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PAPER CHROMATOGRAPHIC AND FLUOROMETRIC EXAMINATION OF THE SEROTONIN CONTENT IN THE NERVOUS SYSTEM AND OTHER TISSUES OF THREE FRESHWATER MOLLUSCS

(Anodonta, Unio, Lymnaea)

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Extensive studies were performed concerning the role of serotonin (5HT) in vertebrates and invertebrates (GARATTINI 1965, ERSPAMER 1966). In the latter group of animals it may function as a stimulating transmitter (WELSH 1957, KOSHTOYANTS 1957). The effect of serotonin has been examined and described in various organs and representants of Molluscs (HILL 1958, KOSHTOYANTS and RÓZSA 1961, AIELLÓ 1957, 1960, GERSCHENFELD and STEFANI 1962, 1962, SALÁNKI 1963, TWAROG 1964, 1966).

That 5HT might have the role of a transmitter was indicated not only by its occurrence only (WELSH and MOORHEAD 1960, DAHL et al. 1966), but also by the fact that the 5HTP decarboxylase enzyme which is involved in synthesis was also demonstrated (WELSH and MOORHEAD 1959, KERKUT and COTTRELL 1963, CARDOT 1966). The monoamino oxidase enzyme, however, which is responsible for the decomposition of 5HT was not demonstrable in the nerve, but only in the other tissues (BLASCHKO and HOPE 1957, KERKUT and COTTRELL 1963).

Examinations were performed concerning the 5HT content of the nervous system and other tissues in a number of molluscan species by chromatographic, fluorometric and histochemical methods (WELSH and MOORHEAD 1960, DAHL et al. 1966, ZS.-NAGY 1967, SAKHAROV and ZS.-NAGY 1967). The results show that in the stage of development of the two classes of Molluscs the serotonin content of the tissues was higher in Lamellibranchs than in Gastropods. Only little data are available concerning the 5HT content of the tissues in freshwater molluscan species. The present investigations were made with the objective to compare the serotonin content of the various tissues in three species.

Method

In the present study the cerebral, visceral and pedal ganglia, the cerebro visceral connective (CVc), the adductors, the tissue of the mantle, gill and the Heart in the *Anodonta cygnea* L.; the cerebral, visceral and pedal ganglia, the CVc, adductors further the tissues of the mantle and the gill in *Unio pictorum* and the pharyngeal ganglia of *Lymnaea stagnalis* were examined. Tissue samples were prepared immediately before the experiment and were stored at ice

temperature for a period not longer than 1 hour before use. The measurements were conducted in the period between October and January.

Paper chromatographic examination

The ganglia of *Anodonta* and *Lymnaea* were examined paper chromatographically. The ganglia were homogenized in 70 percent acetone and centrifuged (10 000 r. p. m.). The supernatant was decanted and the residue was extracted again and centrifuged. The two supernatants were collected and concentrated by evaporation at room temperature to a small volume and dipped onto WHATMAN No 1 paper and developed by ascendent technique at room temperature for 4—12 hours. Solvents of the following composition were used: butanol-acetic acid-water (4 : 1 : 5); 20 percent KCl in water; N-butanol-30 percent methylamine solution (8 : 3). The ganglion extract was developed parallel with the mixture of the extract and authentic substances and with the authentic substances themselves. To develop the spots EHRLICH-reagent and ninhydrin-acetic acid fluorescent reagent was used (ERSPAMER 1966).

Fluorometric examination

Two extraction methods were used. The reagents were prepared according to the method applied by BOGDANSKI (1956).

1. In the case of ganglia and CVc the micro analitical method applied by KUNTZMANN (1961) was used with a few modifications: 5—50 mg tissue was homogenized in 1 ml 0.1 N HCl solution and the homogenizate was transferred into glass-stoppered shaking tube. Thereafter the homogenizator was washed with 0.5 ml water and the wash was added to the homogenase. The pH was adjusted approximately to 10 with anhidrous Na_2CO_3, and 0.5 ml borate buffer (pH 10) was added. Next, 1 g NaCl and 4 ml butanol were added and the tube was shaken for 30 min. After centrifuging 3 ml of the butanol phase was transferred into another test tube containing 6 ml heptane and 1 ml 0.1 N HCl. The tube was shaken. Centrifugation followed and 0.9 ml of the acid phase was transferred into a test tube containing 0.27 ml cc HCl. Excited in quartz cuvette at 300 mμ, fluorescence was measured at 540 mμ (uncorrected instrument values). Blank reagent (in which the tissue aliquot was replaced by water), standard (i.e. serotonin) and ,,internal standard'' (tissue aliquot + serotonin) were taken through the same procedure. A recovery of 85—102 percent was obtained by adding serotonin to the tissue aliquot.

