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BIOLOGICALLY ACTIVE COMPOUNDS IN THE GLOCHIDIA OF ANODONTA CYGNEA L. I. IDENTIFICATION OF TRYPTAMINE AND SOME AMINO ACIDS BY PAPER CHROMATOGRAPHY

KATALIN S.-RÓZSA and ELEMÉR LÁBOS

Biological Research Institute of the Hungarian Academy of Sciences, Tihany, Hungary

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Examinations conducted on agents functioning as either local hormones or transmitters in the stimulatory processes occurring in the early state of the ontogeny of Mollusca are incomplete. It was suggested that 5-HT and tryptamine may, due to their motor increasing effect, be of importance on the veliger larvae of marine gastropods (KOSHTOYANTS, BUZNIKOV and MANUKHIN 1961) and on the glochidia of *Anodonta* (LÁBOS, SALÁNKI and S.-RÓZSA 1964), respectively.

In this present paper we shall report on the chemical analysis conducted by us on active compounds extracted from the glochidia of *Anodonta* and also data obtained by biological testing will be presented.

Method

The glochidia of *Anodonta cygnea* L. were used as test objects. The larvae were obtained from the gill of the mother.

Extraction

The glochidia taken from the gill of full-grown specimens of *Anodonta* were put into Lake Balaton water. The mucous binding substance was removed by repeated washing. 20 g of the washed glochidia was homogenized in 20 ml 50% ice-cold acetone under constant cooling and was placed for 30 mins into a refrigerator at +5° C. Thereafter the homogenized sample was centrifuged for 30 mins at 3500 r.p.m. and the supernatant was evaporated under reduced pressure and taken up in 10% isopropanol solution (1—2 ml). This solution was used for analysis by paper chromatography. In case of biological testing the extract was taken up in snail Ringer solution (MENG 1958) or in Lake Balaton water. Evaporation and development was performed in nitrogen saturated atmosphere.

Chromatographic analysis

The ascending, one-dimensional paper chromatography was employed on Whatman No 1 paper. The following solvents were used: butanol-acetic acid-water (4 : 1 : 5), isopropanol-ammonia-water (8 : 1 : 1), 8% NaCl solution.

Development in NaCl lasted for 1 hour, in the other two solvents for 10—18 hours.

After chromatographic separation the chromatograms were dried and the spots were made visible either with 0.2% ninhydrin (SMITH 1960) at 105° C, or with Erlich- reagent (CURSON and LEEDS 1955), at room temperature. The spots visualized with ninhydrin were viewed under ultraviolet light were both 5-HT and tryptamine appeared as greenish fluorescing spots (RODNIGHT 1959).

Biological testing

Biological testing was performed on glochidia and on the isolated heart preparations of *Helix pomatia* L. In case of glochidia, testing was made as described previously in course of studies on the effect of other agents (LÁBOS, SALÁNKI and S.-RÓZSA 1964), the isolation of *Helix* heart was made as described previously (S.-RÓZSA and GRAUL 1964).

The extract obtained from the glochidia was tested, without chromatographic analysis, in various dilutions in Ringer solution adapted for snail and in Lake Balaton water.

For examining heat stability of the biologically active agents aliquots were taken and kept in a water bath for 15 mins at 100 °C.

The following agents were used as controls and applied dissolved in 10% isopropanol solution: tryptamine (Fluka), 5-hydroxytryptamine creatinin-sulphate (Fluka) and various amino acids.

The experiments were carried out in spring months (March, April) within one season.

Results

1. Chromatographic analysis

On the chromatograms several ninhydrin positive spots were discernible. One of them exhibited greenish fluorescence under ultraviolet light. In the following this spot was identified by running it dissolved in various solvents together with control tryptamine. Upon evidence of data obtained by us the spot revealed by fluorescence may be taken for tryptamine. 5-hydroxytryptamine exhibiting a similar fluorescence cannot be considered because of its Rf value.

It is noteworthy that in case of short term (1—3 hours) development of freshly prepared extracts a fluorescent spot was revealed on every occasion at

Table 1

Presents RF values of one substance of the extract and of control tryptamine in case of different solvents

Solvent	Rf value	
	glochidium extract	tryptamine
Isopropanol-ammonia-water (8 : 1 : 1)	0.71	0.71
Butanol-acetic acid-water (4 : 1 : 5).....	0.75	0.76
NaCl 8%	0.54	0.57

the same height with control tryptamine, in case of long-term development, however, (16—18 hours) a nonfluorescent positive ninhydrin spot was observed in the place of tryptamine.

Similarly, on every occasion, a non-fluorescent spot that stained pinkish-purple with Ehrlich-reagent was seen at the same distance with control 5-HT (Rf = 0.40). This spot cannot be considered identical with 5-HT, for it does not exhibit flurescence under ultraviolet light, not even if short term development technique was applied.

