INTRACELLULAR DISTRIBUTION OF SEROTONIN IN THE CENTRAL NERVOUS SYSTEM AND IN THE HEART OF *HELIX POMATIA*

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According to the generally accepted assumption the transmitter molecules are stored in the nerve terminals and synaptic vesicles. The supposition of GERSCHENFELD (1963) according to which in the central nervous system of Molluscs the dense-core vesicles are the storing places of monoamines is supported by results obtained morphologically (Zs.-NAGY, 1968; COTTRELL and OSBORNE, 1970; JOURDAN and NICAISE, 1970; PENTREATH et al., 1973) as well as by differential and gradient centrifugation (HIRIPI et al., 1973).

The histochemical investigations performed on the heart of *Helix* (COTTRELL and OSBORNE, 1969) and of *Lymnaea* (S.-RÓZSA and Zs.-NAGY, 1967) revealed the localization of monoamines in the neuronal elements and muscle cells. The localization of serotonin in the neuronal elements is confirmed also by autoradiographic examinations carried out on the heart of *Aplysia* (TAXI and GAUTRON, 1969).

Although the physiological and pharmacological examinations performed on the nervous system and heart of *Helix pomatia* (S.-RÓZSA and PERÉNYI, 1966; S.-RÓZSA, 1969; GERSCHENFELD, 1973) support the transmitter role of serotonin, its exact localization in these tissues is yet unknown. Since the nervous tissue can be separated by careful homogenization and subsequent centrifugation into different fractions (WHITTAKER, 1965; 1971) we set the target in our present work to investigate the subcellular localization of serotonin in the nervous and heart tissue of *Helix pomatia* by simply applying this method.

Material and method

For the examinations the cerebral ganglion, the suboesophageal ganglion ring and the heart tissue of *Helix pomatia* were used. During the preparation the tissues were collected in ice-cold physiological solution, they were washed, then after blotting with filter paper their wet weights were taken and homogenization was carried out in 0.2 M iso-osmotic sucrose. Prior to homogenization the tissues were cut up, then in a glass potter (clearance 0.1 mm; 3000 rev/min; 15 strokes) they were homogenized in such volume of sucrose that 5 per cent homogenizate were obtained. The differential and gradient centrifugation was carried out by slightly modifying WHITTAKER's method (1965). The

primary fractions were obtained by differential centrifugation as follows. The nuclear fraction (Nuc) was separated by centrifuging at 900 g for 10 min, the mitochondrial fraction (Mit) at 11,000 g for 60 min, the microsomal fraction (Mic) at 100,000 g for 60 min. The residual supernatant represented the soluble (S) fraction. The subfractions of the mitochondrial fraction were obtained by centrifuging on a sucrose gradient. The mitochondrial fraction was resuspended in iso-osmotic sucrose by careful manual homogenization such that 1 ml of suspension was equivalent to 500 mg of original tissue. 1 ml of the suspension was layered onto a discontinuous sucrose gradient, which was prepared in the case of the ganglion from 1—1 ml of 2.0 M; 1.5 M; 1.2 M; 0.8 M sucrose solutions *(Fig. 1A)*, while in the case of the heart from 1—1 ml of 2.0 M; 1.7 M; 1.5 M; 1.2 M; and 0.8 M sucrose solutions *(Fig. 1B)* before using up, and was stored at 0°C for 1 hr. The centrifuging was carried out at 50,000 g for 2 hr. The fractions were obtained with slicing. Beckman Spinco ultracentrifuge (Model L50; rotors SW 25.1 and L50) was used. The procedure was carried out at 4°C. Small aliquots of the fractions were used for the determination of proteins (LOWRY et al., 1951) as well as for electron microscopic examinations.

For estimating serotonin the normality of the fractions was adjusted to 0.4 by $HClO_4$ and they were diluted to 5 ml then were rehomogenized. After keeping at 0°C for 30 min the homogenizate was centrifuged, and the serotonin content of the supernatant was estimated by the method of SNYDER et al. (1965). The RSA of a fraction was calculated as the percentage of the total recovered protein found in the same fraction.

Samples taken from the fractions for electron microscopic analysis were diluted when necessary to the concentration of iso-osmotic sucrose, then centrifuged at 100,000 g for 1 hr. The pellet was fixed in glutaraldehyde diluted with 3% tap-water or by *Helix* physiological solution for 2—18 hr at room temperature, or at 4°C. After a short washing the pellet was postfixed in 2% OsO_4-collidine at 0°C for 30 min. Following the dehydration the pellets were embedded in Araldite. Sections were cut on an LKB Ultrotome III, micrographs were taken on a TESLA type BS 413 A electron microscope. The sections were stained with uranyl acetate and lead citrate (REYNOLDS, 1963).
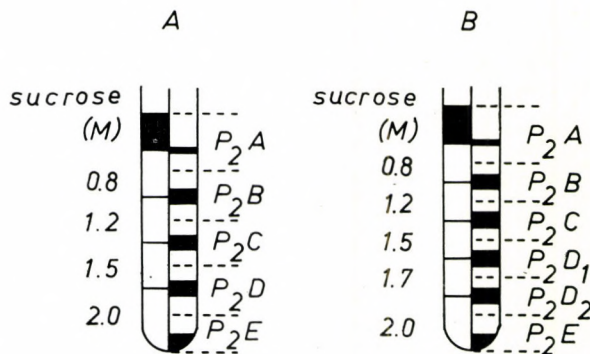

Fig. 1. Arrangement of the gradients before centrifugation (left side) and arrangement of the fractions after centrifugation (right side)

Results

Table I contains the percentage distribution of serotonin in the primary fractions. 70 per cent of serotonin is bound to the tissue elements and 30 per cent of that is present in free form in the soluble fraction. Among the primary fractions of the ganglion the highest concentration of serotonin is found in the nuclear fraction while for the heart in the mitochondrial fraction. The microsomal fraction contains 10—15 per cent serotonin *(Table I)*. The nuclear

TABLE I

Distribution of serotonin in the primary fractions of the ganglion and heart tissue of Helix pomatia

Fractions	Heart	Ganglion
P_1 (Nuc)	24.2	31.9
P_2 (Mit)	43.2	26.5
P_3 (Mic)	14.7	10.45
S (Soluble)	28.6	31.6

fraction of the ganglion contained nuclear fragments, numerous unhomogenized cell fragments, free dense-core vesicles, granules, granular elements of the endoplasmic reticulum, glycogen granules and mitochondria. The dominant structure of the P_2 fraction *(Fig. 2)* is the synaptosoma, in addition a great number of dense-core vesicles, granules of other type, mitochondria and vesicular forms of the granular endoplasmic reticulum also occur. In some areas unique or fascicled collagen fibrils can be found. The glycogen granules or rosettes are uniformly distributed. In the P_3 fraction uniformly distributed free ribosomes and vesicular membrane fragments of smooth surface can be seen *(Fig. 3)*. In the deeper region of the pellet numerous dense-core vesicles also occurred. Elements of the granular endoplasmic reticulum are encountered only rarely *(Fig. 4)*.

