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...PAMINE: LOCALIZATION OF UPTAKE IN ... PEDAL GANGLION OF *QUADRULA* ...STULOSA (PELECYPODA)

ABSTRACT. Uptake of ^{3}H-dopamine in the pedal ganglion of *Quadrula pustulosa* was localized using combined fluorescence and optical light microscopy, in addition to electron microscope autoradiography. Light microscope autoradiograms of the same sections previously prepared for fluorescence microscopy indicated most radioactivity occurred over green, long-lasting fluorescent fiber tracts and nerve cell bodies. Ultrastructural autoradiographic results showed the majority of the radioactivity was localized over synaptic vesicles. Nerve fibers containing neurotubules, and also a limited number of nerve cell bodies were labelled with ^{3}H-dopamine. These results suggest uptake of dopamine to be specific for dopaminergic structures.

Introduction

...mammalian nervous system ...uptake of putative neurotrans- ... functional in terminating ... and maintaining levels of ... (Iversen, 1967). In the ... uptake has received relatively ... and only recently has bio- ...ence indicated uptake in the ... have pharmacological and ...erties similar to mammalian ...ester *et al.*, 1971; Myers and ... Morphological localization ...monoamines has been largely ...5-hydroxytryptamine (5-HT) ...gastropods. Autoradiographic ...into uptake of 5-HT have ...mulation to be both specific ...Iversen, 1969; Pentreath and ... and non-specific (Ascher *et* ... nervous tissues. In a pelecypod, ... Sweeney (1972) presented auto- ... evidence for the specificity of ...H-dopamine (DA) into neuronal structures. Kerkut *et al.* (1967) and Sedden *et al.* (1968) used gastropods to study dihydroxyphenylalanine and 5-hydroxytryptophan uptake and observed specific increases in characteristic DA or 5-HT fluorescence, depending on the precursor used.

The fluorescence method of Falck *et al.* (1962) and autoradiography are both sensitive and specific histochemical techniques for the demonstration and localization of monoamines. In the molluscs, Welsh (1972) has recently reviewed the fluorescence histochemical results that describe the widespread distribution of DA and 5-HT. Combined procedures for fluorescence and optical light microscope (OLM) autoradiography have proven useful for localizing newly accumulated 5-HT in relation to endogenous stores. Fuxe *et al.* (1965) used fluorescence and electron microscopy to study monoamine terminals in rat brain. Hammarström *et al.* (1966) have studied localization of labelled 5-HT in relation to endogenous 5-HT of mouse gastrointestinal tract.

The combined techniques have been utilized here in an effort to further understand the nature of uptake of DA in the pedal ganglion of a freshwater pelecypod and its specificity for neuronal structures. In addition, uptake has been localized on the subcellular level using electron microscope autoradiography.

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Materials and Methods

Pedal ganglia of *Q. pustulosa* were obtained as previously described (Myers and Sweeney, 1972). Preparation of ganglia for light microscopy was by routine histological procedures. Tissues were fixed in Bouin's fixative, embedded in Paraplast (Curtin Scientific), sectioned at 3 μ and stained with hematoxylin and eosin.

For fluorescence microscopy, freshly dissected ganglia were coated with talc, frozen in liquid nitrogen, and dried for 25–26 hr at −50°C in an Edwards–Pearse Tissue Freeze Drier. Monoamine fluorescence was induced by exposure of whole ganglia to formaldehyde vapors (paraformaldehyde stored at least 1 week at 58% R.H. prior to use) for 1·5–2 hr at 80°C. The tissues were vacuum embedded in Paraplast for 1 hr, sectioned at 3 μ on a Reichert Ultramicrotome, and transferred to a glass slide coated with a thin film of albumin affixative. After applying a drop of immersion oil-xylene (1 : 1) and a coverslip, sections were examined and photographed under a Leitz fluorescence microscope.

For combined fluorescence microscopy and OLM autoradiography, ganglia were labelled by incubation in saline containing ascorbate (1 mg/5 ml) and made up to 1 μM ^{3}H-DA (15 min incubation at 25°C). After 15 min wash in saline, ganglia were processed for fluorescence microscopy and photographed, as described above. To prepare the same sections for autoradiography, coverslips were removed by allowing slides to soak in xylene. When slides were removed from the xylene, a drop of 50% Entellan (E. Merck AG, Damstadt, Germany) was applied to the sections and the excess drained off. Sections were then coated with Kodak NTB-3 Nuclear Emulsion by a roller method, exposed for 7–14 days, developed in Dektol (Kodak), and stained according to Montreuil-Langlois (1962). OLM autoradiograms were photographed using a Zeiss Photomicroscope equipped with phase optics, correlating radioactive structures with fluorescent structures.

Both labelled and unlabelled ganglia were prepared for the electron microscope according to the method of Gupta *et al.* (1969). Ganglia were fixed with 2% glutaraldehyde in cacodylate buffer (pH = 7·60) and post-fixed in 1% osmium tetroxide. The
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Ganglia were labelled with ^{3}H-DA accor
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Autoradiograms were exposed 4 week
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processing. Average grain size was less
500 Å with sizes ranging from very
electron-dense particles to not more
700 Å. Tissues were examined in an
3-H electron microscope.