Development in NaCl, when it did not last beyond 1 hour, produced colour reactions indicative of indolalkylamine decomposition products.

1. bluish-grey Rf = 0.75
2. blue Rf = 0.41
3. yellow Rf = 0.65

Out of them the 1st and 3rd fades quickly and the stability of the 2nd lasts longer. These facts suggest that in the glochidia an important role is played by indolalkylamine metabolism.

In the course of tryptamine-determinations the single amino acids were, as expected, best resolved in the solvent: butanol—acetic acid—water. In the followings the single amino acids were run as controls together with the separated and eluated spots. In this way five different amino acids were identified. Phenylalanine was identified on ninhydrin pretreated paper and with 1% $NaHCO_3$ treatment after which the colour reaction of other amino acids disappeared and only that of phenylalanine remained. Tyrosine was detected with Ehrlich-reagent and the other amino acids with ninhydrin. The Rf values of these amino acids are summarized in *Table 2*.

Table 2

Identification of amino acids in
butanol : acetic : acid : water (4 : 1 : 5) mixture

Spot of glochidium extract	Rf	Amino acid	Rf
1	0.15	tyrosine	0.14
2	0.20	valine	0.20
3	0.36	tryptophan	0.36
4	0.39	leucine	0.38
5	0.50	phenylalanine	0.51

The other five compounds having high Rf values (0.59—0.80—0.92——0.96—0.98) could not be identified with amino acids. It cannot be excluded that there are in the extract peptides which are separable and detectable on paper with identical methods (WELSH and FRONTALI 1966, JAEGER 1966).

2. Biological testing

5, 10, 20 and 30fold dilutions of the extract produced alike stimulation on isolated *Helix* heart preparations. Subsequently, the effect of 1 : 10 dilution was studied. The increase of amplitude and frequency produced

is similar to the activity increasing effect of biologically active amines. Initial sharp rise which is characteristic of the effect of 5-HT was never observed *(Fig. 1)*.

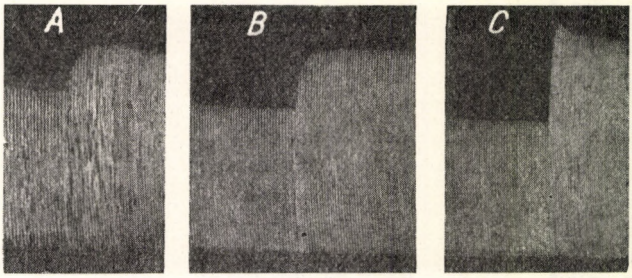


Fig. 1. Biological testing conducted on the isolated heart preparation of *Helix* *A* — tryptamine (10^{-5} M), *B* — extract of glochidium (diluted to 1 : 10), *C* — 5-hydroxytryptamine (10^{-5}M)

1. ábra. Biológiai tesztelés izolált *Helix* szíven.
A — triptamin (10^{-5} M), *B* — glochidium extraktum 10×-es hígításban
C — 5-hydroxytryptamine (10^{-5} M)

If the extract was applied dissolved in 5 ml Lake Balaton water the glochidia responded with an initial increase of activity, within 10—15 mins, however, this phenomenon ceased *(Fig. 2A)* and 80—90% of the animals was closed by this time. In case the extract was used after dilution the sharp initial increase of activity was missing and a gradual increase of activity was observed which lasted for more than 20 mins *(Fig. 2B)*. This activity increase was different from that produced by tryptamine *(Fig. 2D)*, namely, its level was the same in the first 10 mins and in the interval between 10—20th mins. Greater dilutions produced similar effect.

Heat treatment of the extract of glochidia increased the effectiveness of the active compounds in them. Thus on *Helix* heart the activity increasing effect of the extract increased by 20—40% after boiling, and in the case of glochidia, too, a characteristic, more than 20 min long activity was produced. This effect is more pronounced when using a 1 : 5 dilution *(Fig. 2C)*. On the other hand the ratio of closures in the boiled extract decreases by 20—45%. A greater ratio of closure was not produced even if the boiled extract was applied for more than 20 mins.

Discussion

In the freshly collected acetone extracts of glochidia tryptamine was demonstrable. From among biologically active agents tryptamine is the most effective in producing an increase in the rhythmic activity of glochidia (LÁBOS, SALÁNKI and S.-RÓZSA 1964). After detection with ninhydrin the component present in the extract exhibited greenish fluorescence under ultraviolet lamp and stained purple with Ehrlich reagent and was located at the same level with tryptamine. 5-HT was not demonstrable in the extract.

