Z. Zellforsch. 141, 33—54 (1973)

Evidence for the Existence of Serotonin-, Dopamine-, and Noradrenaline-Containing Neurons in the Gut of *Lampetra fluviatilis**

H. G. Baumgarten*, A. Björklund**, L. Lachenmayer, A. Nobin** and E. Rosengren***

Department of Neuroanatomy, Institute of Neurology, University of Hamburg, Federal Republic of Germany, and Departments of Histology and Pharmacology, University of Lund, Sweden

Received March 16, 1973

Summary. Monoamine-containing neurons in the gut of *Lampetra fluviatilis* are characterized by histochemical, electron microscopical and biochemical methods. Strongly yellow fluorescent, probably serotonin-containing, intrinsic neurons are found along the entire length of the intestine. Their processes aggregate to form large bundles of mainly non-terminal axons, constituting a subepithelial fibre plexus. This subepithelial, ganglion cell comprising plexus is connected to a wide-meshed subserosal plexus which has ganglion cells of different size and few varicose, single axons. Intermingled with both plexus there occur — in the anterior and middle but not in the preanal portion of the lamprey intestine — scattered green fluorescent intrinsic perikarya, emanating faintly green fluorescent, poorly varicosed axons.

The formaldehyde-induced neuronal fluorophores conform to serotonin (yellow fluorescent compound), noradrenaline, and dopamine (green fluorescent substance), as revealed in microspectrofluorimetric recordings. The electron microscopical analysis of the yellow fluorescent intrinsic neurons in the terminal hindgut shows nerve cell pericarya and axons equipped with a typical population of occasional small granular and many large granular vesicles (750–1600 Å). The number and opacity of cores of the small and the osmiophilia of the cores of the large granular vesicles are significantly increased following short-term treatment with 5,6-dihydroxytryptamine. Long-term treatment with 5,6- or 5,7-dihydroxytryptamine provokes severe signs of ultrastructure impairment and eventual degeneration in the supposed serotonin-containing axons, besides indications of piling-up of organelles in the non-terminal axons due to arrest of axonal transport.

Chromatography of acid extracts from the lamprey intestine, gills and kidney reveals the presence of serotonin (besides another unidentified indoleamine) and dopamine and noradrenaline in the gut, but only dopamine in the brain. The detection of serotonin, noradrenaline and dopamine in the lamprey gut is confirmed by chemical determinations.

The occurrence of intrinsic serotonin-, noradrenaline- and dopamine-containing neurons in the gut of *Lampetra fluviatilis* deviates from the established pattern of innervation of the vertebrate intestine and is considered to be a remnant of an autonomic innervation principle common in invertebrates.

Key words: Intestine, *Lampetra fluviatilis* — Serotonin-, dopamine-, noradrenaline-containing neurons — Identification by histochemistry — Electron microscopy — Biochemistry.

Introduction

Serotonin has been suggested to play a transmitter role in neurally mediated control of peristalsis in the mammalian gut (Bülbring and Gershon, 1967). Fluorescence histochemistry of the mammalian gut, however, has failed to provide any

* Supported by grants from the Deutsche Forschungsgemeinschaft.
** Supported by grants from the Swedish Medical Research Council (No. 14X-712 and 14X-56.
*** The authors are indebted to Lilan Bengtsson, Gertrude Stridsberg, Eva Svensson and Rolf Frank for skilful technical assistance.

evidence for separate serotonin-containing nerves in Auerbach's (or Meissner's) plexus of various species (Norberg, 1964; Jacobowitz, 1967; Baumgarten, 1967; Baumgarten, Holstein and Owman, 1970; Costa and Furness, 1971; review by Burnstock, 1969).

In chemical determinations, Robinson and Gershon (1971) detected small concentrations of endogenous serotonin (5-hydroxytryptamine,5-HT) in longitudinal muscle strips with Auerbach's plexus adherent, of the guinea-pig ileum. The localization of these small amounts of endogenous 5-HT was not established, and it does not seem unlikely that such low concentrations might have been caused by platelet or mast cell 5-HT or by a leakage of enterochromaffin-cell 5-HT during the preparative removal of the muscle strips. After catecholamine depletion and monoamine oxidase inhibition, a weak diffuse UV-labile, formaldehyde-induced yellow fluorescence appeared in Auerbach's plexus. The structural localization of this fluorescence was not clearly observable, however. Robinson and Gershon (1971), moreover, reported that indoleamine fluorescence could be induced in Auerbach's plexus by the application of 5-hydroxytryptophan (5-HTP). Because of the wide-spread occurrence of unspecific l-amino-acid decarboxylase (see Pletscher, Gey and Burkard, 1966; Molinoff and Axelrod, 1971) most tissues will synthesize serotonin after the administration of its precursor,5-HTP, and the induction of serotonin fluorescence in tissues upon application of 5-HTP does not necessarily reflect storage of 5-HT under physiological conditions.