Results

Morphology of the pedal ganglion

A limited description of the morphol
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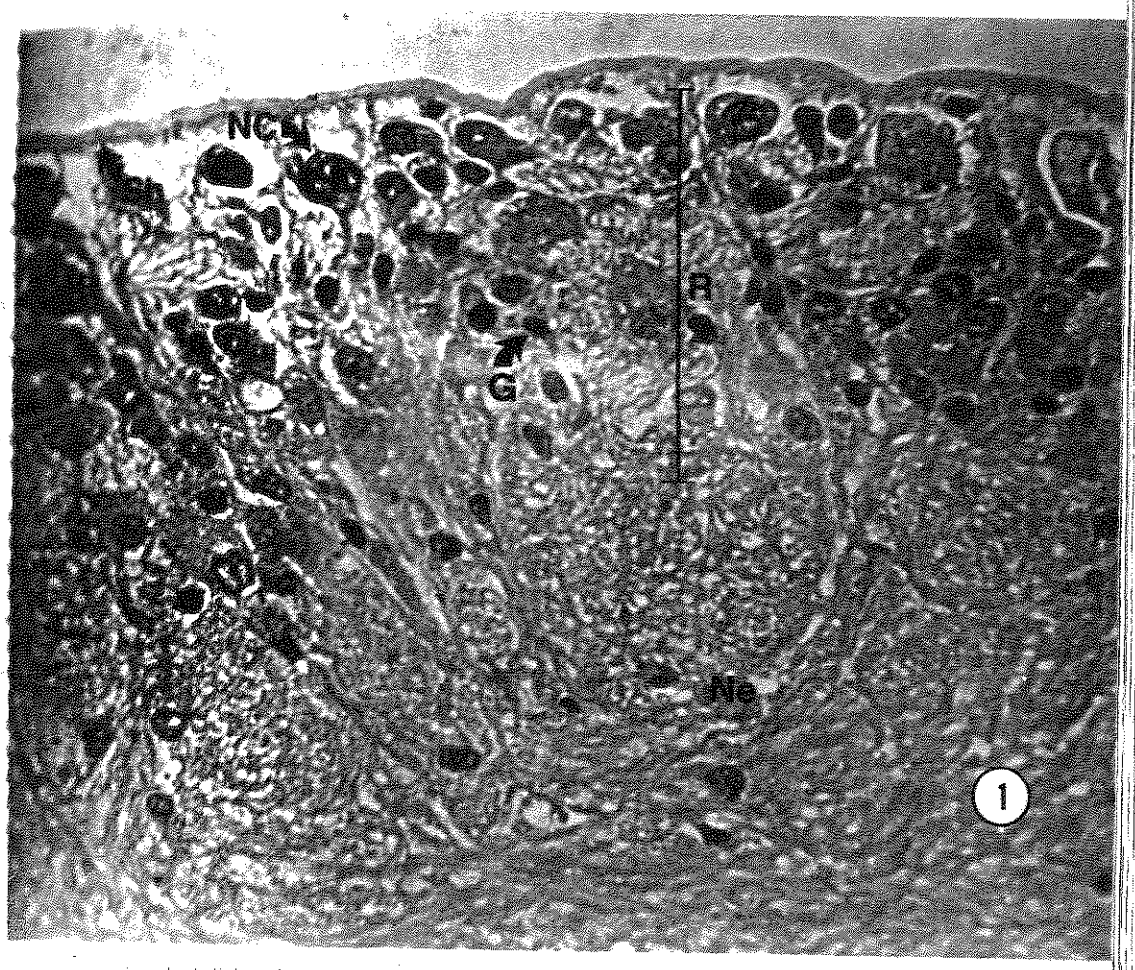

Fig. 1. A light microscope section of the pedal ganglion showing its anatomical organization. The ganglion is surrounded by a sheath (Sh). Below the sheath is the cortical layer (R) characterized by nerve cells (NC) and glial cells (G). The interior of the ganglion, the neuropile (Ne) is a mass of nerve fibers and some glial cells. ×400.

vulgnea (Zs.-Nagy, 1964). Nerve
are characterized by their mono-
processes and the presence of
in the perikaryon (Zs.-Nagy and
Bullock, 1970). Fibers contain neuro-
and neurofilaments, or in many in-
are packed with vesicles of varying
density, and appearance (Fig. 2).
functions appear to be exclusively
; no axo-somatic synapses were
observed. Synapses were character-
thickened pre- and post-synaptic
Generally two types of synapses
were observed: those containing a mixture of dense-cored vesicles (750–1000 Å) of varying density plus some 'clear' vesicles (Fig. 3), and those containing smaller, clear synaptic vesicles (500–700 Å; Fig. 4). Synapses containing only clear synaptic vesicles of the smaller size are possibly cholinergic, since it is known that molluscan ganglia contain acetylcholine. Synapses containing a mixture of dense-cored and clear vesicles most likely contain monoamines (Pfeifer, 1968). The varying degree of density in vesicle cores within a single population

is apparently dependent upon the monoamine content of the vesicles (Zs.-Nagy, 1967). The larger dense-cored vesicles that were observed (1400–1700 Å) are similar to those described by Gerschenfeld (1963) as 'neurosecretory' vesicles.

