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BIOCHEMICAL, FLUORESCENCE MICROSCOPIC AND ULTRASTRUCTURAL STUDIES ON BIOGENIC MONOAMINES IN THE GUT OF HELIX POMATIA

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Abstract

Authors studied the innervation of the gut in *Helix pomatia* with simultaneous chemical, fluorescence microscopic and electron microscopic methods. On the basis of the chemical measurements it was determined that adrenaline, noradrenaline, dopamine and serotonin are all present in the snail gut in a significant amount. Serotonin was demonstrable in the largest amount (1.26 μg/g wet weight), at the same time, the amount of noradrenaline was lower by two orders (0.05 μg/g wet weight). The fluorescence microscopic studies confirmed the results of the chemical determination and demonstrated a large amount of fluorescent fibres, fibre bundles and neurons in the complete intestinal tract. Electron microscopically neurons, synaptic neuropil and terminal nerve fibres were found in the snail gut muscle. Dense-core vesicles with diameters of 100 nm were observed both in the nerve cell bodies and the axon terminals directly neighbouring the muscle fibres.

The observations obtained by the above complex methods serve unambiguous evidence for the aminergic innervation of the snail gut.

Key words: Helix pomatia, gut, biogenic monoamine, fluorescence studies, ultrastructure.

Introduction

The scientific interest towards molluscs, and within this snails has been increased significantly in last few decades. Besides scientific viewpoints, there are also economical causes of this interest: snails are valuable export goods and they are also animals causing damage in agriculture and so they, cannot be underestimated. From scientific point of view snails have particularly proved to be good test objects for neurobiological researches, as large, well accessible neurons have been found in the central nervous system, which have been used for complex neurophysiological and neuromorphological studies. The majority of the neurobiological studies have been done on the central nervous system (ELEKES, 1983; KISS et al., 1977; OSBORNE et al., 1982; S-RÓZSA et al., 1974), but studies have also been continued on the peripheral nerve areas (BENEDECZKY, 1977; ERDÉLYI et al., 1972; HERNÁDI et al., 1983; KISS et al., 1982; S-RÓZSA et al., 1964).

ÁBRAHÁM (1940) had turned his attention to the rich innervation of the snail gut quite a long time ago. Despite this, the first ultrastructural studies (ÁBRAHÁM, 1983; HALASY et al., 1983; TÁNCZOS et al., 1979) had only commenced with great delay, but supported well the early light microscopic observations. During the course of electronmicroscopic studies nerve cell bodies and large amounts of nerve terminals were found between the muscle fibres. On the basis of the ultrastructural characteristics HALASY et al. (1983) separated 3 types of nerve terminals. The majority of the

terminals contained neurosecretory granules therefore it was assumed that these correspond to peptidergic terminals. Nerve terminals containing dense-core vesicles were also detected, which were thought to originate from aminergic neurons. All these facts rendered the occurrence of aminergic innervation in the snail gut probable, thus the present study carried out by biochemical measurements and fluorescence microscopic studies simultaneously, aimed to verify the presence of aminergic innervation and concluding from this we wish to discuss its possible role in the regulation of the intestinal activity.

Materials and Methods

I. Electronmicroscopy

Our studies were carried out on mature *Helix pomatia* L. *(Mollusca: Gastropoda)* individuals collected from humid meadows at the environs of Szeged in Summer, 1983. The animals were dissected and the 1 mm^3 sized pieces cut from the various sections of the intestinal tract were fixed in cold state in 2.5% glutaraldehyde diluted with phosphate buffer, resp. Karnovsky-fixative, for 2 hours besides 7.3 pH. Then the tissue blocks were washed in phosphate buffer containing 7.5% saccharose, then postfixed in 2% osmium tetroxide set to 7.5 pH with phosphate buffer for 2 hours at 4 °C. Then the material was dehydrated in ascending alcohol series. The tissue blocks were contrasted with saturated uranyl acetate in 75% ethanol in dark for 1 hour. The blocks were embedded in Spurr-embedding material and then sections prepared, recontrasted with lead citrate and then studied under TESLA BS 500 electronmicroscope.