Studies on the ^{3}H-5-HT uptake by longitudinal muscle strips from the guinea-pig ileum indicate that this occurs in structures not identical with the adrenergic nerves (Ross and Gershon, 1972). From the results of radioautographic labelling of neuronal elements in the myenteric plexus by ^{3}H-5-HT, Ross and Gershon (1972) concluded that non-adrenergic nerves equipped with large granular vesicles (corresponding to those described by Baumgarten, Holstein and Owman (1970) as p-type fibres in the mammalian colon) were responsible for specific uptake of tritiated serotonin. In all probability, nerve fibres characterized by such large opaque vesicles belong to the so-called intrinsic inhibitory non-adrenergic, non-cholinergic neurons that have been claimed by Burnstock and co-workers to synthesize and store ATP (see review by Burnstock, 1972) rather than endogenous 5-HT. The results of Robinson and Gershon (1971) and Ross and Gershon (1972) suggest that intrinsic perikarya of Auerbach's plexus in mammals are able to bind and to retain serotonin, but this does not necessarily mean that the neurons in question are serotonergic.

True serotonin neurons possess a high affinity, selective uptake mechanism for serotonin (Shaskan and Snyder, 1970) and related compounds such as 5,6-dihydroxytryptamine (Baumgarten, Evetts, Holman, Iversen, Vogt and Wilson, 1972). 5,6-Dihydroxytryptamine (5,6-DHT), which makes central 5-HT neurons degenerate after its intraventricular injection (Baumgarten, Björklund, Lachenmayer, Nobin and Stenevi, 1971; Baumgarten. Lachenmayer and Schlossberger, 1972; Baumgarten, Björklund, Holstein and Nobin, 1972; Björklund, Nobin and Stenevi, 1973), is taken up into the noradrenaline-containing nerve terminals of Auerbach's plexus in the rat and causes a partial chemical sympathectomy, but leaves other myenteric neurons unaffected (Baumgarten, Göthert, Holstein and Schlossberger, 1972). In order to unmask specific uptake sites for indoleamines in

the guinea-pig myenteric plexus of the ileum, animals were injected with repeated doses (75 mg/kg) of 6-hydroxydopamine (6-OH-DA). After degeneration of the adrenergic nerve terminals, induced by the 6-OH-DA treatment, 6-hydroxytryptamine or 5,6-dihydroxytryptamine was given to the adrenergically denervated animals, but no distinct staining of separate indoleamine concentrating neuronal elements was revealed at short term experiments (Baumgarten, unpublished observations).

Recently, Furness and Costa (1973) described a set of noncholinergic excitatory nerves in the guinea-pig proximal colon; the stimulation of these nerves was mimicked by exogenous 5-HT. The 5-HT-induced contractions of the smooth muscle were blocked by phentolamine (an unspecific blocker substance) and by methysergide (a compound which also inhibits uptake of indoleamines into noradrenergic nerves; Baumgarten, unpublished findings). Further evidence for the possibility of tryptaminergic transmission in the guinea-pig colon was gained by the finding that the 5-HT mediated contractions became tachyphylactic upon repeated application of exogenous 5-HT. These observations, however, merely indicate that the substance released from the nerves is probably chemically similar to 5-HT. However, extensive studies of drug-induced changes in the fluorescence characteristics of nerves in the proximal colon of the guinea-pig produced no indication of other than adrenergic structures (Costa and Furness, 1971).