Fluorescence microscopy

Data have been interpreted on the premise that yellow-green fluorescence is a result of 5-HT, and that green fluorescence is a result of DA presence (Kerkut *et al.*, 1967; Sedden *et al.*, 1968). Likewise, rapidly fading fluorescence is characteristic of 5-HT containing structures and longer-lasting fluorescence is characteristic of DA containing structures (Cottrell and Osborne, 1970; Jaeger *et al.*, 1971). Results indicated two distinct types of fluorescence in the pedal ganglion. A less intense, yellow-green fluorescence which tended to fade rapidly was attributed to 5-HT containing structures. This type of fluorescence was observed in a majority of the cell bodies that fluoresced in the periphery of the ganglion. Fewer fiber tracts directly below the cell body layer displayed fluorescence indicative of 5-HT. The other characteristic fluorescence appeared to be green and longer-lasting, most likely a result of DA. However, this type of fluorescence occurred less often in cell bodies than the fluorescence due to 5-HT. Fig. 5 is a section of the bi-lobed pedal ganglion showing peripheral nerve cells of each lobe and the inner neuropile. DA fluorescence appeared primarily in fiber tracts directly below the cell body layer (Fig. 5). Fluorescence from 5-HT was often poorly recorded graphically since it faded rapidly examining sections, and also because long exposure times for the film (2.5 Decreased fluorescence intensity ing long exposures was a result of the sections (3 μ) taken, imperative for bined fluorescence and OLM autoradiography. Glial cells generally did not fluorescence. Control ganglia not to formaldehyde displayed little autofluorescence.

Combined fluorescence microscopy and OLM autoradiography

Autoradiograms of ganglia labelled ^{3}H-DA were carefully correlated fluorescent micrographs of the same All fluorescing structures were not sarily radioactive. A sampling of autograms showed 15% (88 cells out of the nerve cell bodies were radioactive. Similarly, examination of fluorescence graphs and autoradiograms showed able correlation between cell bodies were both radioactive and fluorescent to DA content (Figs. 6a and 6b). This lation is in conformance with the vation that fewer nerve cell bodies long-lasting green fluorescence. The of the green fluorescing fiber tracts the cell body layer were also radioactive (Figs. 7a and 7b). This suggests newly up ^{3}H-DA is mostly confined to dopamine fiber tracts.

Fig. 2. An electron micrograph demonstrating characteristic appearance in the neuropile. Nerve fibers (Nfb) contain neurotubules (Nt) and neurofilaments (Nf) or many times are packed with dense-cored (D) or clear (C) vesicles.

Fig. 3. A characteristic synapse (S) with a mixed population of dense-cored and synaptic vesicles measuring 750–1000 Å. Nerve fibers also contain neurofilaments and occasional large granular neurosecretory vesicles (Ns) which measure 1400 × 54,000.

Fig. 4. An electron micrograph of a synapse populated by small clear vesicles (C) measuring 500–700 Å. Neurotubule (Nt). × 47,000.

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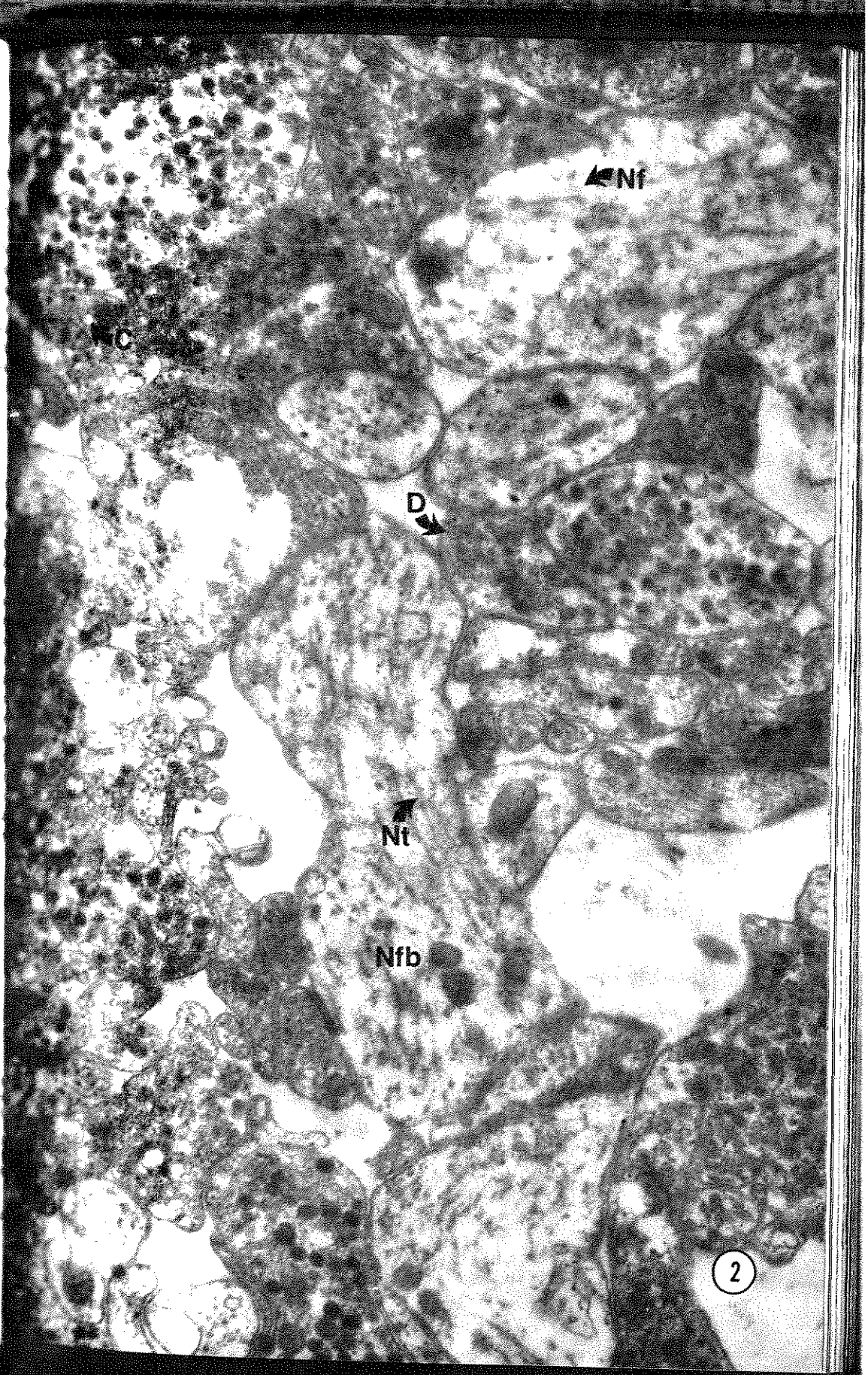