2. In the case of muscle, mantle, gill and the Heart the method described by BOGDANSKI (1956) was used. In the case of the muscle and the mantle butanol extraction was preceded by protein precipitation with $ZnSO_4$ (ERSPAMER 1966).

Reagent blank, standard and ,,internal standard'' were simultaneously also taken through the same procedure as the sample. Measurement was performed at 540 mμ with excitation at 300 mμ (uncorrected instrument readings). A recovery of 86—105 percent was obtained.

The measurements were performed in Aminco—Bowman spectrophotofluorometer. Excitation and emission spectra were taken with Cimagraphe (Type 30/40 GZ) recorder. When taking the emission spectra GG14 colour filter was used for filtering out reflection which presented itself at 360 mμ maximum.

Results

Examination by paper chromatography

When examining the ganglion extract of *Anodonta* two spots could be discerned on the chromatographic paper, whereas in case of *Lymnaea* one spot only *(Fig. 1)*. Rf values of the ganglion extracts and of the authentic substances

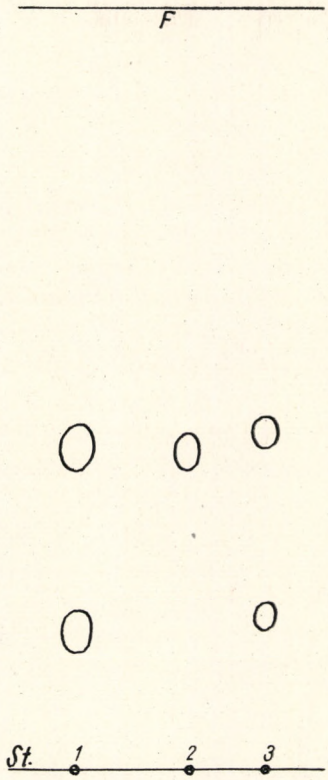

Fig. 1. 1. Ganglion extract of *Anodonta*, 2. Ganglion extract of *Lymnaea*. 3. 5HT and 5HTP chromatograms. Developing by ascending technique. The solvent used: *n*-butanol: acetic acid: water (4 : 1 : 5). Development with EHRLICH reagent

1. ábra. 1. *Anodonta* ggl, 2. *Lymnaea* ggl. extraktum, 3. 5HT és 5HTP kromatogramja Futtatás felszálló kromatogramként *n*-butanol : ecetsav : viz (4 : 1 : 5), futtatószerben. Előhívás EHRLICH reagenssel

developed simultaneously are illustrated in *Table 1.* The Rf values of the ganglion extract were somewhat lower as compared to the authentic substances, nevertheless it was possible to identify them as 5HT and 5HTP in case of *Anodonta* and as 5HT in case of *Lymnaea*. If, namely, 5HT and 5HTP was added to the extracts the intensity of the corresponding spots increased, and the Rf values were in that case too smaller than those of 5HT and 5HTP.

Table 1.
Rf values of 5HT and 5HTP of the ganglion extracts in *Anodonta*, *Lymnaea* and control

Solvents	*Anodonta* RF_1	ggl. RF_2	*Lymnaea* ggl. Rf	Authentic 5HT Rf	Authentic 5HTP Rf
Butanol: acetic acid: water (4 : 1 : 5)	0.39—0.47	0.17—0.21	0.39—0.46	0.45—0.47	0.20—0.22
20 percent KCl in water	0.33—0.38	0.38—0.42	0.32—0.37	0.36—0.38	0.40—0.44
Butanol: 30 percent methylamine (8 : 3)	0.57—0.60	—	0.56—0.60	0.59—0.61	—

Examination by fluorometry

Besides identifying serotonin by paperchromatography it has been identified also on basis of its excitation and emission spectrum. In a 3 N HCl solution serotonin has maximum fluorescence at 550 mμ when excited at 295 mμ (UDENFRIEND, BOGDANSKI and WEISSBACH 1955).