Thus, we are facing a complicated, uncertain, and even contradictory picture concerning the role of 5-HT and possible existence of tryptaminergic nerves involved in control of the smooth muscle motility in the mammalian intestine. Contrary to this, serotonin-containing nerves innervating peripheral tissues have recently been clearly demonstrated in invertebrates (Burnstock and Robinson, 1967; Ehinger, Falck and Myhrberg, 1968; Ehinger and Myhrberg, 1972; Myhrberg, 1972). Among the lower vertebrate species, cyclostomes might retain certain features of invertebrates, especially concerning the organization of their visceral organs. If this is so, the invertebrate principle of peripheral serotonergic innervation might also be retained in these early forms of vertebrates. Studies on the distribution of monoamines in the lamprey brain (Baumgarten, 1972) provided evidence that certain vessels in the head region of the river lamprey are indeed innervated by serotonin-containing nerves. Subsequent work established that yellow fluorescent, probably 5-HT-containing, nerves are wide-spread in this species and that the gut has the densest network of intrinsic 5-HT-containing nerves so far detected.

The present paper reports biochemical, histochemical, microspectrofluorometric and electron microscopical evidence for the existence of serotonin-containing neurons in the lamprey gut.

Material and Methods

Animals. More than 250 adult lampreys *(Lampetra fluviatilis)*, caught in rivers of Northern Sweden during winter and stored for up to three months in aquaria were used for the present study.

Fluorescence Microscopy and Microspectrofluorometry. Pieces from different levels of the gut were dissected out and rapidly frozen in propane to the temperature of liquid nitrogen. After freeze-drying, they were processed for the fluorescence microscopical visualization of biogenic monoamines according to the Falck-Hillarp method (for technical details, see Björk-

lund, Falck and Owman, 1972; Baumgarten, 1972). The sections were mounted either on microscope slides for fluorescence microscopy or on cover slips for microspectrofluorometric analysis.

The microspectrofluorometric analysis was perfored with a modified Leitz microspectrofluorometer, according to Björklund *et al.* (1972). The spectral identification of noradrenaline and dopamine was carried out according to the method of Björklund, Ehinger and Falck (1968). All spectra were corrected and expressed as relative quanta versus wavelength.

Electron Microscopy. Freshly dissected pieces from the midgut and proximal and terminal hindgut of decapitated lampreys of either sex were immersed in 6% ice-cold glutaraldehyde (phosphate buffered; total osmolarity about 950 mosmol) for $^1/_2$ hr and postfixed for 3 hrs in phosphate-buffered (0.1 M) OsO_4-solution (1%) containing 0.1 M sucrose at pH 7.2. Embedding was performed in Epon 812. Sectioning, staining, and electron microscopy were performed as described earlier (see Baumgarten, Björklund, Holstein and Nobin, 1972). Besides control specimens, gut pieces from animals pretreated with either 5,6- or 5,7-dihydroxytryptamine[1] were analyzed microscopically. Micrographs were taken in a Philips EM 300. For drug treatment schedules, see "Pharmacological treatments".

Monoamine Determinations. 5-Hydroxytryptamine (5-HT), was determined fluorimetrically according to the method of Bertler (1961) and noradrenaline (NA) and dopamine (DA) according to Bertler *et al.* (1958) and Häggendal (1963). The determinations on the gut were made either on pooled whole gut samples or on pooled dissections of the proximal and distal portion of the gut, respectively.

Chromatographic analysis. Serotonin was identified by thin layer chromatography. The entire gut, the kidneys, and the gills were pooled, the samples were homogenized in a mixture of acetone and 0.1 N hydrochloric acid (95:5, vol/vol), centrifuged, and the supernatants reduced to a small volume that was spotted onto silica gel thin layer plates (Kieselgel H, Merck, Darmstadt, W. Germany). The plates were developed unidimensionally in methyl-acetate-isopropanol-ammonia 9:7:4 (with 2 mg/100 ml EDTA added). After development, the plates were reacted with the formaldehyde spray reagent according to Seiler and Wiechmann (1964; see Björklund *et al.*, 1970). Reference compounds were run parallel on the chromatograms.

Catecholamines were identified by paper chromatography. 2 samples of 30 pooled lamprey guts, one sample of 10 lamprey brains, and 2 pooled rat brains were analyzed. The samples were homogenized in 0.4 N perchloric acid. The catecholamines were adsorbed onto alumina at pH 8.6 eluted with 0.1 N hydrochlorid acid, and then spotted onto paper chromatograms. The chromatograms were run in phenol-0.1 N HCl 9:1. After development, the papers were reacted with gaseous formaldehyde (2 hrs at +80°C) according to Björklund *et al.* (1970). Reference compounds were run parallel on the chromatograms.