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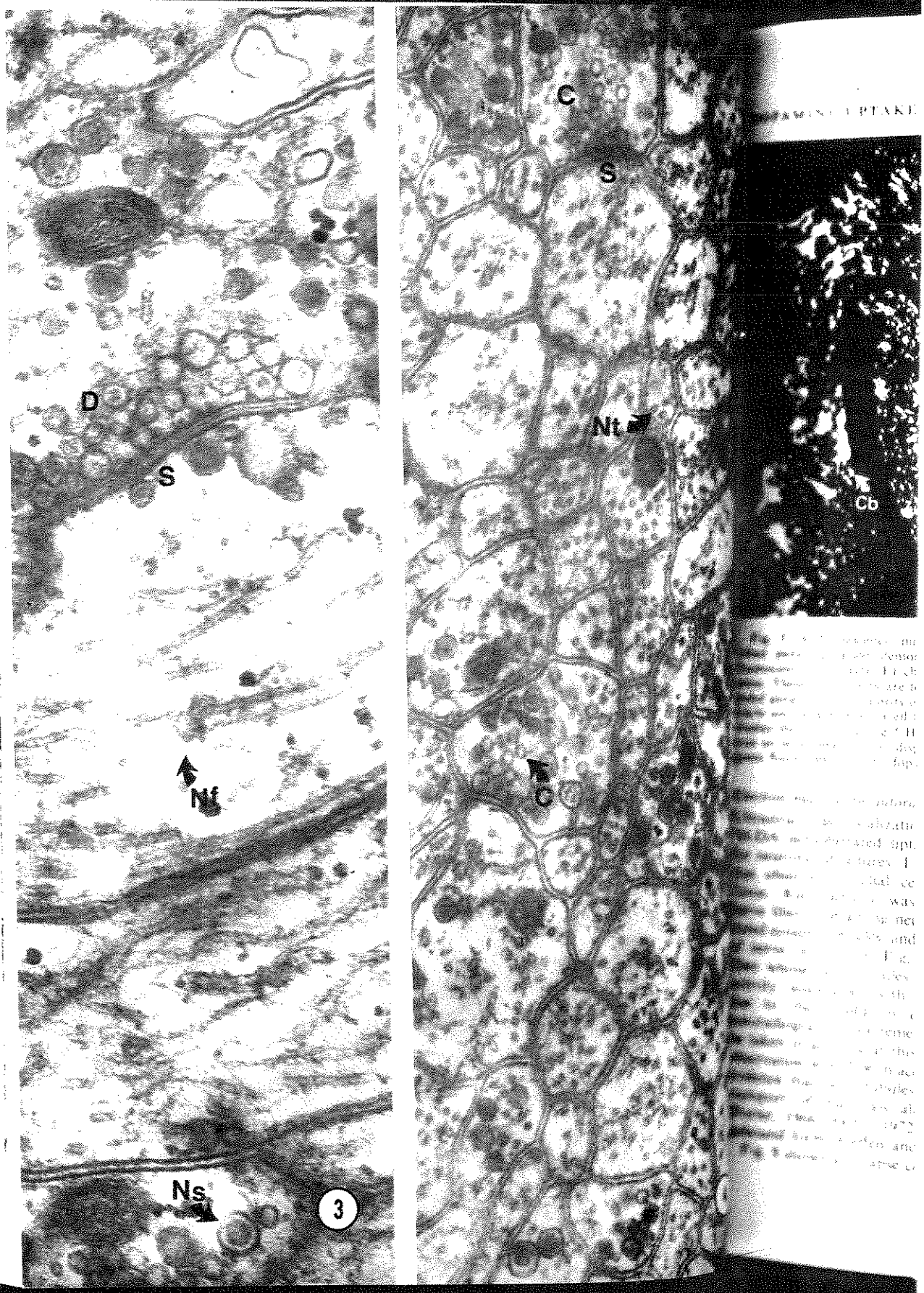
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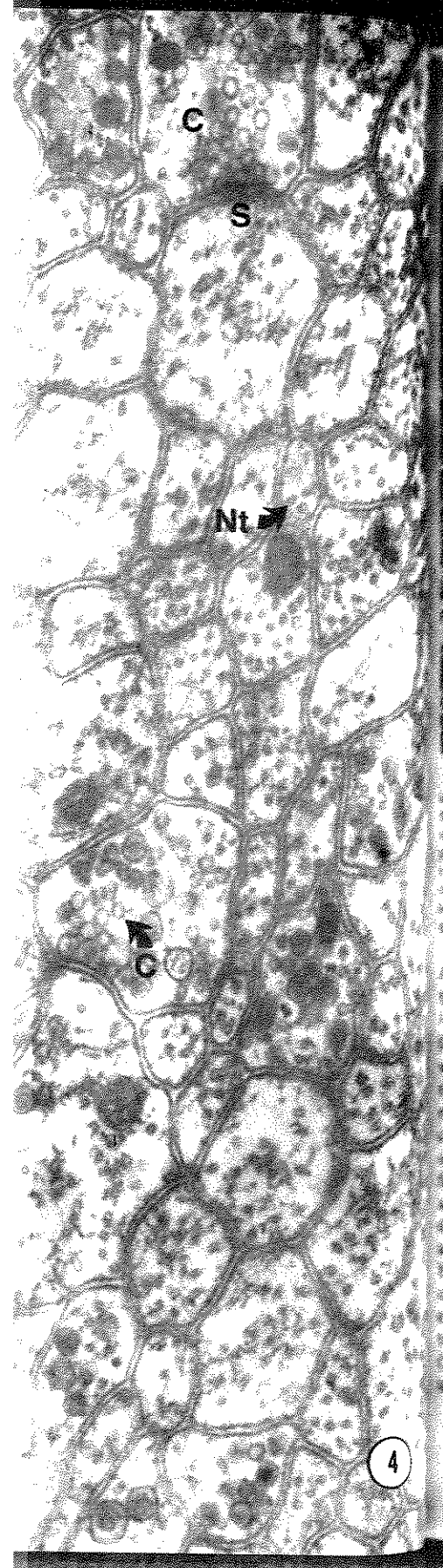