In the nuclear fraction of the heart collagen fibrils to a high extent, numerous fibrous and filamentous structures presumably constituting the contractile elements, mitochondria, membrane fragments of unknown origin can be observed. In some areas tubular elements are fairly visible, rather resonably the elements of the sarcoplasmic reticulum. In the mitochondrial fraction nerve endings containing dense-core vesicles or elementary and glycogen granules could be observed. This fraction is characterized by a great number of free dense-core vesicles or granules, glycogen granules. Mitochondria, elements of the granular endoplasmic reticulum, smooth membrane elements are also abundant *(Fig. 5)*. The structure of the microsomal fraction: the upper layer of the pellet generally is characterized by a dense, fibrous fundamental structure containing vesicles of small and larger size, membrane fragments, sometimes mitochondria and granules. Sporadically some collagen fibrils occur too *(Fig. 6)*. The denser layer of the pellet contains numerous rounded, smooth-surfaced membrane profiles as well as a great number of elongated tubular formations, which might be considered to be the constituting elements of the sarcotubular system. The numerous free ribosomes are uniformly distributed *(Fig. 7)*.
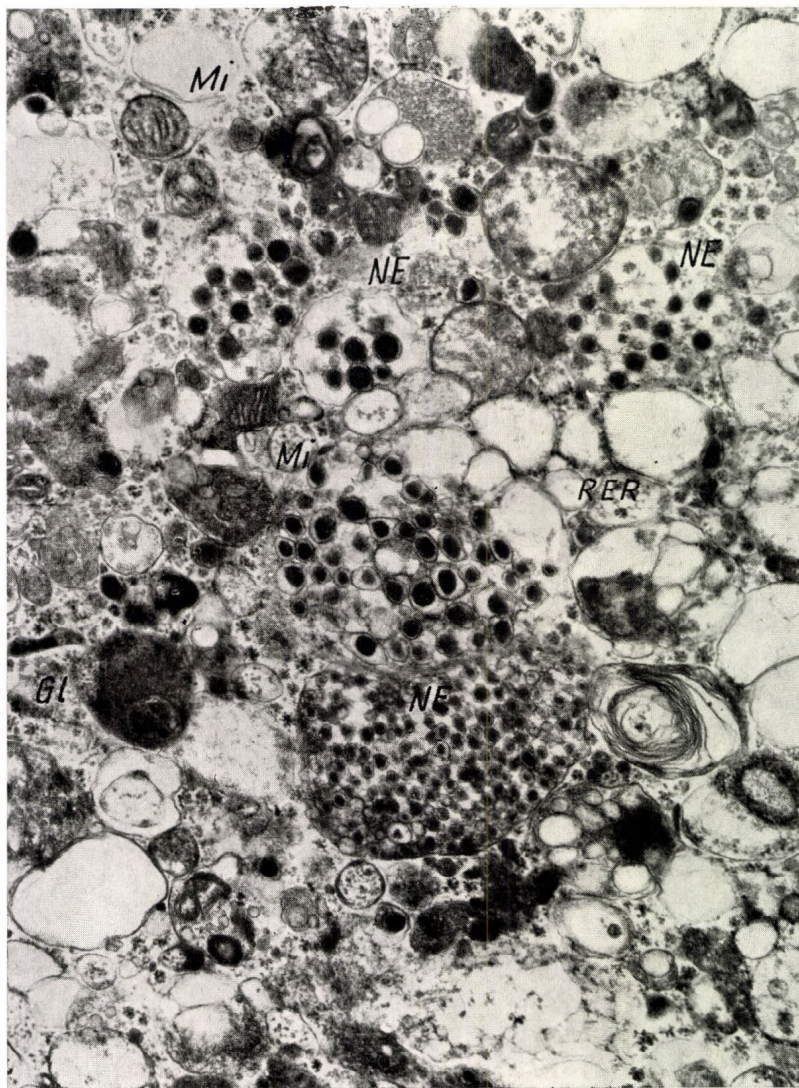
Fig. 2. Electron micrograph of the mitochondrial (P₂) fraction of the ganglion. NE — nerve endings, Mi — mitochondria, GL — glycogen granules or rosettes, RER —granular endoplasmic reticulum. ×22,000

Table II shows the percentage distribution and RSA values of serotonin found in the subfractions of the mitochondrial fraction. The 17 per cent serotonin content of the P_2A subfraction is practically not bound to tissue elements but it is present in free state in the supernatant fluid. The protein concentration of this fraction is minimal and when measuring serotonin in the supernatant, and in other part of the fraction containing tissue elements separately, the quantity of serotonin is found to be 15 per cent in the super-
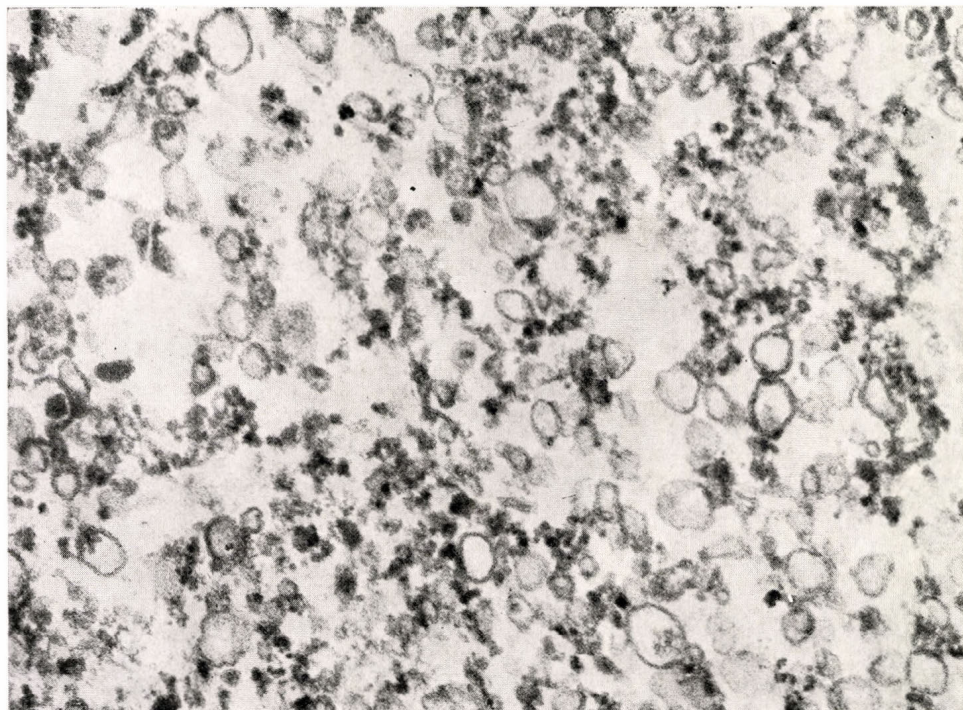
Fig. 3. Electron micrograph of the microsomal (P$_3$) fraction of the ganglion. ×35,000

natant fluid while only 1—2 per cent in that part, which contains tissue ele-
ments. The 10—12 per cent serotonin content of subfractions P$_2$B and P$_2$E
has a low RSA value. 60 per cent of the serotonin content of the mitochondrial
fraction is present in subfractions P$_2$C and P$_2$D displaying a RSA of more
than 1.0.