II. Fluorescence microscopic studies

The saccharose-phosphate-glyoxylic acid (SPG) method (de la Torre et al., 1976) was used for the histochemical demonstration of the monoamines. The alimentary tract was dissected in whole length or in small pieces, then incubated in the reaction mixture containing 6.8 gr saccharose, 3.2 g KH_2PO_4 and 1 g glyoxylic acid at 4 °C for 15 min. At the end of the incubation period the submucous layer was stripped off, the muscle layer was stretched out on slides, the moisture removed by blotting paper and the specimens dried by cold air for about 1/2 hour. The completely dry samples were treated with heat for 4 min. at 95 °C. The preparations were covered with paraffin oil and studied with Leitz Orthoplan microscope equipped with indirect illumination and HBO 50 high-pressure mercury-vapour lamp. Green fluorescence characteristic of catecholamines was detected by applying E-3 filter-block. Pictures were prepared on FORTE-PAN 400 ASA black and white film. The control preparations were treated as described previously, but glyoxylic acid was left out of the reaction mixture.

III. Chemical Studies

Adrenaline and noradrenaline were determined according to the fluorimetric method of Anton and Sayre (1962), the dopamine measured according to Schellenberger and Gordon (1971) and serotonin demonstrated according to Snyder et al. (1965). Perkin—Elmer HPF-44B type fluorescence spectrophotometer was used for the measurements.

Results

I. Electronmicroscopy

One of the types of the nerve fibres occurring in the smooth muscle layer of *Helix pomatia* gut contains so-called large dense-core vesicles, the average diameters of which were 100 nm. During the course of the present study attention was paid to these nerve fibres and terminals, thought to be aminergic on the basis of the literary data (Gabella, 1979; Mercer et al., 1981). Such nerves could frequently be observed