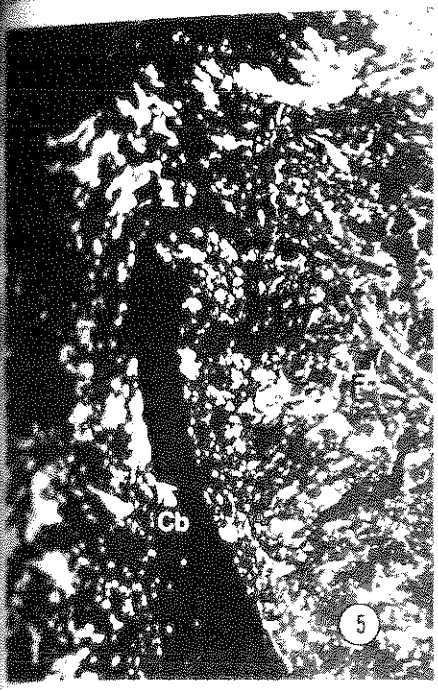

...escence micrograph of the bi-... demonstrating the bright ... (Cb) characteristic of dopa-... are found throughout the ... majority of the fluorescing fiber ... Cell bodies (Cb) primarily ... 5-HT fluorescence, how-... neuron displays the long-lasting ... to dopamine. × 400.

... autoradiography

... localization of uptake of ... demonstrated uptake to be specific ... structures. Little or no uptake ... in glial cells or connective ... radioactivity was associated with ... containing neurotubules, dense-... vesicles, and a limited number ... cell bodies. Fig. 8 shows a nerve ... neurotubules may have radio-... associated with them. However, ... the resolution of ultrastructural ...graphy, extreme caution should ... in arriving at this conclusion. This ... would be in accordance with the ... that neurotubules may function in ... of materials along nerve fibers ...; Ochs, 1972) in a bound or ...rm (Geffen and Livett, 1971). ... shows a synapse containing vesicles labelled with ^{3}H-DA. Both dense-cored and some clear synaptic vesicles are labelled. The density of the core varies from one vesicle to another, even to the extent of being 'clear'. Very few of the small, clear vesicles (500–700 Å) were labelled. Fig. 10 shows a nerve cell body which accumulated ^{3}H-DA. These radioactive cell bodies occurred infrequently in thin sections. Grains were often generally dispersed in the cytoplasm or associated with the endoplasmic reticulum in cell bodies that were radioactive. Association of radioactivity with the Golgi apparatus was not observed.