In the case of the ganglion according to the electron microscopic examina-
tions the main components of subfraction P$_2$A were the larger and smaller
membrane profiles. Dense-core vesicles and granules are also observed sporad-
ically. Fraction P$_2$B is characterized by a very great number of free dense-core
vesicles and granules. There are many smooth membrane profiles, which partly
might have been depleted synaptosomes. In some places intact nerve endings
could be seen containing vesicles of different type. The elements of the endo-
plasmic reticulum carrying ribosomes occurred, though rarely. The pre-
dominant structure of the fraction P$_2$C is the synaptosoma *(Fig. 8)*. The
vesicule population of the nerve endings is of heterogeneous appearance
(Fig. 9). Beside the typical dense-core vesicles, its elements and neuro-
secretory granules sometimes terminals could be seen, which might be con-
sidered to be of cholinergic nature. This subfraction is characterized by a mass
of free dense-core vesicles, granules. Mitochondria can be seen both in free
form and in nerve endings and the same arrangement is found concerning
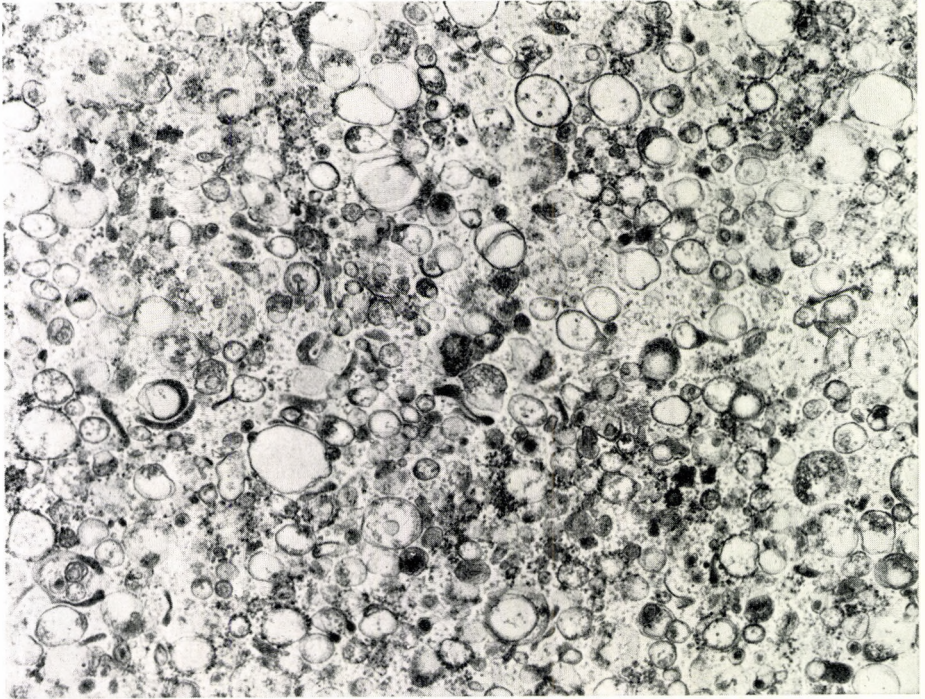the glycogen granules. Elements of the granular endoplasmic raticulum also

Fig. 4. Electron micrograph of the microsomal (P_3) fraction of the ganglion. $\times 35,000$

TABLE II

Distribution and RSA of serotonin in the subfractions of the mitochondrial fraction obtained from the ganglion of Helix pomatia

Fractions	5HT %	RSA
P_2A	17.1	—
P_2B	12.4	0.84
P_2C	25.8	1.48
P_2D	34.2	1.22
P_2E	10.4	0.38

occur. Subfraction P_2D shows an accumulation of more damaged nerve endings as compared to those of P_2C, most of them are full of dense-core vesicles *(Fig. 10)*. Among the numerous free vesicles glia granules are also abundant. Elements of the granular endoplasmic reticulum are frequently encountered. Glycogen granules can be found in large quantities similarly to those of P_2C. Mitochondria occur to a lesser extent. Fraction P_2E contains sporadically nerve endings, free dense-core vesicles and vesicles of other type. Elements of the reticulum with ribosomes and numerous collagen fibrils are also characteristic here.
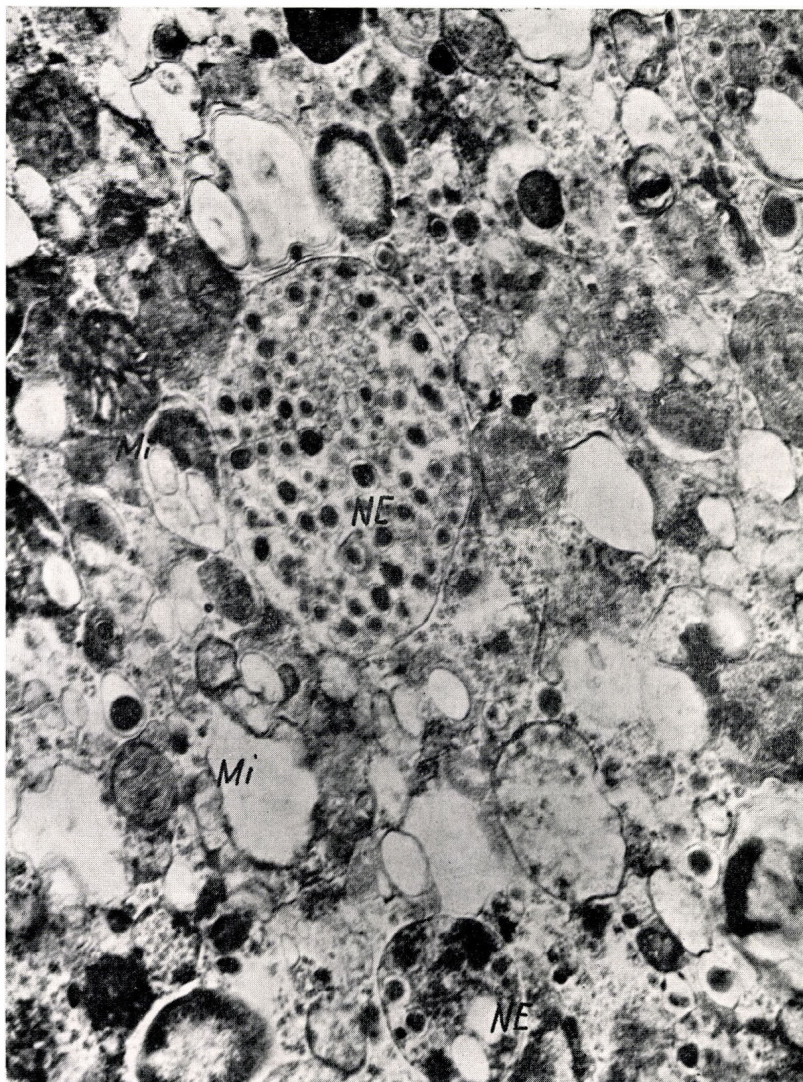
Fig. 5. Electron micrograph of the mitochondrial (P$_2$) fraction of the heart. NE — nerve endings, Mi — mitochondrium. ×25,000