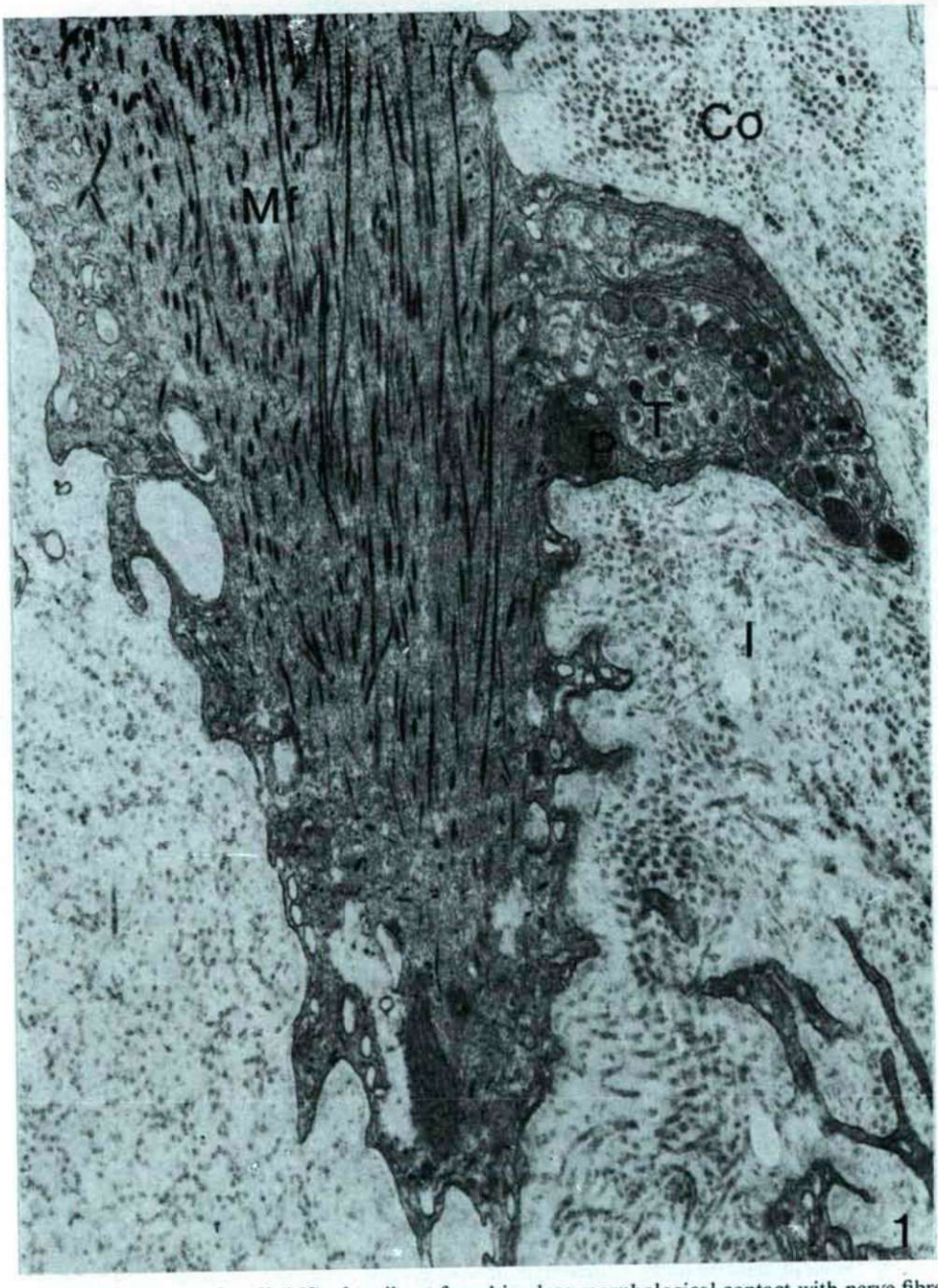


Fig. 1. Smooth muscle cell (Mf) of snail gut found in close morphological contact with nerve fibre (T) containing dense-core vesicles. The muscle fibre admits a mitochondrium-containing process (P) towards the terminal. I = interstitium, Co = collagen fibre ×20 000