The small, irregular, autoradiographic grain size (less than 500 Å) resulted from physical development, in addition to the properties of the emulsion. In this process the developer dissolves the silver bromide crystals, leaving only the latent image upon which silver ions present in the solution are then attached (Kay, 1965). Grains were distinguished from precipitate contamination by their size and also by the concentration at specific loci. Background was assessed as being extremely low. This was based upon the absence of grains dispersed randomly over the section and also their absence in areas of emulsion not over tissue. Also any excessive image softness is due to the fact that the electron beam, in addition to penetrating a specimen and supporting membrane, must also penetrate any remaining emulsion base.

Discussion

Results from the combined fluorescence microscopy and OLM autoradiography, and the electron microscope autoradiography strongly suggest chemical selectivity for uptake of ^{3}H-DA into dopaminergic neurons. DA is the only catecholamine present in the pedal ganglion (Myers and Sweeney, 1972). In addition, there appears to be morphological specificity of uptake into defined subcellular structures. Earlier results (Zs.-Nagy, 1967; Sweeney, 1968) with other pelecypods are confirmed here in that DA fluorescence was confined mostly to the nerve fibers in the neuropile and to few cell bodies. Most fluorescing cell bodies in this study displayed characteristic 5-HT fluorescence, a compound known to occur in high concentrations in pelecypods (Zs.-Nagy, 1967; Hiripi, 1968; Salanki, 1972).

The significance of uptake of DA is dependent on evidence that uptake is confined to dopaminergic structures and is not occurring in non-nervous tissue components or serotonergic tissue. Various methodological procedures for morphological localization of uptake have proven useful. Sedden *et al.* (1968) injected the DA precursor (DOPA) and the 5-HT precursor (5-hydroxytryptophan) into ganglia of *Helix*, observing in the former case an increase in green fluorescence in cells and neuropile, and in the latter case an increase in yellow-green fluorescence in these structures. Kerkut (1967) also observed an increase in green or yellow-green fluorescence after injection of DOPA or 5-hydroxytryptophan in *Helix* brain. These experiments cannot definitely ascertain whether DOPA uptake occurred only in dopaminergic tissue or if 5-hydroxytryptophan uptake occurred only in serotonergic tissue.

Autoradiographic methods using labelled precursors may encounter difficulties in distinguishing precursor from product in the developed autoradiogram. Likewise, use of the labelled amine for autoradiographic localization does not determine if uptake is restricted to specific types of nervous tissue even if radioactivity is associated with specific morphological structure. Ascher *et al.* (1968), using OLM autoradiography, reported that in *Aplysia* and *Helix* ganglia most of the radioactivity resulting fr accumulation was localized in the co tissue sheath surrounding the These results are in contrast to tho and Gautron (1969) who used microscope autoradiography to dem accumulation of 5-HT to be cardiac nerve fibers in *Aplysia*. M Sweeney (1972) have also used OL radiography to demonstrate neuron mulation of ^{3}H-DA in a fres pelecypod.

By adapting fluorescence and graphic methods to the same tissue results were mutually supportive taining specificity and localizing ^{3}H-DA. Since both DA and 5-HT fluo were observed in *Q. pustulosa* pedal this method provided evidence specificity of uptake of ^{3}H-DA into minergic neurons. All fluorescent str were not radioactive, however, the of labelled fibers displayed green cence. This would be expected if uptake sites were limited in number

The three types of synaptic vesic cribed as clear, dense-cored, and secretory are similar to those repo other molluscan nervous tissues (Ge feld, 1973). Evidence that neuro-tran substances are contained in, or ass with, these vesicles was first reported Robertis *et al.* (1962). Using rat

Figs. 6a and 6b. A fluorescence micrograph and light microscope autorad the same tissue section showing fluorescent cell bodies and nerve fibers that radioactive from the uptake of ^{3}H-dopamine. Arrows correlate structures that both fluorescence and radioactivity. 6a, ×455; 6b, ×724.

Figs. 7a and 7b. A fluorescent micrograph and light microscope autorad the same tissue section showing a fluorescent nerve fiber tract that has also ^{3}H-dopamine (arrows). There was good correlation between structures that long-lasting green fluorescence due to dopamine and those that were radioac result of ^{3}H-dopamine uptake. 7a, ×455; 7b, ×680.

Fig. 8. An electron microscope autoradiogram of pedal ganglion nerve fiber Radioactivity from uptake of ^{3}H-dopamine is associated with nerve fibers co both neurotubules (Nt), and synaptic vesicles measuring 750–1000 Å. Stars wi indicate representative autoradiographic grains. Small spherical grains resul use of a physical developer. Synapse (S). ×37,000.