As far as the heart is concerned, the preliminary investigations showed that when for the separation of fractions the same gradient was used as in the case of the ganglion, fraction P$_2$D consisted of two fractions of different density, which could be separated to subfractions P$_2$D$_1$ and P$_2$D$_2$ by increasing the quantity of the gradients in such a way that the RSA value of the more dense subfraction P$_2$D$_2$ showed a considerable increase as compared to the RSA of the fraction P$_2$D. In the case of the ganglion, the separation
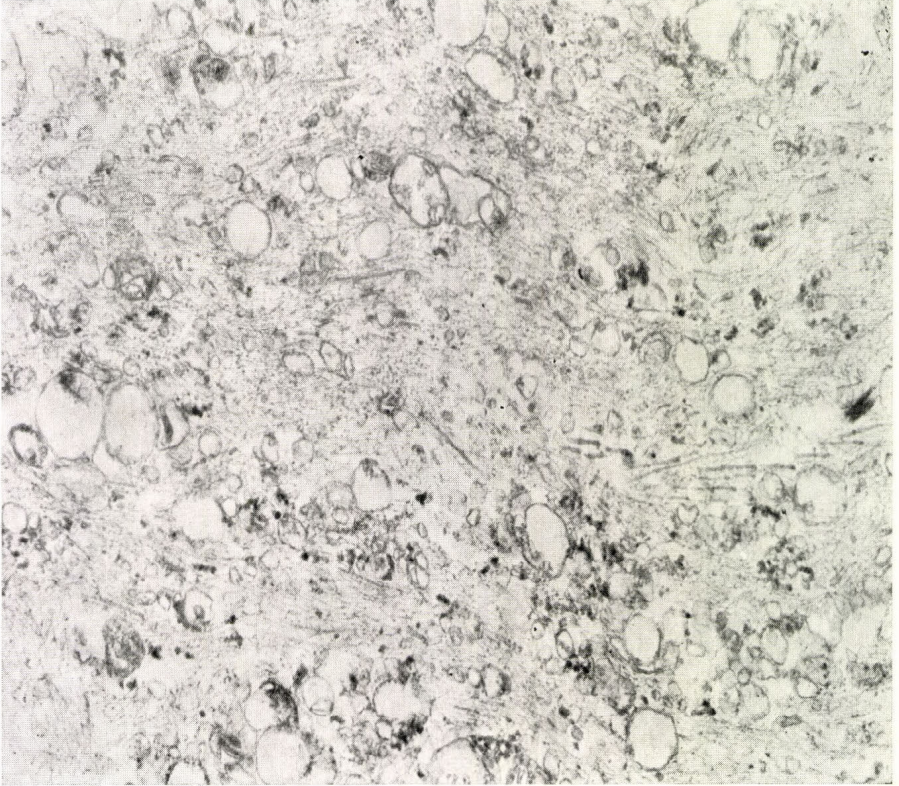
Fig. 6. Electron micrograph of the microsomal (P₃) fraction of the heart. Smooth membrane profiles in the typical filamentous fundamental structure. ×22,000

of the fraction P_2D in the same way did not result in any fraction having higher RSA value. Similarly to that of the ganglion, the subfraction P_2A contained only a few tissue elements, and its serotonin content of 9 per cent was practically present dissolved in the supernatant fluid.

Subfractions P_2B and P_2C contain minimal amount of serotonin with low RSA. The 20 per cent serotonin content of the fraction P_2D_1 has nearly identical RSA value with that of the subfraction P_2D of the ganglion. The highest concentration of serotonin is present in subfractions P_2D_2 and P_2E. However, in fraction P_2E serotonin is bound with a low RSA, while in fraction P_2D_2 with a very high RSA *(Table III)*.

According to the ultrastructural analyses, subfraction P_2A consists of smooth membrane fragments and empty membrane vesicles. Fraction P_2B is similar to P_2A, but here a few vesicular elements can be found within the empty membrane profiles now and then indicating their synaptosomatic origin. Some free dense-core vesicles frequently with eccentric core can also be seen as well as a number of mitochondria. Subfraction P_2C is characterized by a great number of mitochondria as an exclusive structural component in some places *(Fig. 11)*. In addition, smooth membrane fragments, dense-core
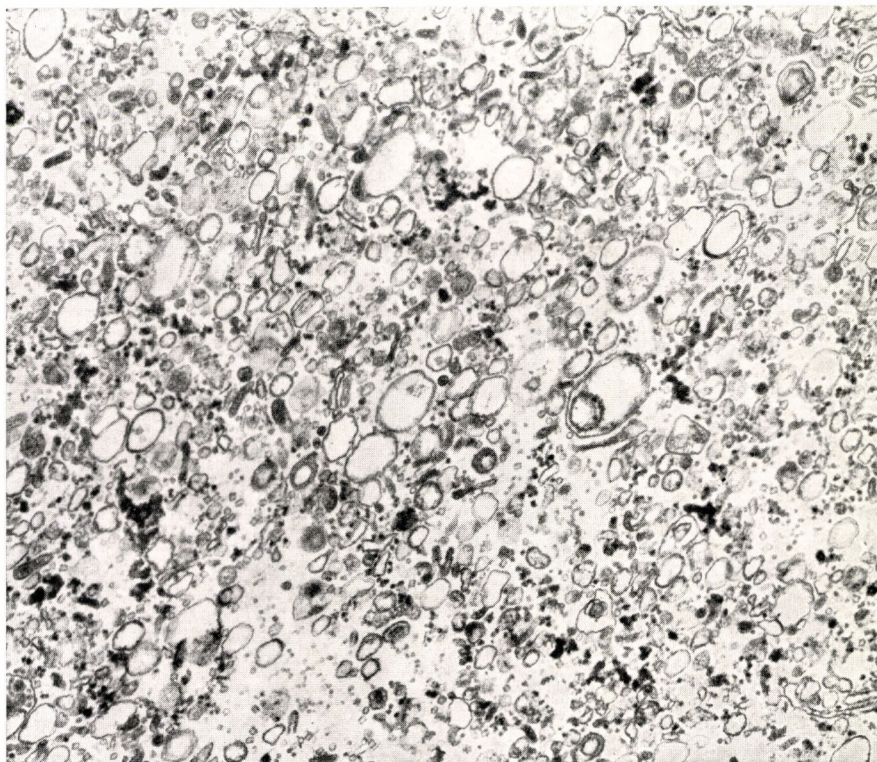
Fig. 7. Electron micrograph of the microsomal fraction of the heart. The frequent tubular formations presumably correspond to the elements of the sarcotubular system.
× 30,000

vesicles of relatively frequent occurrence and granules are also seen. The predominant components of fraction P_2D_1 are the mitochondria occurring en masse cohered completely in some places. Rarely a few nerve endings with dense-core vesicles and generally free granules of major size can be seen (*Fig. 12*). Subfraction P_2D_2 is the only one, where besides a rather large quantity of mitochondria often encountered nerve endings in shrunken state