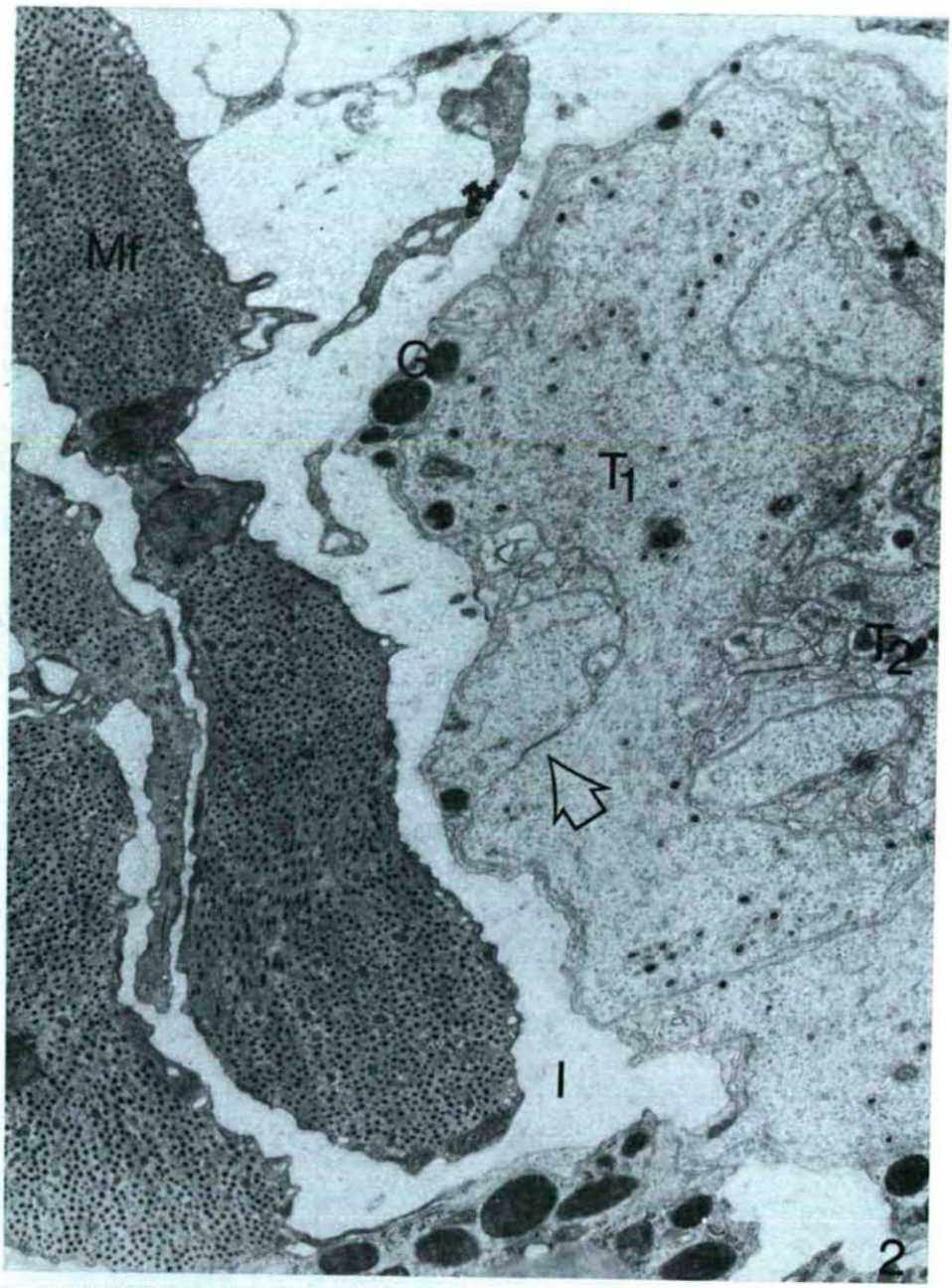


Fig. 2. Detail of neuropil in the muscle layer of snail gut. Mf = smooth muscle cell, T_1 = dense-core vesicle-containing nerve fibre, T_2 = nerve fibre containing neurosecretory granules, I = interstitium, G = glial process with glial granules. The arrow points to a close junction between two nerve fibres. ×15 000

in the direct neighbourhood of the smooth muscle fibres (Fig. 1), as well as in the neuropil (Fig. 2). The terminals — which lost their glial covering and the number of dense-core vesicles increased in them — were usually in tight morphological contact with the processes of smooth muscle cells, which also contained mitochondria (Fig. 1). The sarcolemma and the terminal membrane attached on large surfaces, but neither of them showed synaptic membrane specialization.

The nerve fibres of similar type found in the neuropil (Fig. 2) contain dense-core vesicles in relatively lower amount than in the terminals, however, a significant amount of microtubuli and neurofilaments can be found in them. The surface of the nerves facing to the interstitium was covered by glial processes. Up to now, synaptic connections could not be observed among the nerves situated within the neuropil.

In the interstitium between the muscle fibres neurons were also found, the perikaryon of which also contained large amounts of dense-core vesicles (Fig. 3). On the basis of the vesicle-type similarity, the above-mentioned nerves could be regarded as the processes of these neurons. The most characteristic organelles of the cells were the rough surfaced endoplasmic reticulum as well as the Golgi-apparatus, in the cisternae of which material of similar density to the contents of the dense-core vesicles could be observed, especially at higher magnification (Fig. 3. insert). It was also characteristic to these cells that mitochondria were present in large amounts. Quite frequently other types of fibres, containing neurosecretory granules could be observed close to the soma (Fig. 3. arrow).

II. Fluorescence microscopic studies

The histofluorescence characteristic to monoamines only appeared in the samples treated with glyoxylic acid. This means that the observed fluorescence really originated from the monoamines present in the tissues. Regarding the fluorescence intensity and the distribution of the fluorescent nerve elements three main segments were found in the snail gut.

In the foregut an extremely rich network of the green fluorescent nerve elements was observed (Fig. 4). The fluorescence was rather intensive and well localized. One part of the fluorescent fibres had independent course in the form of thin varicose fibres (Fig. 4. arrow), while at other places the fibres were arranged in thick bundles (Fig. 4., 5. arrow heads). Nerve cell bodies of various sizes and shapes were observed in close connection to the nerve bundles (Fig. 4., 5. filled arrows). At some places single, strongly fluorescent cells were also seen with no visible connection to nerve fibres. These were uniform in size, but varied in shape (Fig. 6., 7a, b). In most of the cells the fluorescence intensity was the same on the whole cell surface (Fig. 6., 7a), while in a few cases the fluorescence was not observed above the nucleus (Fig. 7b). The fluorescence intensity of the nerve fibres running in the stomach was lower than in the foregut, and formed a loose network (Fig. 9). Around the fibres a large number of relatively small cells were observed in which the fluorescence was limited to the cytoplasm (Fig. 9a). These cells were very uniform regarding both size and shape, all of them appeared to be unipolar. Most of the hindgut fibres were thin and varicose (Fig. 8). The fluorescence of the fibres were localized, but the intensity was lower than that of experienced in the foregut. Fluorescent cell bodies were only rarely observed in this part of the gut.