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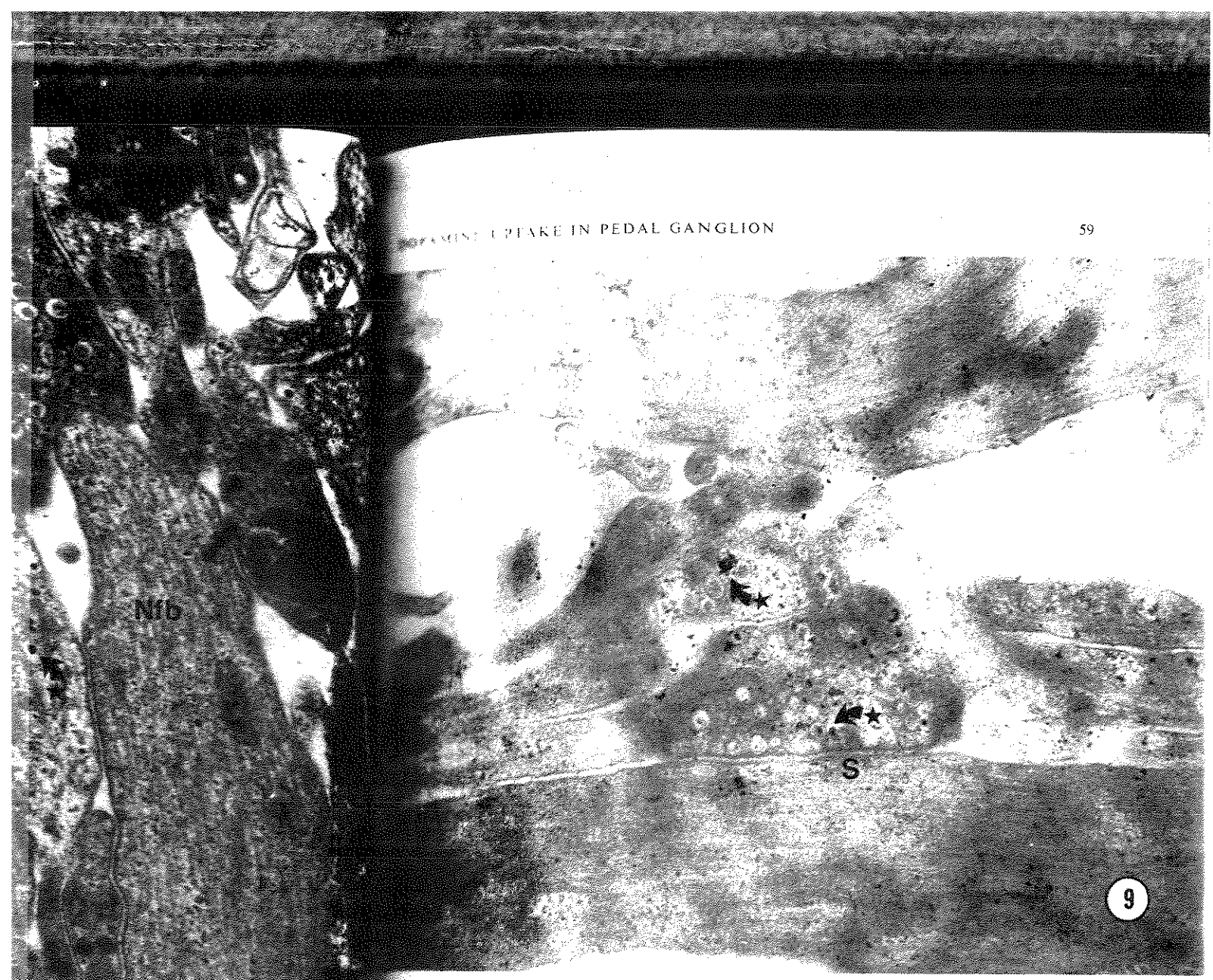

Fig. 1. An electron microscope autoradiogram of a synapse (S) populated by vesicles of varying density which measured 750–1000 Å and have taken up ^{3}H-dopamine. Stars with arrows denote representative autoradiographic grains. Small grain radioactivity size permits resolution of radio-activity over individual synaptic vesicles. ×40,000.

able to correlate acetylcholine synaptic vesicles. Because molluscan have been shown to contain ine (Bacq, 1935; Kerkut and Cot- , one may only speculate that populated by clear vesicles (500– *Q. pustulosa* are cholinergic. vesicles (750–1000 Å), in which of the core appeared to vary, abundantly in the nerve fibers and profiles of the pedal ganglion. In instances the vesicles appeared 'clear' though their size was still in the range dense-cored vesicles. Zs.-Nagy (1967), using a pharmacological method, demonstrated the dense-core is related to DA content in neurons of *Anodonta cygnea* and that the degree of density is dependent upon the DA levels in the vesicle. However, other factors could possibly affect the appearance of vesicle cores, such as the type and time of fixation (Tranzer and Thoenen, 1967). Despite certain precautions, it is reasonable to associate catecholamine content with granular vesicles (Geffen and Livett, 1971). This proves valuable in ascertaining specificity of uptake of DA in *Q. pustulosa* where this amine is the only catecholamine