TABLE III

Distribution and RSA of serotonin in the subfractions of the mitochondrial fraction obtained from the heart tissue of Helix pomatia

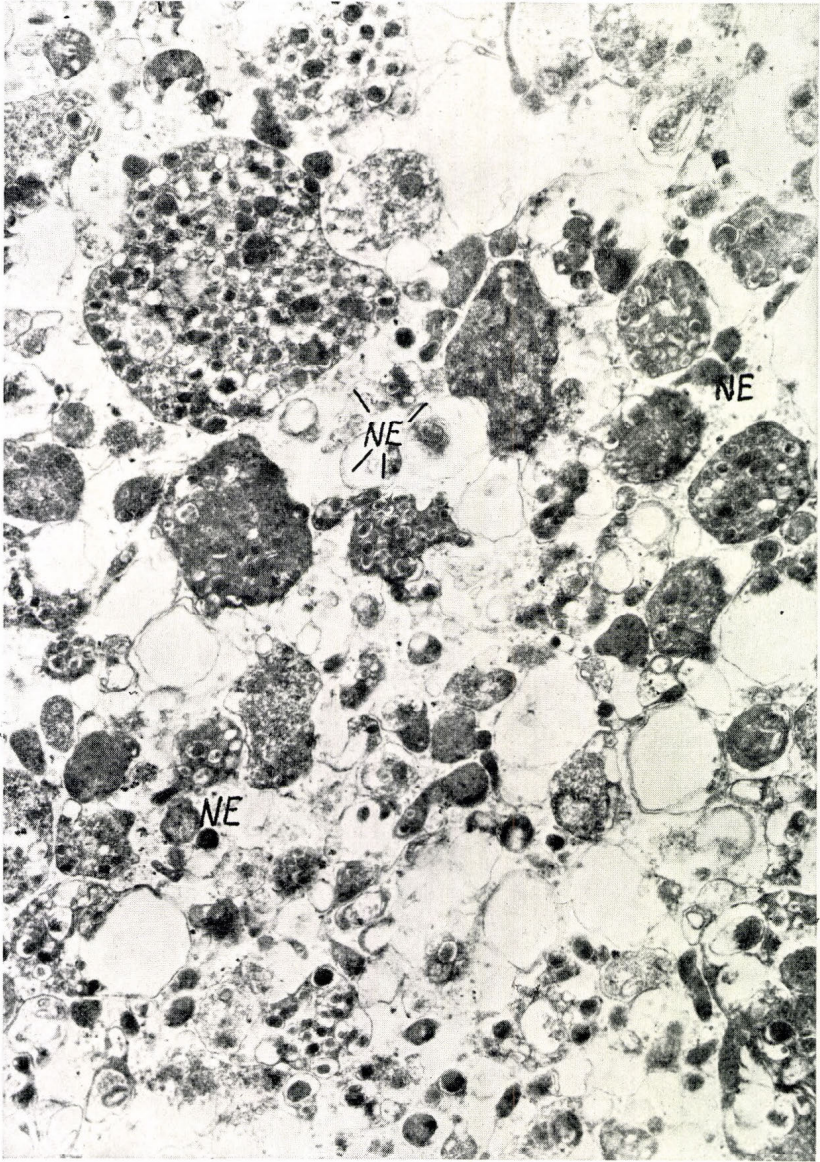| Fractions	5HT %	RSA
P_2A	9.00	—
P_2B	4.28	0.79
P_2C	10.00	0.40
P_2D_1	20.60	1.16
P_2D_2	25.20	3.17
P_2E	26.80	0.73

Fig. 8. Electron micrograph of the subfraction P_2C of the ganglion. Nerve ending — NE — is the predominating structure. $\times 24,000$

are characteristically observed *(Figs. 13 – 14)*. The synaptosomes contained dense-core vesicles mixed with empty ones or elementary neurosecretory granules. Membrane profiles containing no vesicular component frequently occur, but the glycogen granules within them unequivocally indicate their synaptosomatic origin. Freely occurring glia (interstitial) granules are also
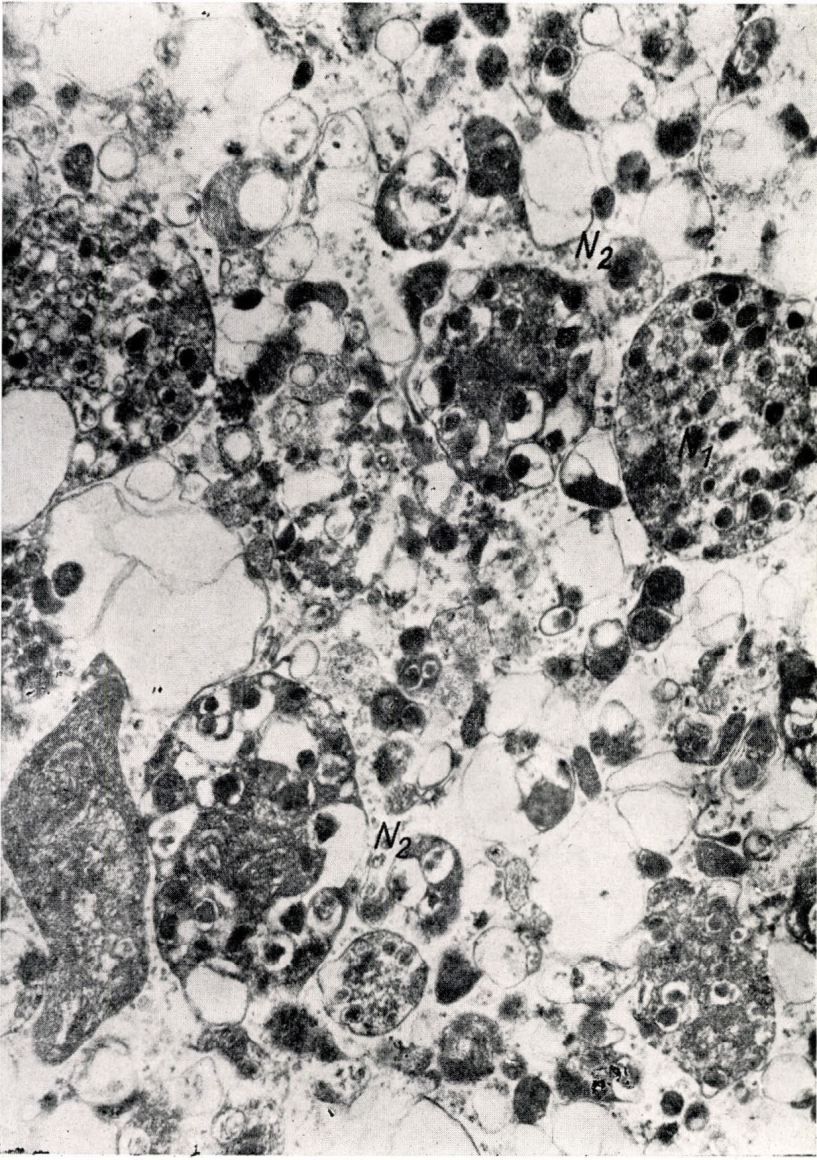
Fig. 9. Electron micrograph of the subfraction P_2C of the ganglion. Nerve endings containing vesicles of different type (N_1, N_2). $\times 35,000$