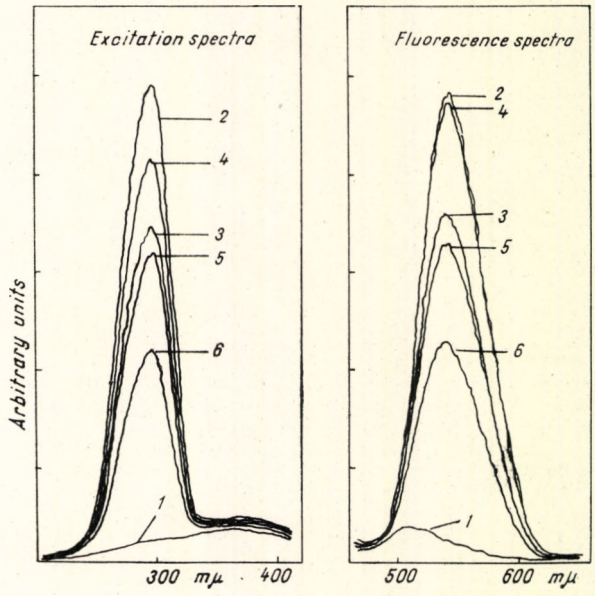

Fig. 2. Spectra of the extracts of the ganglia and the CVc of *Unio*:
1. blank reagent, 2. standard 5HT, 3. cerebral ganglia, 4. pedal ganglia, 5. visceral ganglia, 6. CVc

2. ábra. Unio ggl. és CVc extraktumok spektrumai:
1. reagens blank, 2. standard 5HT, 3. cerebrális ggl., 4. pedális ggl., 5. viscerális ggl., 6. CVc

The maxima of both excitation and emission spectra of the tissues examined by us well corresponded to the maxima of authentic serotonin, and they were at 298—300 mμ and 538—540 mμ respectively (all values are uncorrected instrument readings). Such a spectrum is presented in *Figs. 2* and *3*.

Table 2 summarizes concentration values of serotonin in the various tissues, expressed in μg 5HT/g fresh weight unit. In the case of ganglia and the CVc the 5HT content of a pair of ganglia and CVc are also given. The values are the averages of 5—20 measurements.

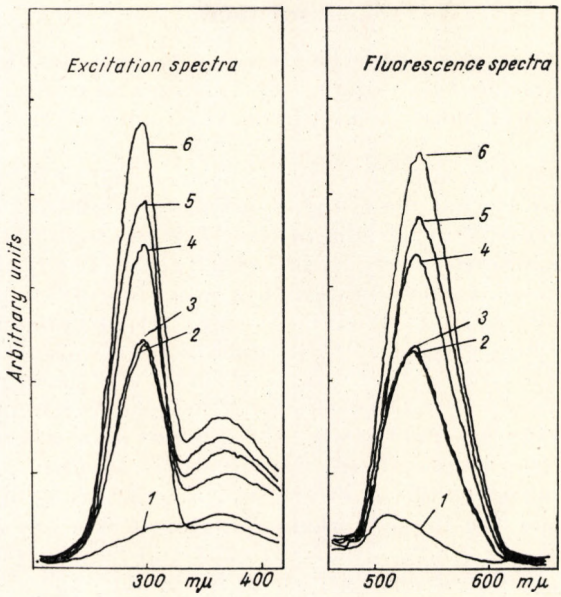

Fig. 3. Spectra of mantle and muscle extracts of *Anodonta:*
1. blank reagent, 2. muscle, 3. mantle, 4. muscle + 5HT, 5. standard 5HT, 6. mantle + 5HT

3. ábra. Anodonta köpeny és izom extraktumok spektrumai: 1. reagens blank, 2. izom, 3. köpeny, 4. izom + 5HT, 5. standard 5HT, 6. köpeny + 5HT

Table 2.
Serotonin concentration in the various tissues (fluorimetric measurement)

	ANODONTA		*UNIO*		*LYMNAEA*
	μg 5HT/g wet weight	μg 5HT/ specimen	μg 5HT/g wet weight	μg 5HT/ specimen	ggl. μg/g wet weight
Cerebral ganglion	72.5	0.19	112	0.19	
Visceral ganglion	43.5	0.23	66	0.20	14
Pedal ganglion	66.5	0.30	108	0.31	
CVc	38.0	0.34	53.5	0.096	
Muscle	0.33		0.22		
Mantle	0.37		0.33		not
Gills	0.25		0.90		examined
Heart	0.0		not examined	not examined	

As the results show the 5HT-content of the ganglia of *Unio* is the highest. As regards ganglion tissue the values were higher in the case of *Unio* than in case of *Anodonta*. 5HT-content of the muscle and the mantle was nearly the same in both species, whereas in the gill of *Unio* almost four times as much 5HT was demonstrable than in the gill of *Anodonta*.