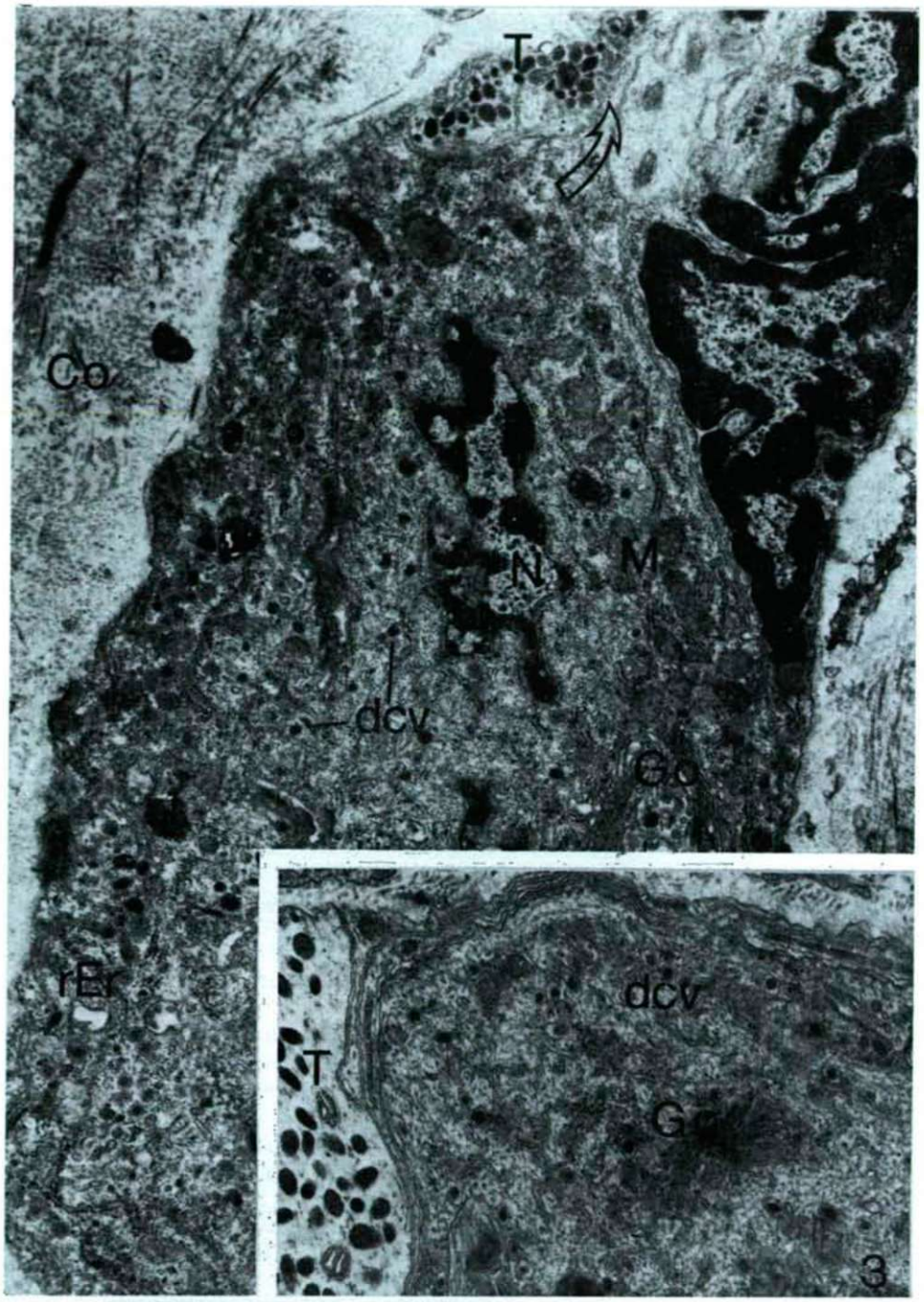
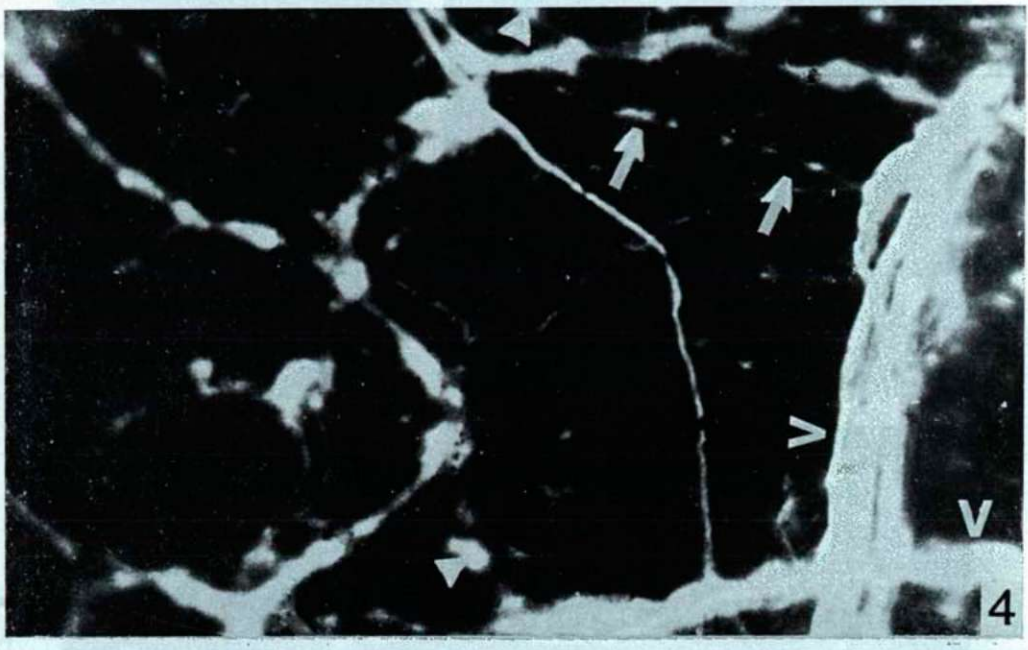