characteristic. Sometimes the nerve endings are completely filled with an intensively dense medium. Collagen fibrils appear in groups. Subfraction P_2E contains sporadically occurring mitochondria, elementary collagen fibrils, smooth membrane profiles and membrane fragments.
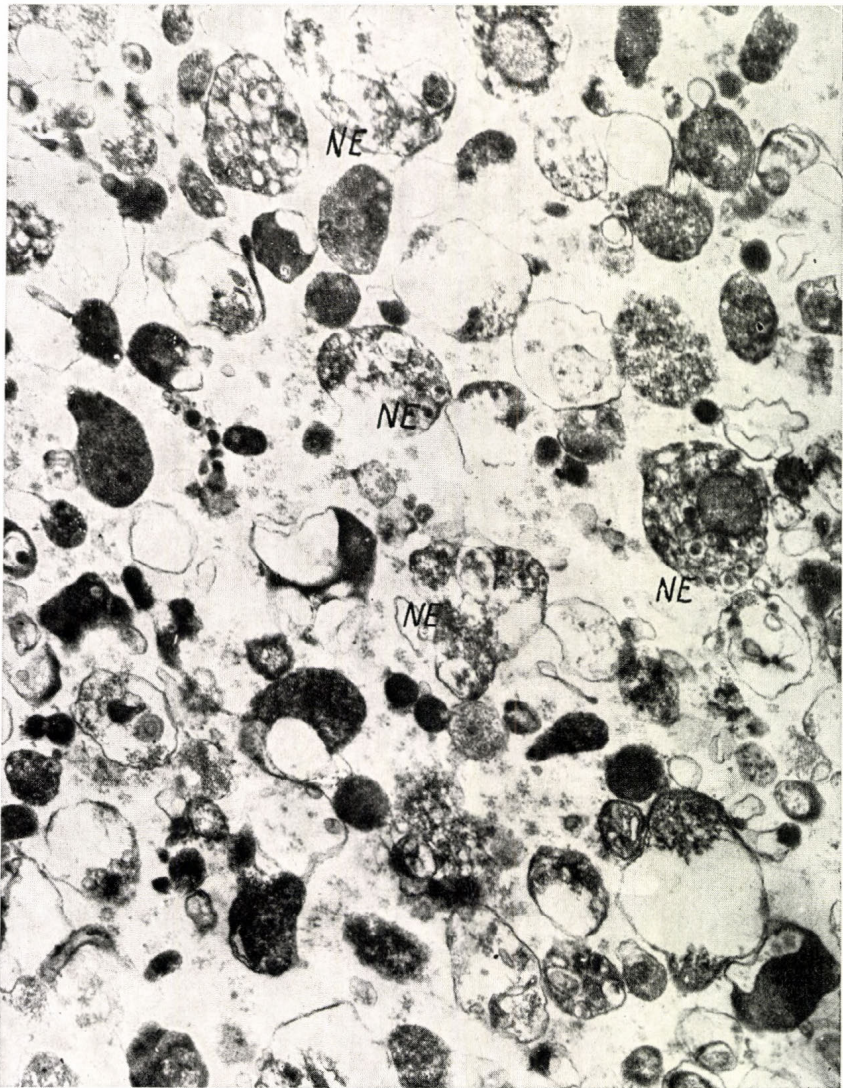
Fig. 10. Electron micrograph of the subfraction P_2D of the ganglion. NE — nerve ending. $\times 31,500$

Discussion

Our results show that after the primary fractionation of the homogenizate obtained both from the ganglion and from the heart 70 per cent of serotonin is bound to the tissue elements. It is in agreement with the ratio of free 5HT to bound 5HT described for the mammalian brain (WHITTAKER, 1965; 1971). However, it is conspicuous, that the percentual serotonin content of the nuclear fraction in *Helix* is a relatively high value, especially as far as
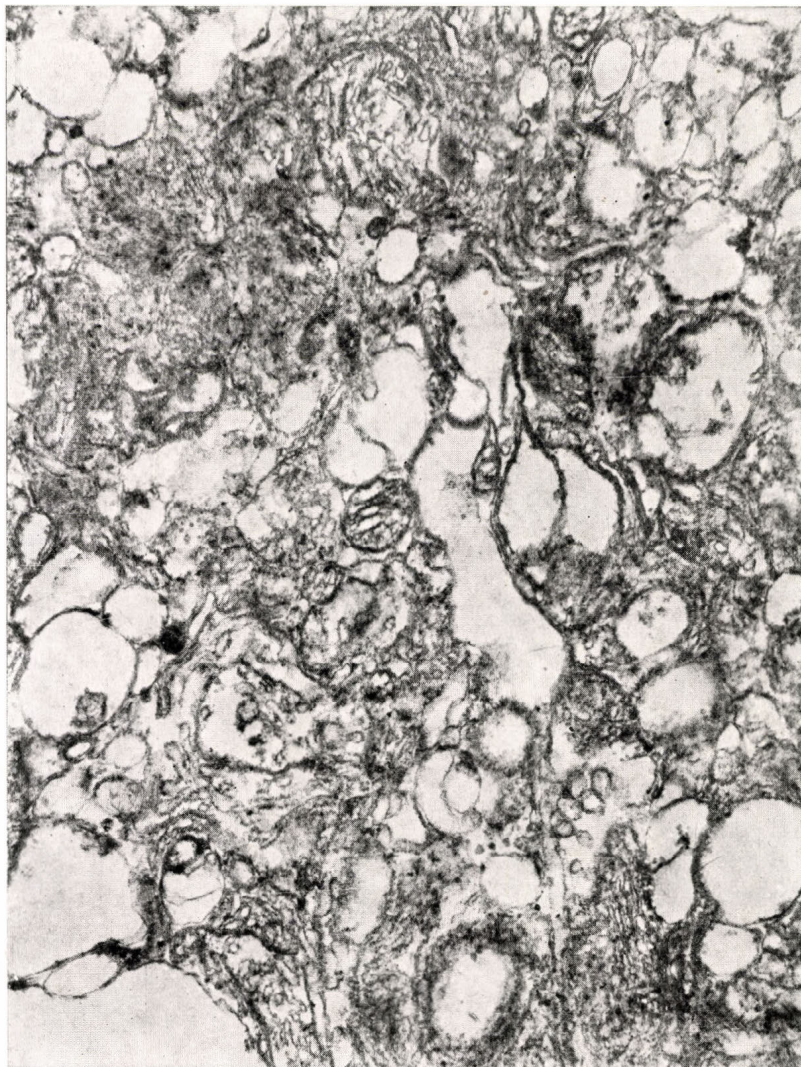
Fig. 11. Electron micrograph of the subfraction P$_2$C of the heart. ×35,000