In the *Lymnaea* only the ganglia were examined concerning their 5HT content. The value obtained was considerably lower than in the case of *Anodonta* or *Unio*. In the heart serotonin was not demonstrable.

Discussion

The paperchromatographic Rf values of the ganglion extracts well conform to those of authentic serotonin. The maxima of excitation and emission spectra similarly well correspond to the maximum of the spectrum of the authentic serotonin.

As evidenced by quantitative data obtained, out of the species examined the 5HT content of the ganglia of the *Unio* was the highest, it was in somewhat less amount present in the ganglia of *Anodonta*, whereas the 5HT content of the *Lymnaea* was approximately by one order lower. The results obtained well conform to previous literary data (WELSH and MOORHEAD 1960, DAHL et al. 1966, KERKUT and COTTRELL 1966) indicating that serotonin is of more general occurrence in the nerve tissues of the mussel, than in those of the snail. 30—60 μg/g 5HT was demonstrated by DAHL (1966) in the ganglia of *Anodonta piscinalis*, whereas in those of *Helix pomatia* only 4 μg/g. As examined by KERKUT and COTTRELL (1963) the 5HT content in *Helix aspersa* was of the same value (0.5—4 μg/g). Also histochemical examinations confirm this observation. A fluorescence indicative of the presence of serotonin was observed in nearly every nerve cell of the mussel, whereas in the nervous system of the snail only in a few cells (ZS.-NAGY 1967, SAKHAROV and ZS.-NAGY 1967). Out of the ganglia of the Lamellibranchs highest 5HT content expressed in wet weight unit was demonstrable in the cerebral ganglia, lower values were obtained in case of pedal ganglia, whereas in the visceral ganglia the 5HT concentration values amounted only to about 60 percent of that of the cerebral ganglia. As evidenced by DAHL (1966) 5HT content (62 μg) was the highest in the pedal ganglia of *Anodonta piscinalis*, in comparison to the other ganglia of the same species, i.e. it was less in the cerebral ganglia (58 μg) and in conformity to our results by about 50 percent less in the visceral ones (30 μg).

5HT content of CVc is relatively high. Nearly the same 5HT concentration was measured in the CVc and in the ganglia. This is in contradiction to histochemical data evidencing that the neuropil of the ganglia and the axons themselves do not contain 5HT (ZS.-NAGY 1967). This suggested, therefore, that the high 5HT content of the CVc is the result of the transport of 5HT originating from the ganglia.

The concentration of serotonin is low in the adductors, nevertheless, it is easily demonstrable. With regard to this it is assumable, that it is involved in the neuromuscular transport as it has been suggested previously on basis of physiological examinations (TWAROG 1954, 1966).

In the mantle and the muscle the concentration of 5HT was the same. The low 5HT content of the latter tissue is ascribed to the presence of nerve elements, by which it is thought the low concentration might be explained.

Considerable difference in the serotonin content of the gill was found between *Anodonta* and *Unio*. The concentration of serotonin in the gill of the former was 0.25 μg, while in the latter 0.9 μg. Serotonin concentration of the same order (0.1—1.0 μg/g) was demonstrated by AIELLO (1962) in the gill of MYTILUS. This agent appears to be involved in the regulation of the movement of the cilia.

It is only the tissue of the heart, out of the tissues examined in which serotonin was not demonstrable. Were serotonin present in the heart its concentration would possibly fall below 0.02 μg/g. In one instance about 8 g heart tissue obtained from 120 animals was extracted by the method described. Had serotonin been present in a concentration of 0.02 μg/g this would have presented itself in a final concentration of 0.10—0.16 μg/1.5 ml; serotonin is in such concentration namely demostratable by the applied method. Also the high activity of monoaminooxidase might explain the absence of serotonin. With regard to this, however, we have no data at our disposal.

Summary

Examinations on the concentration of serotonin by paper chromatography and fluorometric method in the central nervous system and other tissues of *Anodonta cygnea* L., *Unio pictorum* and *Lymnaea stagnalis* L. have led to the following conclusions:

1. 5HT was demonstrable in the ganglia, the cerebrovisceral connective (CVc) of the gill, the adductors and the mantle of *Anodonta* and *Unio*. In the heart, however, its presence could not be demonstrated.