Pharmacological Treatments. Half of the total number of animals processed for fluorescence microscopy received 200 mg/kg i.p. nialamide 6 hrs before killing. 10 animals were pretreated with reserpine, 10 mg/kg i.p., 24 hrs before decapitation. 10 animals were given 15 mg/kg i.p. 5,6-dihydroxytryptamine (creatinine sulfate H_2O), 20 hrs before processing for fluorescence and electron microscopy (4 animals for each technique); 2 animals were fixed for electron microscopy 2 hrs after the i.p. injection of 5,6-DHT. 10 animals were pretreated with one injection of 45 mg/kg i.p. 5,7-dihydroxytryptamine (creatinine sulfate $^1/_2$ H_2O) 24 hrs before processing for either fluorescence or electron microscopy (5 animals per technique).

Results

Chromatographic Analysis. On the silica gel thin layer chromatograms of the acid gut extracts, a yellowish fluorescent spot was detected with the formalin spray reagent that was isographic with 5-hydroxytryptamine. It was clearly separated from the blue tryptophan spot and also from several closely related

1 The generous supply of 5,7-dihydroxytryptamine by Dr. A. Manian, National Institute of Mental Health, Bethesda/Maryland, USA, is gratefully acknowledged.

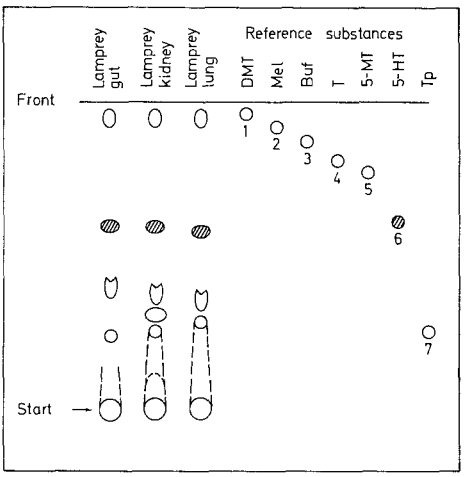


Fig. 1

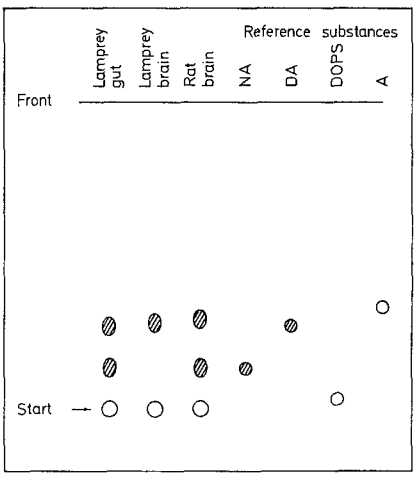


Fig. 2

Fig. 1. Indoleamine chromatography on silica gel thin layer developed in methylacetate-isopropanol-ammonia (9:7:4); the spots were visualized with formalin according to Seiler and Wiechmann (1964). *Note added in proof:* In Fig. 1 Lamprey *lung* means Lamprey *gills*

Fig. 2. Catecholamine chromatography on paper, developed in phenol-0,1 N HCl 9:1; the spots were visualized by formaldehyde gas treatment according to Björklund *et al.* (1970). The spots were observed as fluorescence under UV-light (365 nm). Abbreviations: *DMT* N,N-dimethyltryptamine; *Mel* N-acetyl-5-methoxytryptamine (melatonin); *Buf* N,N-dimethyl-5-hydroxytryptamine (bufotenin); *T* tryptamine; *5-MT* 5-methoxytryptamine; *5-HT* 5-hydroxytryptamine; *Tp* tryptophan; *NA* noradrenaline; *DA* dopamine; *DOPS* 3,4-dihydroxyphenylserine; *A* adrenaline

indoleamines, such as NN-dimethyltryptamine, N-acetyl-5-methoxytryptamine (melatonin), tryptamine, and 5-methoxytryptamine (Fig. 1). Fluorescent spots isographic with 5-HT were also found in acid extracts from lamprey kidney and gills. In addition, in the extracts from the lamprey gut, kidney, and gills, a second yellowish spot was observed on the developed chromatograms which was isographic with NN-dimethyltryptamine or possibly N-acetyl-5-methoxytryptamine (melatonin) (Fig. 1).