the ganglion is concerned. The high serotonin concentration of this fraction is accounted for the difficulty to homogenize both the ganglion for its strong connective tissue and the heart for its fibrous structure, thus, the frequent occurrence of unhomogenized cellular particles, supported by the morphological analyses, increases the serotonin content of this fraction. Though the serotonin content in the nuclear fraction decreases by using improved and more powerful homogenization, this decrease in fact causes an increase in the percentual serotonin content of the soluble fraction but not in the mitochondrial
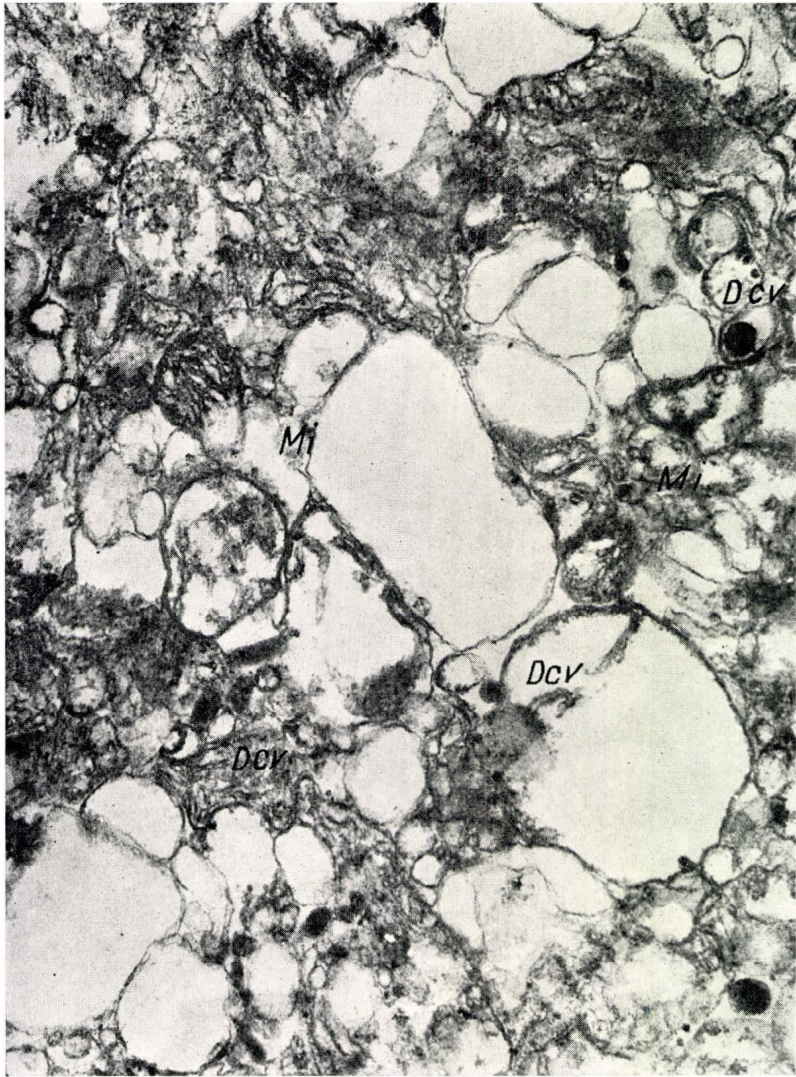
Fig. 12. Electron micrograph of the subfraction P_2D_1 of the heart. DCV — dense-core vesicle, Mi — mitochondrium. $\times 35{,}000$

one. In the case of the ganglion, the high percentual serotonin content may be due to the fact that the elements of connective tissue sedimented in the nuclear fraction contain serotonin in a considerable concentration (JUORIO and KILLICK, 1972).

Since the serotonin content of the microsomal fraction is relatively low and the mitochondrial fraction contains those tissue elements, which may take part in the storage of the transmitter, the subfractions of the latter fraction were investigated.
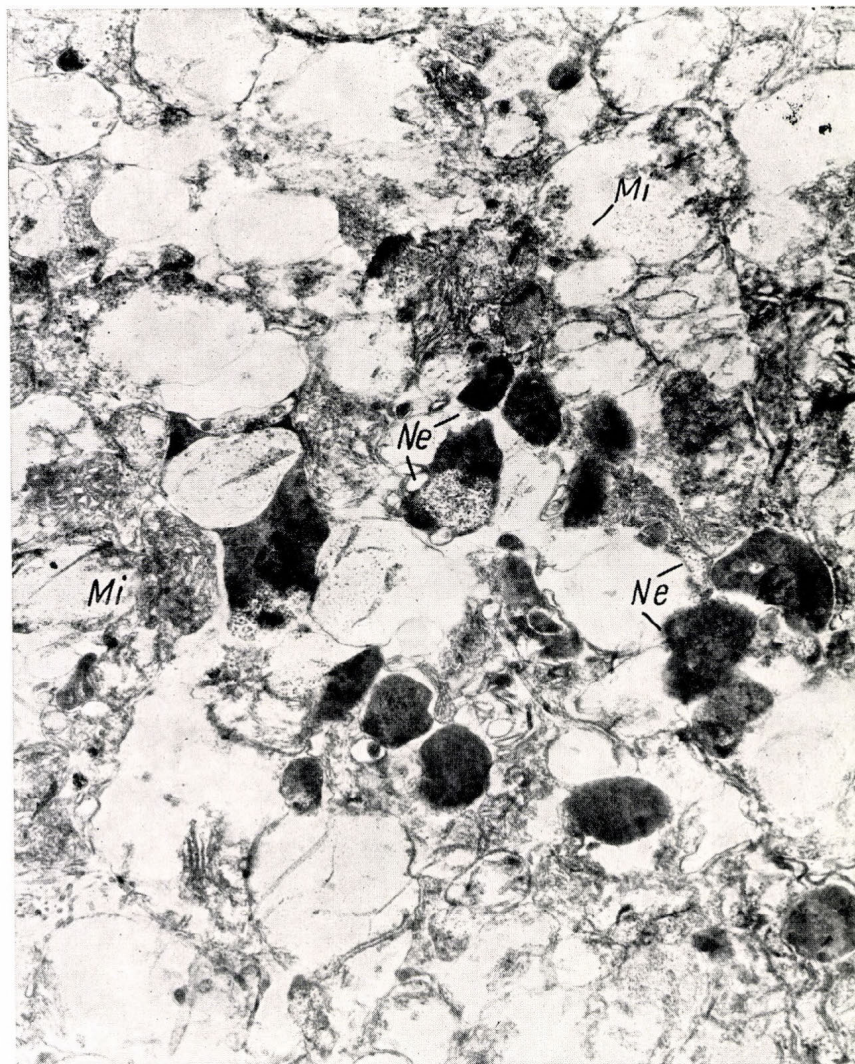
Fig. 13. Electron micrographs of the subfraction P_2D_2 of the heart. NE— nerve ending, Mi — mitochondrium. ×22,000