Tyrosine, valine, tryptophan, leucine and phenylalanine were also identified in the acetone extract by parallel development with amino acids. The

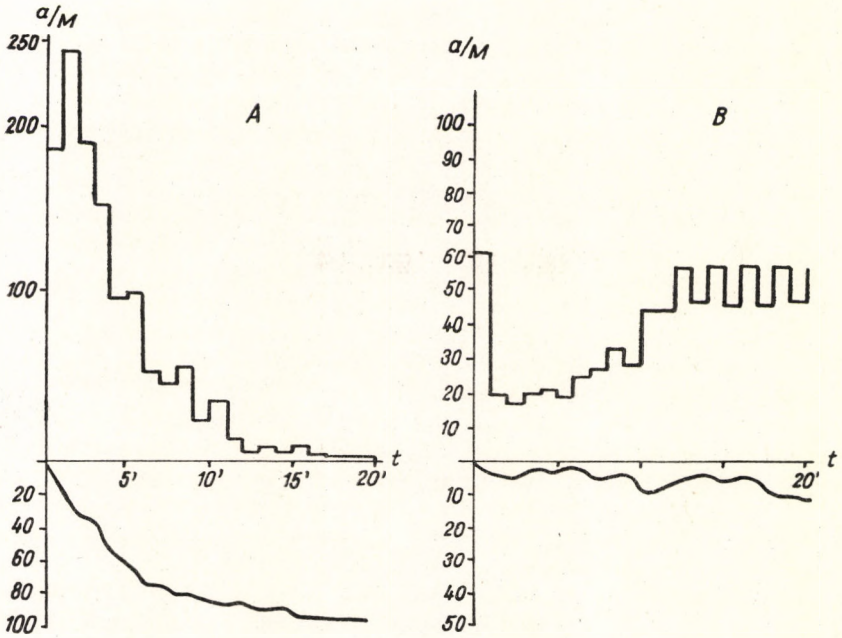
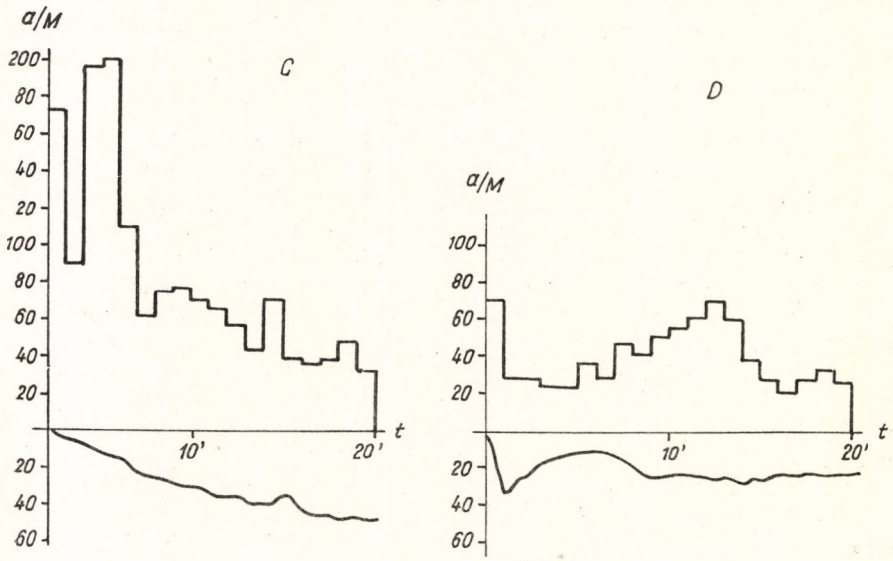


Fig. 2. Biological testing on glochidia. *Abscissa:* time in minutes. *Ordinate:* number of contractions per minutes. The lower curve expresses the ratio of closured in percentage of glochidia. *A* — glochidium extract diluted to 1 : 5, *B* — glochidium extract diluted to 1 : 10, *C* — 5 fold diluted glochidium extract after boiling for 10 minutes, *D* — 10 100 μg/ml tryptamine

2. ábra. Biológiai tesztelés glochidiumokon. *Abszcissza:* idő percekben, *ordináta:* a kontrakciók percenkénti száma. Az alsó görbe a bezárt glochidiumok arányát adja %-ban. *A* — glochidium extraktum 5-ös hígításban, *B* — glochidium extraktum 10-es hígításban, *C* — 5-ös hígítású glochidium extraktum 10 perces forralás után, *D* — 100 μg/ml triptamin



other five components were not identified for lack of control. In the case of the nonidentified factors the investigations have to be extended for peptides which are applied as stimulatory factors on molluscs (JAEGER 1966, WELSH and FRONTALI 1966). It is not out of question that besides biologically active amines peptides, too, are involved in the stimulatory effects produced on glochidia.

