, however, be excluded that the serotonin present in the cerebral ganglion fails to react to the formadehyde treatment for some special reason, hence fluorescing products do not appear. The fluorescence intensity of serotonin fluorophore is about three times lower than that of catecholamines of the same concentration (JONSSON, 1971), nevertheless, owing to that in *locust* the concentration of serotonin is about 50 percent higher than the concentrations of noradrenaline and dopamine together (HIRIPI and S.-RÓZSA, 1973), it should be expected to find areas containing either only yellow fluorescence at some places or emission curves indicating the presence of catecholamine-serotonin mixture. The question is further complicated by the findings that in Trichoptera species serotonin was not found in the head (KLEMM and BJÖRKLUND, 1971) and in the supra- and suboesophageal ganglia (KLEMM, 1968; 1971) either biochemically or histochemically. In the central nervous system of *Periplaneta americana* serotonin was detected by GERSCH et al. (1961), while FRONTALI and NORBERG (1966) failed to observe serotonin in the supra- and suboesophageal ganglion using the fluorescence histochemical method. Due to the above contradictions one can draw no definitive conclusion regarding the cellular localization of serotonin on the basis of data available.

The green fluorescence observed in the locust brain behaved upon HCl-treatment similarly to dopamine in model experiments (BJÖRKLUND et al., 1968). Therefore, one can state that the great majority of the fluorophore originate from dopamine, and only small portion may do so in noradrenaline. According to biochemical measurements (HIRIPI and S.-RÓZSA, 1973), the quantity of noradrenaline occurring in the locust is 6 times less than that of dopamine. Even in other species dopamine proved to be predominant among the catecholamines in both biochemical and microspectrofluorimetric as well as electron histochemical examinations (FRONTALI and HÄGGENDAL, 1969; KLEMM and BJÖRKLUND, 1971; MANCINI and FRONTALI, 1970).

Summary

The cerebral ganglion of the *Locusta migratoria migratorioides* was investigated except the optic lobe. Only green fuorescence was found after FALCK—HILLARP formaldehyde reaction in the regions investigated. In the mushroom body green fluorescence was observed in the pedunculus, alpha and beta lobes, but was not in the globuli cell layer and the calyx neuropile. The most intense fluorescence was detected in the central body. Varicose, fluorescing fibres are present now and then in the protocerebral neuropile as well as in the tritocerebrum, in some places they are grouped. We failed to observe any fluorescing structures in the deutocerebrum.

The microspectrofluorimetric analysis of the fluorophore revealed that the fluorescence originates only in catecholamines, fluorophores of catecholamines and serotonin do not occur simultaneously in the regions investigated. The exposure to HCl-vapours proved that the catecholamines consist mainly of dopamine, in accordance with other species.

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A *LOCUSTA MIGRATORIA MIGRATORIOIDES* R. F. (INSECTA, ORTHOPTERA) AGYA MONOAMIN TARTALMÁNAK FLUORESZCENS MIKROSZKÓPOS ÉS MIKROSPEKTROFLUORIMETRIÁS VIZSGÁLATA KÜLÖNÖS TEKINTETTEL A PROTOCEREBRUMRA

B.-Muskó Ilona, Zs.-Nagy Imre és Deák György

Összefoglalás

A *Locusta migratoria* cerebrális ganglionja képezte vizsgálat tárgyát, kivéve az optikus-lebenyt. A vizsgált területeken csak zöld fluoreszcencia fordult elő a FALCK—HILLARP formaldehid reakció után. A zöld fluoreszcencia a gombatest részei közül megfigyelhető volt a pedunculusban, az alfa- és béta-lebenyben, de hiányzott a globulussejtek rétegében és a kalyxban. A legintenzívebben a corpus centrale fluoreszkált. Elszórt, varikózus, fluoreszkáló axonok láthatók a protocerebrális neuropilben, valamint a tritocerebrumban, helyenként tömörülve. A deutocerebrumban nem sikerült fluoreszkáló területeket megfigyelni.

A fluoreszcencia mikrospektrofluorimetriás vizsgálata azt mutatta, hogy csak katecholaminoktól származik, szerotonin és katecholaminok fluoroforja keverten nem fordult elő a vizsgált területeken. A HCl-kezelés azt bizonyította, hogy megegyezően más rovarfajokkal, a katecholaminok túlnyomó többségét dopamin képezi.