Fig. 3. Intramural neuron from the gut muscle of snail. Dense-core vesicles can be seen in the perikaryon (dcv). N=nucleus, rEr=rough surfaced endoplasmatic reticulum, Go=Golgi-apparatus, M=mitochondrium. The arrow indicates the close junction between the neuron and a nerve fibre (T) containing neurosecretory granules. Co=collagen fibres ×15 000
Insert: Magnified detail of a similar neuron with dense-core vesicles (dcv) and well developed Golgi-apparatus (Go). A nerve fibre (T) of the neurosecretory type is observable besides the cell. ×30 000



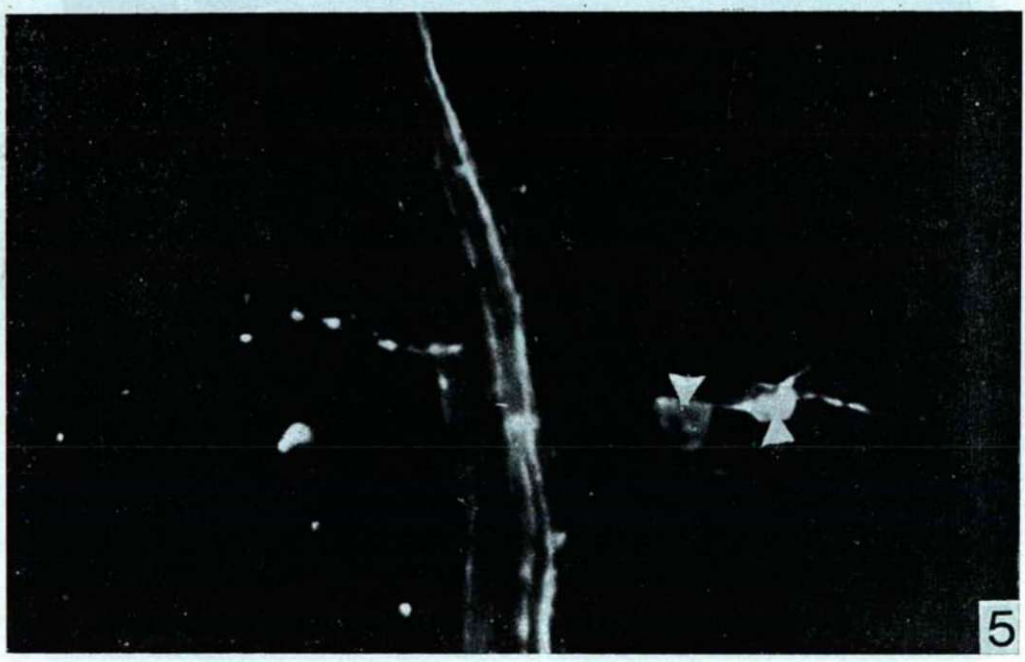
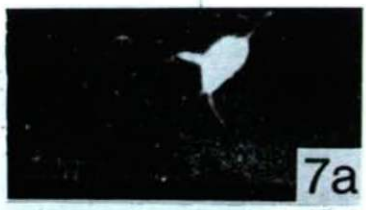