The order of 5HT concentration in the case of *Anodonta:* cerebral ggl. (72.5 μg/g), pedal ggl. (66.5 μg/g); visceral ggl. (43.5 μg/g); CVc 38 μg/g; mantle (0.37 μg/g); muscle (0.33 μg/g); gill (0.25 μg/g), in the case of *Unio:* cerebral ggl. (112 μg/g); pedal ggl. (108 μg/g); visceral ggl. (66 μg/g); CVc (53.5 μg/g); gill (0.9 μg/g); mantle (0.33 μg/g); muscle (0.22 μg/g).

2. The ganglia of the *Lymnaea* contain less 5HT than those of the Lamellibranchs.

3. The high serotonin content of the CVc speaks in favour of the possibility of serotonin transport.

4. The low serotonin-content of the adductor indicates that the presence of this compound is connected to the nerve elements, and it is suggested that it might be related to its functioning as a neuromuscular transmitter.

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Összefoglalás

SZEROTONIN PAPIRKROMATOGRÁFIÁS ÉS FLUORIMETRIÁS VIZSGÁLATA HÁROM ÉDESVIZI MOLLUSCA *(ANODONTA, UNIO, LYMNAEA)* IDEGRENDSZERÉBEN ÉS MÁS SZÖVETEIBEN

Hiripi László

Papírkromatográfiásan és fluorimetriásan vizsgálva a szerotoninmegoszlást *Anodonta cygnea* L., *Unio pictorum* és *Lymnaea stagnalis* L. központi idegrendszerében és egyéb szöveteiben azt találtuk, hogy

1. *Anodonta* és *Unio* ganglionjai a cerebro-viscerális connectivum (CVc), a kopoltyú, záróizmok és köpeny tartalmaznak 5HT-t, a szívben azonban nem sikerült kimutatni.

Az 5HT koncentráció sorrendje: *Anodonta* esetében: cerebrális ggl. (72,5 μg/g); pedális ggl. (66,5 μg/g); viscerális ggl. (43,5 μg/g) CVc (38 μg/g); köpeny (0,37 μg/g); izom (0,33 μg/g); kopoltyú (0,25 μg/g).

Unio esetében: cerebrális ggl. (112 μg/g); pedális ggl. (108 μg/g); viscerális ggl. (66 μg/g); CVc (53,5 μg/g); kopoltyú (0,9 μg/g); köpeny (0,33 μg/g); izom (0,22 μg/g).

2. *Lymnaea* ganglion kevesebb 5HT-t tartalmaz, mint a Lamellibranchiatáké.

3. A CVc magas szerotonin-tartalma szerotonin transzport lehetőségét támasztja alá.

4. A záróizom alacsony szerotonin-tartalma a szerotonin idegelemekhez kötött jelenlétére utal, ami feltételezett neuromuscularis transmitter szerepével függhet össze.

ИССЛЕДОВАНИЕ СЕРОТОНИНА ПРИ ПОМОЩИ БУМАЖНОЙ ХРОМАТОГРАФИИ И ФЛУОРИМЕТРИИ В НЕРВНОЙ СИСТЕМЕ И ДРУГИХ ТКАНЯХ ТРЕХ ВИДОВ ПРЕСНОВОДНЫХ МОЛЛЮСКОВ (*ANODONTA*, *UNIO*, и *LYMNAEA*).

Л. Хирипи

При исследовании распределения серотонина методами бумажной хроматографии и флуориметрии в нервной системе и других тканях беззубки, перловицы и большого прудовика установлено, что:

1. Ганглии, церебро-висцеральные коннективы (ЦВК), жабры, запирательные мышцы и мантия беззубки и перловицы содержат серотонин, однако в сердце его выявить не удалось. По содержанию серотонина в мг/г ткани беззубки распределены следующим образом: церебральные ганглии 72,5; педальные ганглии 66,5; висцеральные ганглии 43,5; ЦВК 38; мантия 0,37; мышцы 0,33; жабры 0,25. Содержание в тканях перловицы: церебральные ганглии 112;педальные ганглии 108; висцеральные ганглии 66; ЦВК 53,5; жабры 0,9; мантия 0,33;мышцы 0,22.

2. Ганглии большого прудовика содержат меньше серотонина, чем ганглии исследованных пластинчатожаберных.

3. Высокое содержание серотонина в ЦВК указывает на возможный транспорт серотонина в ЦНС.

4. Низкое содержание серотонина в запирательной мышце указывает на то, что он локализован здесь в нервных элементах. Обсуждается предполагаемая роль серотонина в нервно-мышечной передаче.