Analysis of the catecholamines in lamprey gut and brain were performed by paper chromatography. The formaldehyde gas treated chromatograms showed, in the extracts from gut and brain, an intensely fluorescent spot which co-chromatographed with dopamine (Fig. 2); in addition, in the extracts from lamprey gut, a weak fluorescent spot was found isographic with noradrenaline (Fig. 2). No adrenaline could be detected in these extracts.

Chemical Analysis. In several tissues and organs from the lampreys, significant amounts of serotonin could be detected fluorimetrically (see Table); the values obtained from the various tissues are listed in Table 1. The highest concentration was found in the gut, whereas lower concentrations were found in brain and still lower levels in kidney, gills, striated muscle and skin.

Table 1. Concentrations of 5-HT, NA and DA in different tissues of the lamprey. Values are ± S.E.M. Figures within brackets give number of determinations

Tissue	5-HT (μg/g)	NA (μg/g)	DA (μg/g)
Gut		0.13 (2)	1.57 (2)
Proximal Gut	13.20 ± 2.35 (4)	0.27 (2)	0.70 (2)
Distal Gut	8.93 ± 1.87 (4)	0.06 (2)	0.63 (2)
Brain	2.06 ± 0.30 (6)	0.02 ± 0.01 (6)	0.57 ± 0.06 (6)
Heart[a])	0.04 (1)	2.84 (2)	1.60 (2)
Skin	0.14 (1)	0 (1)	0 (1)
Kidney	0.33 (1)	0 (1)	0 (1)
Muscle	0.11 (1)	0.21 (1)	0 (1)
Gills	0.46 (1)	0.05 (1)	0 (1)
Dorsal body wall	0,26 (1)	5.97 (1)	0 (1)

a) Adrenaline: 42,21 μg/g (1).

The chromatographic analysis showed detectable amounts of both dopamine and noradrenaline, and the fluorimetric determinations confirmed these results (see Table 1). In the gut, the catecholamines occurred in considerably lower concentrations than serotonin.

Fluoroscence Microscopy. Along the entire length of the gut, the mucosal epithelium exhibited a strong yellow-brownish autofluorescence (Fig. 3, 4), present in freeze-dried tissue before or after formaldehyde treatment. The autofluorescence was insensitive to irradiation with blue excitation light and was stored in large cytoplasmic granules of mucosal epithelial cells. It most likely derived from a lipochrome pigment. Small yellow-brownish granules were randomly distributed all over the subepithelial connective tissue layer (Fig. 3). They might correspond to lipid droplets seen in fibroblasts with the electron microscope.

Formaldehyde treatment failed to induce any monoamine fluorescence in the mucosal epithelial cells; thus the intestinal epithelium in Lampetra lacks monoamine-containing enterochromaffin cells.

Freeze-dried gut tissue exposed to formaldehyde (80°C, 1 hr) revealed monoamine fluorophores bound to nervous structures and cells without processes. Two types of fluorophores could be distinguished: a bright yellow fluorescent compound which faded rapidly upon irradiation with blue excitation light, and a clearly green fluorescent substance showing a more retarded photodecomposition. Thus,

Fig. 3. Longitudinal section from lamprey hindgut showing brightly fluorescent lipochrome granules of the mucosal epithelial cells (left side of the picture) and the subepithelial connective tissue layer showing yellow-fluorescent medium sized 5-HT perikarya, associated with brightly yellow fluorescent axons. The small fluorescent dots spread over the connective tissue layer conform to small autofluorescent granules stored in fibrocyte-like cells. ×280

Fig. 4a—c. Longitudinal section from the lamprey hindgut showing details of the subepithelial 5-HT plexus. a) bundles of undulating, highly yellow fluorescent, mainly smoothly contoured nerve fibres (control specimen), b) ganglion cells associated with and contributing to axons of the 5-HT plexus, c) heavily twisted axons of the subepithelial plexus after 5,7-DHT treatment. Note distending of individual highly fluorescent axons. ×280