... Myers and Sweeney, 1972), and is ... stored in the dense-cored vesicles. ... to the evidence obtained on ... microscope level are the ultrastruc- ... autoradiographic data that may pre- ... possibility that DA could be taken ... storage sites. Results here ... uptake occurred primarily into the ... vesicles. Although 5-HT occurs ... concentration in molluscan ganglia, ... evidence for localization in dense- ... of peripheral nerves is lacking. ... results differ between gastropods ... where in the latter 5-HT ... mainly restricted to perikarya ... the neuropile. Cottrell and ... (1970) have employed a cyto- ... method to show 5-HT to be ... granulated vesicles occurring ... cells of *Limax*, while Jourdan ... (1970) have presented similar ... properties of *Aplysia*. Taxi and ... have used electron micro- ... autoradiography to demonstrate ... was localized in nerve ... of *Aplysia* heart. Pentreath ... (1972) have used electron ... autoradiography to localize ... in nerve fiber processes of ... However, using density ... fractionation, Zs.-Nagy (1965) and ... Maget (1967) have shown 5-HT ... associated with dense-cored vesicles ... Thus their results have led ... to conclude 5-HT is not stored ... vesicles, but in association ... other cell particles such as endo- ... reticulum. These data strengthen ... that uptake of DA in this ... is specific for dopaminergic ... dense granular vesicles of peri- ... would contain primarily DA. ... was also shown to be ... nerve fibers containing neurotubules, and also with a limited number of cell bodies. Although most of the label occurred over vesicles, there is uncertainty as to the fate of this 'unpackaged' radioactivity. Radioactive nerve cell bodies were relatively difficult to find using the electron microscope. The grains were generally dispersed in the cytoplasm or associated with the endoplasmic reticulum. This association implies involvement of the newly accumulated amine in the packaging process, although definite association with the Golgi apparatus was not observed. With reference to the 'unpackaged' grains occurring over neurotubules and cells, results do not appear unusual for several reasons. The chosen time schedule for incubation of ganglia in ^{3}H-DA (15 min) may not have been ideal for localization in specific organelles of the perikaryon. In addition, labelling with an end product, as opposed to a precursor, could possibly bypass the need for a cellular 'packaging' process. Thirdly, the possibility exists that organelles located in the nerve cell body are not functional in packaging end product neurotransmitter. Cell bodies often contained abundant rough endoplasmic reticulum and sometimes vesicles associated with the Golgi apparatus. Zs-Nagy (1967) and Gray (1970) reported the role of Golgi in origin of synaptic vesicles in molluscs. In the nerve cell body, therefore, it is not precisely clear how newly accumulated amine is treated. The low rate of formation of dihydroxyphenylacetic acid from DA in this preparation (Myers and Sweeney, 1972) decreases the possibility DA is simply metabolized.

The question of specificity of uptake, both chemical and morphological, carries especial significance if the process is to be considered important in synaptic physiology. Uptake of DA in this preparation has been shown to have kinetic properties not unlike uptake

... electron microscope autoradiogram of a nerve cell body which took ... Grains are dispersed throughout the cytoplasm or are associated ... Mars with arrows indicate examples of grains. Cytosomes (Cy).

in mammalian preparations and sensitivity to selected pharmacological agents (Myers and Sweeney, 1973). The morphological results reported here were imperative in order to support the chemical data that suggest uptake is an active and selective process in molluscan nervous systems. More extensive experimentation into competition studies using amines or their analogs is necessary, along with other anatomical and chemical evidence, in order to evaluate precisely the role of uptake.

Summary

Fluorescence microscopy, optical light microscopy, and electron microscope autoradiography, were used to localize uptake of ^{3}H-dopamine in the pedal ganglion of a freshwater pelecypod. Combined histochemical and optical light microscope autoradiographic techniques demonstrated a correlation between green fluorescent nerve fiber tracts and cell bodies, and radioactivity resulting from uptake of ^{3}H-dopamine. The long-lasting green fluorescence, presumably due to ganglion dopamine, occurred primarily in fiber tracts directly below the cell body layer. Fewer cell bodies displayed long-lasting green fluorescence. Likewise radioactivity from [illegible] ^{3}H-dopamine occurred primarily [illegible] tracts, with only 15% of the [illegible] radioactive. Ultrastructural autoradiography indicated most of the radioactivity associated with synaptic vesicles [illegible] fibers containing neurotubules. Grains appearing over cell bodies occurred [illegible] dispersed in the cytoplasm, often [illegible] with endoplasmic reticulum. These morphological data support neurochemical evidence that uptake of dopamine may be an active process in pedal ganglion physiology. In addition, the results indicate that ^{3}H-dopamine is specific for dopaminergic structures in the pedal ganglion.

Acknowledgements

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Likewise radioactivity from [illegible] ^{3}H-dopamine occurred primarily [illegible] tracts, with only 15% of the [illegible] radioactive. Ultrastructural autorad[illegible] indicated most of the radioact[illegible] associated with synaptic vesicles [illegible] fibers containing neurotubules. [illegible] pearing over cell bodies occurred [illegible] dispersed in the cytoplasm, often [illegible] with endoplasmic reticulum. These [illegible] logical data support neurochemical [illegible] that uptake of dopamine may be an [illegible] process in pedal ganglion physi[illegible] addition, the results indicate [illegible] ^{3}H-dopamine is specific for dop[illegible] structures in the pedal ganglion.

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This work constitutes a portion [illegible] doctoral thesis submitted to the [illegible] College, University of Illinois, [illegible] Illinois.

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