Fig. 4. Fluorescence induced by glyoxylic acid in the foregut of snail gastrointestinal tract. The thin varicose fibres (arrow) and the thick nerve bundles (arrow-heads) form a complicated network, at places with the small cells which are in close contact with the fibres (filled arroheads). ×580

Fig. 5. Fluorescent nerve bundle in the foregut. The thin varicose nerve fibre originating from the bundle as well as two neurons (filled arrow-heads) in the direct neighbourhood are observed. ×620



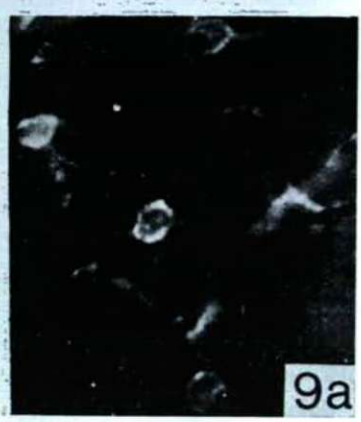


Fig. 6. and 7. Main types of neurons observed in the foregut wall. Most of the cells are multipolar, but bi- and unipolar types can also be found among them. The fluorescence is occasionally limited to the cytoplasm. ×620

Fig. 8. Fibres running in the hindgut wall. Varicosities (arrows) can well be seen in one part of the thinner fibres. ×620

Fig. 9. Detail of fluorescent nerve fibres found in the stomach wall. ×580

Fig. 9a. Groups of unipolar neurons can be seen at places among the fibres. In these the fluorescence is limited to the cytoplasm and its intensity is much lower than in the cells of the foregut. ×680

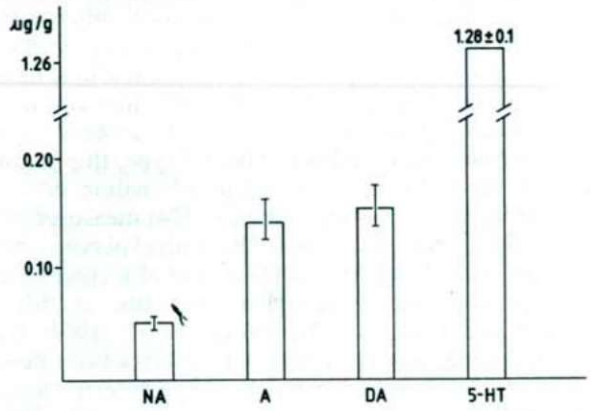


Fig. 10. Amount of monoamines in the snail gut expressed in µg/g wet weight.

III. Chemical studies

The snail gut contained monoamines in significant amount (Fig. 10). The 5-HT occurred in the largest number (1.25 µg/g wet weight), while the amount of noradrenaline was found to be lower by two orders (0.05 µg/g wet weight). The concentration of adrenaline and dopamine, was also significant (0.14 and 0.16 µg/g).

Discussion

The fluorescence microscopic studies have in many cases demonstrated the presence of monoamine (MA)-containing neurons in the central nervous system of snail (Dahl et al., 1966; Jaeger et al., 1970; Marsden and Kerkut, 1970; Sakharov and Zs-Nagy, 1968; Sedden et al., 1968). It is likely that in the central nervous system of the snail the dense-core vesicles are storing the MA, as this has been confirmed by several electronmicroscopic, electronhistochemical and autoradiographic experiments (Cottrell and Osborne, 1970; Gerschenfeld, 1963; Jourdan and Nicaise, 1970; Kuhlmann, 1970; Pentreath and Cottrell, 1973; Pentreath et al., 1973, 1974). The histochemical studies carried out on *Helix* (Cottrell and Osborne, 1969) and *Lymnaea* hearts (S.-Rózsa and Zs-Nagy, 1967) have demonstrated that MA-s are present both in the nerve and muscle elements. In the heart of *Aplysia* the localization of serotonin (5-HT) in nerve elements has also been verified by electronmicroscopic autoradiographic studies (Taxi and Gautron, 1969). On the basis of earlier and newer biochemical as well as physiological results it is presumable that MA-s function as neurotransmitters in the nervous system and heart of *Helix* (Gerschenfeld, 1973; Hiripi and Salánki, 1973; Juorio and Killick, 1972; Nemcsók et al., 1975; S-Rózsa, 1969; S-Rózsa and Perényi, 1966). Since large number of data prove the fact that the monoamines also act as mediators in the mammalian gut (Burnstock, 1983; Furness et al., 1980; Gabella, 1979; Komuro et al., 1982), the aim of our study was to attempt the demonstration of the presence of adrenaline, noradrenaline, dopamine and serotonin in the gut of *Helix pomatia*, as well as the probable neurotransmitter role of these in the regulation of the intestinal activity.