In the case of the ganglion, among the mitochondrial subfractions, P_2C and P_2D have the highest 5HT content with highest RSA, while subfractions A, B and E contain a lower quantity of serotonin with low RSA value. On the basis of ultrastructural analyses we may suppose that subfractions C and D may bind 5HT more specifically than the other three subfractions. Since nerve endings and free dense-core vesicles occur in fractions C and D in the greatest number, it is obvious to suppose that these structures take part in the binding of 5HT. It is in agreement with the results of our previous investigations performed on mussel (HIRIPI et al., 1973), where the primary binding of 5HT
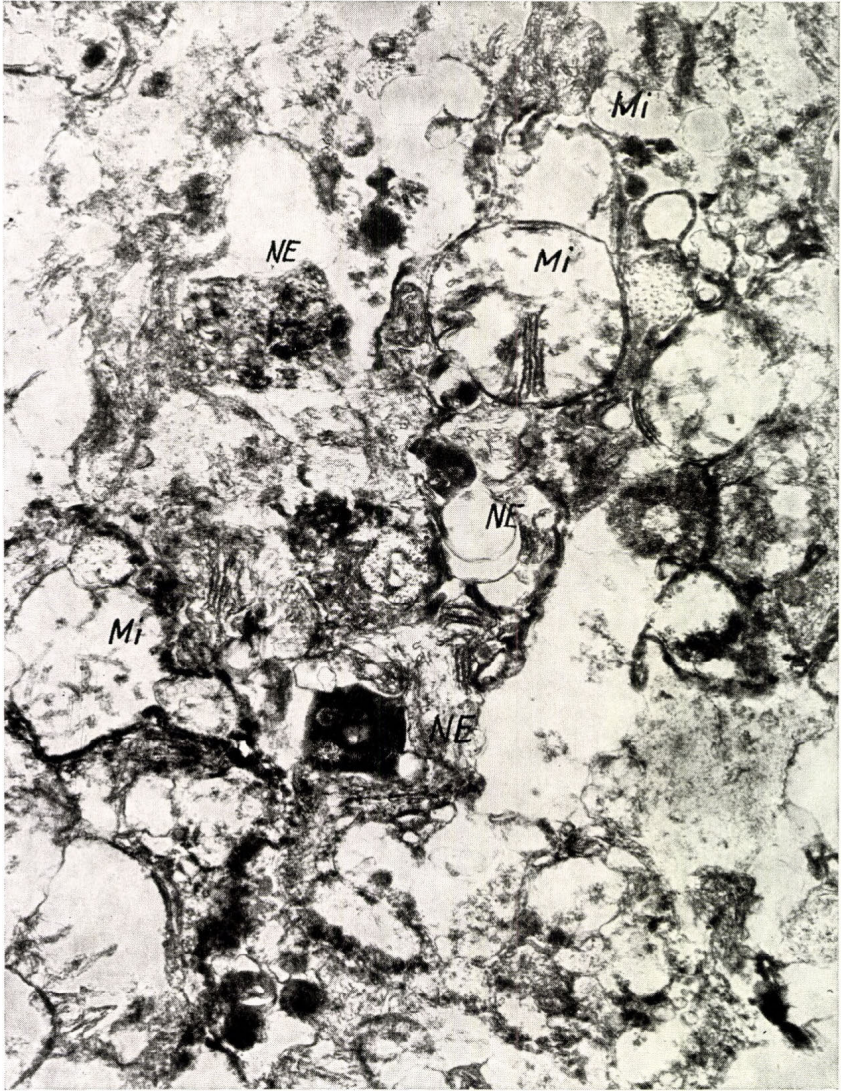
Fig. 14. Electron micrograph of the subfraction P_2D_2 of the heart. NE — nerve ending, Mi-mitochondrium. ×22,000

in synaptosomes and vesicles was also observed. The vesicular binding of 5HT in the central nervous system of the snail is suggested also by normal electron microscopic, autoradiographic and electron-hystochemical examinations (COTTRELL and OSBORNE, 1970; JOURDAN and NICAISE, 1970; PENTREATH and COTTRELL, 1973; PENTREATH et al., 1973; WEINREICH et al., 1973).

Among the mitochondrial subfractions of the heart, subfraction P_2D_2 shows a significantly high RSA of serotonin. The electron microscopic anal-

yses revealed that this is the only subfraction in the heart, where a large quantity of nerve endings occurs. On the basis of this fact, it can be supposed that serotonin is bound to the nerve endings also in the case of the heart. In the heart of *Aplysia* the examination of the accumulation of radioactive serotonin by electron-microscopic autoradiography also indicates the 5HT content of the nervous elements (TAXI and GAUTRON, 1969). However, it is conspicuous, that the 5HT content and RSA in subfraction P_2D_1 of the heart is hardly lower than those of the subfractions P_2C and P_2D containing mainly synaptosoma. Considering the almost exclusive mitochondrium content of subfraction P_2D_1 of the heart the possibility of 5HT binding also to the mitochondria cannot be disregarded.

Though the RSA value characterizing the serotonin content of the subfraction P_2E is low, the fact cannot be neglected, that its percentual serotonin content corresponds to that of fraction P_2D_2. Since this fraction consisted mainly of elementary collagen fibrils, the possibility should not be excluded that the muscle cells may store a considerable amount of the serotonin. This confirms the results of the histochemical investigations showing localization of serotonin in the muscle elements (S.-RÓZSA and ZS.-NAGY, 1967; COTTRELL and OSBORNE, 1969).

Summary

Examination of the subcellular localization of serotonin in the nervous and heart tissue of *Helix pomatia* by differential and gradient centrifugation revealed that

1. In the primary fractions of the ganglion and heart homogenizates 70 per cent of 5HT is present in bound, 30 per cent in free form. In the ganglion the 5HT content of the nuclear fraction is relatively high.

2. In the nervous tissue the highest proportion of bound 5HT is contained by the dense-core vesicles of the synaptosoma.

3. 5HT is primarily localized in the nerve endings also in the case of the heart and mitochondria also contain 5HT in a considerable quantity.

4. In the heart 5HT is also localized in the muscle elements.

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SZEROTONIN INTRACELLULÁRIS MEGOSZLÁSA
HELIX POMATIA KÖZPONTI IDEGRENDSZERÉBEN ÉS SZIVÉBEN

Hiripi László, Elekes Károly és S.-Rózsa Katalin

Összefoglalás

Szerotonin szubcelluláris lokalizációjának vizsgálata differenciál és gradiens centrifugálással *Helix pomatia* ideg és szívszövetében igazolta, hogy

1. A ganglion és szív homogenizátum primér frakcióiban az 5HT 70%-a kötött, 30%-a szabad formában van jelen. Ganglionban a nuclearis frakció 5HT tartalma relatíve magas.

2. Idegszövetben a kötött 5HT a legnagyobb hányadát a szinaptoszóma dense-core vezikulái tartalmazzák.

3. Az 5HT a szív esetén is elsődlegesen idegvégződésekre lokalizált, de emellett a mitochondriumok is jelentős mennyiségű 5HT-t tartalmaznak.

4. Az 5HT szívben az izomelemekre lokalizáltan is megtalálható.