Data obtained by biological testing show that in the acetone extract of glochidia stimulatory factors are predominating. Not even highly concentrated extract produced inhibition on *Helix* heart. The concentrated extract produced on the glochidia also considerable tonicity. The increase of rhythmic activity produced is similar to that produced by ions (initial effect), and by tryptamine (an effect lasting for more than 10 mins). On heat treatment the stimulation produced by the extract both on *Helix* heart and on the rhythmic activity of glochidia increased. At the same time, however, its effect on closures of glochidia decreased. This implies that the factor which produces tonic effects is not heat stable and changes on the effect of heating. The observation, however, that the effect produced by the stimulatory factor increases upon heat treatment suggests that a portion of it may be present in the extract also in bounded form which is activated on the effect of heating. It might be also inferred, as an explanation to this phenomenon, that due to the decreased tonicity, there are more glochidia in the condition of producing rhythmic activity.

The presence of tryptamine in the extract of glochidia supports our previous supposition that this agent might be considered as a local hormone in these larvae (LÁBOS, SALÁNKI and S.-RÓZSA 1964). It has to be emphasized that three amino acids: tyrosine, phenylalanine and tryptophan that are the precursors of important, biologically active amines were also detected by us. The presence of tyrosine and phenylalanine emphasizes again the role played by the catecholamines to which we attributed previously an inhibitory function (LÁBOS 1966). It is also imaginable that depending on changes in the receptor structures and enzyme systems different agents may take the main role in mediation in the different states of ontogeny. This might explain in the case of *Anodonta* the difference of mediation observed among full-grown specimens and in specimens in different states of ontogeny.

Summary

1. Tryptamine is detectable in the acetone extract of glochidia by paper chromatography. This serves as a new evidence for the suggestion that this agent functions as a local hormone in these larvae.

2. From among the amino acids tyrosine, valine, tryptophan, leucine and phenylalanine were identified in the extract of glochidia by paper chromatography.

3. It was established by biological testing that the activity of some stimulatory factors in the extract of the glochidium increases on the effect of heat. The factor producing tonic contraction is heat-sensitive and its activity decreases on heat-treatment.

4. It is assumed that in the different states of ontogeny different biologically active amines are functioning as transmitters in the case of *Anodonta* species.

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BIOLÓGIAILAG AKTÍV ANYAGOK ANODONTA CYGNEA GLOCHIDIUMAIBAN I. TRIPTAMIN ÉS NÉHÁNY AMINÓSAV PAPÍRKROMATOGRÁFIÁS IDENTIFIKÁLÁSA

S.-Rózsa Katalin és *Lábos Elemér*

1. Glochidiumok acetonos extraktumában papírkromatográfiásan kimutatható triptamin, amely újabb érv ezen ágens lokális hormon szerepére nézve ezen lárvákon.

2. Aminósavak közül a tirozint, valint, triptofánt, leucint és fenilalanint identifikálták papírkromatográfiásan, glochidiumok extraktumából.

3. Biológiai tesztelés eredményeként megállapítást nyert, hogy a glochidium-extraktum bizonyos serkentő anyagainak aktivitása hő hatására növekszik. A tónusos zárást létrehozó faktor hőérzékeny, aktivitása hőkezeléssel csökken.

4. Feltételezik, hogy a fejlődés különböző szakaszaiban más-más biológiailag aktív amin tölt be transzmitter szerepet *Anodonta*-kon.

БИОЛОГИЧЕСКИ АКТИВНЫЕ ВЕЩЕСТВА В ГЛОХИДИЯХ БЕЗЗУБКИ I. ИДЕНТИФИКАЦИЯ ТРИПТАМИНА И НЕКОТОРЫХ ДРУГИХ АМИНОКИСЛОТ ПОСРЕДСТВОМ БУМАЖНОЙ ХРОМАТОГРАФИИ

Каталин Ш.-Рожа и *Элемер Лабош*

1. — Триптамин был выявлен в ацетоновых экстрактах глохидиев беззубки при помощи бумажной хроматографии. Наличие этого агента подчеркивает его значение как локального гормона в глохидиях.

2. — Помимо триптамина были идентифицированы следующие аминокислоты: тирозин, валин, триптофан, леуцин и фенилаланин.

3. — В результате биологического тестирования (на сердце виноградной улитки и глохидиях) было установлено, что активность стимулирующего вещества экстракта глохидиев увеличивается при нагревании, а фактор, вызывающий тоническое закрытие глохидиев, является термолабильным, его активность при нагревании снижается.

4. — Предполагается, что на разных стадиях развития беззубки разные биологически активные амины выступают в качестве медиаторного вещества.