According to our results, each of the assumed monoamines were revealed in the snail gut. However, rather significant differences appeared in the concentration of the various monoamines. Serotonin was present in highest amount, noradrenaline could be demonstrated in the lowest concentration. The absolute amount of monoamines was rather low when comparing these values with the data measured in other molluscs. Since the tissue in question is of peripheral, visceral type, this is not surprising. Nevertheless, the ratio of serotonin, dopamine and adrenaline shows great similarity to the ratios measured in shells. SALÁNKI et al. (1974) measured 40 μg/g serotonin, 25 μg/g dopamine and 5 μg/g noradrenaline in the central nervous system of *Anodonta cygnea*. Adrenaline, noradrenaline and dopamine were also successfully demonstrated in the gut of animals phylogenetically of higher order, thus in fish, *Amphibia,* birds and mammals (EULER and FANGE, 1976; BRODIE et al., 1964; BOGDANSKI et al., 1963; MANUKHIN et al., 1969; NORTH, 1965). The results of our fluorescence microscopic studies — according to which, especially in the foregut, highly intensive and well localizable fluorescence could be detected both in the nerve fibres and in certain neurons — support by far the results of the chemical determination. In the possession of the data obtained with the two study methods, the statement can be regarded as grounded (HALASY and BENEDECZKY, 1983) that conformably to vertebrates, the monoamines play role as mediators in the gastrointestinal tract of snails as well. The fact that the majority of the fluorescent perikaryons were in close morphological contact with the fluorescent nerve fibres means that at least a part of the fluorescent fibres is intrinsic in origin. Nevertheless, the fact that cells having no visible morphological contact with fluorescent nerve fibres were also observed suggests that the other part of fluorescent fibres has extrinsic origin. This presumption is also supported by the data of the chemical measurements since in the snail gut the serotonin content was the highest, therefore it is probable that the large part of the separately fluorescent single cells may correspond to enterochromaffine cells of serotonin content. Naturally, it cannot either be ruled out that one part of the separately fluorescent cells are also neurons, however, in the processes the concentration of the monoamines was actually rather low — due to this the process system proving the neuron nature could not be indicated. Our electronmicroscopic results can also be well interpreted by the results of the chemical measurements and the fluorescence microscopic observations. First of all, what seems to be likely is that the terminals containing large dense-core vesicles found in the neighbourhood of the muscle fibres function with mediators of monoamine character. The circumstance that the large dense-core vesicles were not only found in the axon-terminals but also in the neuropils and in the perikaryon of certain neurons strengthens our earlier assumptions, namely that a rather developed local neuron network can be found in the snail gut similarly to the gut of vertebrates, the morphological features of which strongly resemble the Auerbach's plexus. However, while in the neuropil of the mammal myenteric plexus a large amount of mainly axo-axonic synapses were observed (KOMURO et al., 1982), so far synapses were hardly observed by us in the myenteric plexus of the snail gut. It seems that the morphological signs of the interrelationship between the neurons are less expressed in the snail gut compared to mammals. The neuromuscular junction itself, however, is essentially the same as in the vertebrates of higher order. Directly besides or somewhat further from the smooth muscle fibres, a large number of axon terminals filled with dense-core vesicles can be observed. They never establish synaptic contact with the sarcolemma, thus the possibility of fast stimulus-

transfer is not ensured. Since the cells in question are smooth muscle cells ensuring the peristaltic movement of the gut wall, this is not even necessary. It is probable that the mediator substances empty continuously into the extracellular space through exocytosis and their effect displays slowly but long-lastingly on the muscular tension. The classic determination of GERSCHENFELD (1973) according to which monoamines play significant role as neurotransmitters in the snail, is supplemented by our studies with the fact that the monoamines may also take part in the regulation of the function of the gastrointestinal tract, the physiological relations of which are to be clarified by further studies.

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