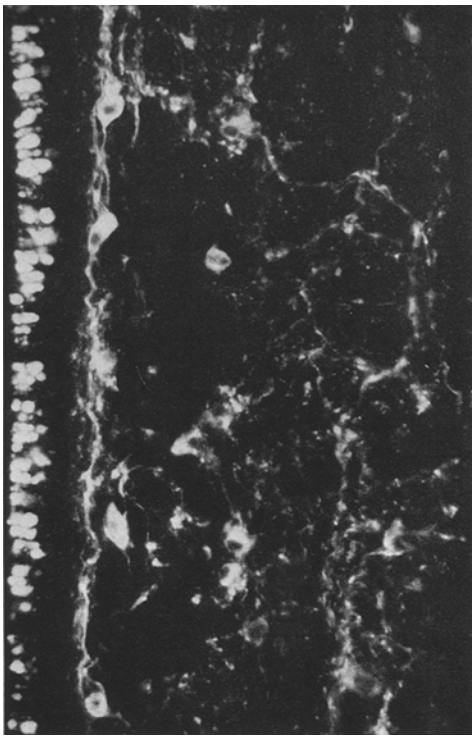


Fig. 3

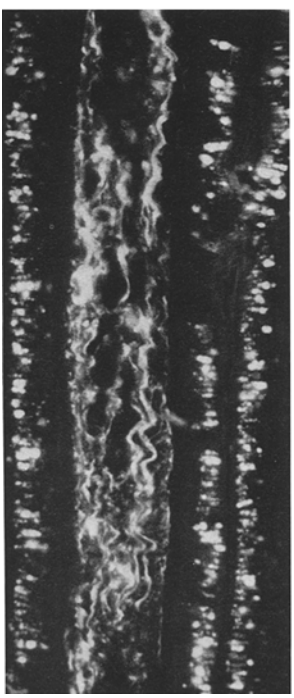


Fig. 4a

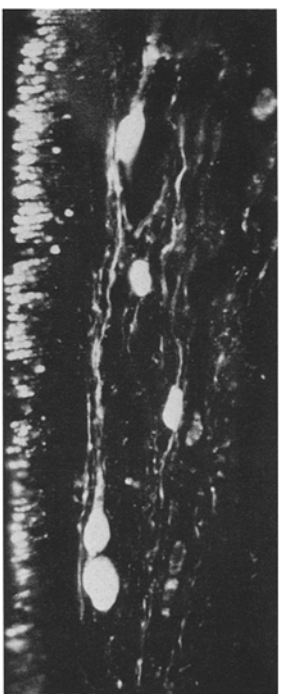


Fig. 4b

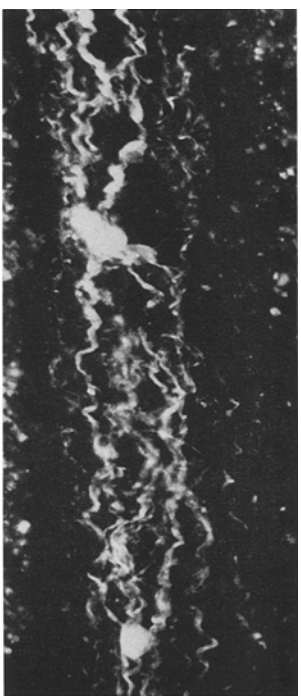


Fig. 4c

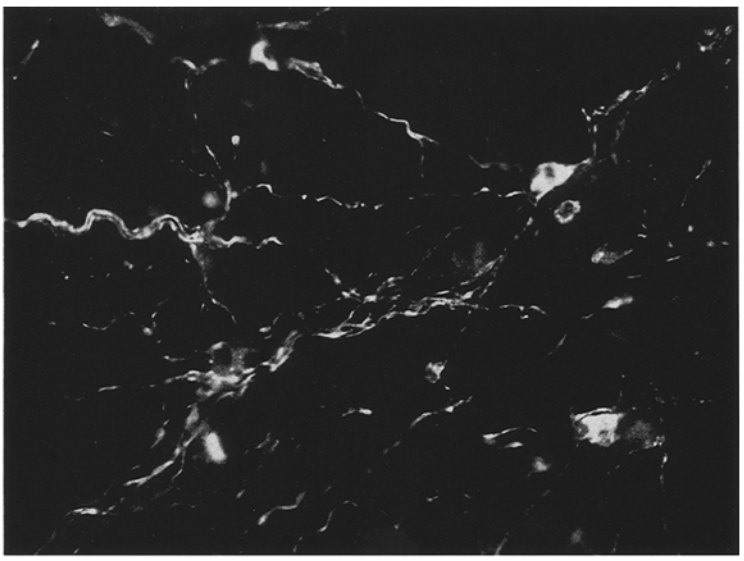


Fig. 5

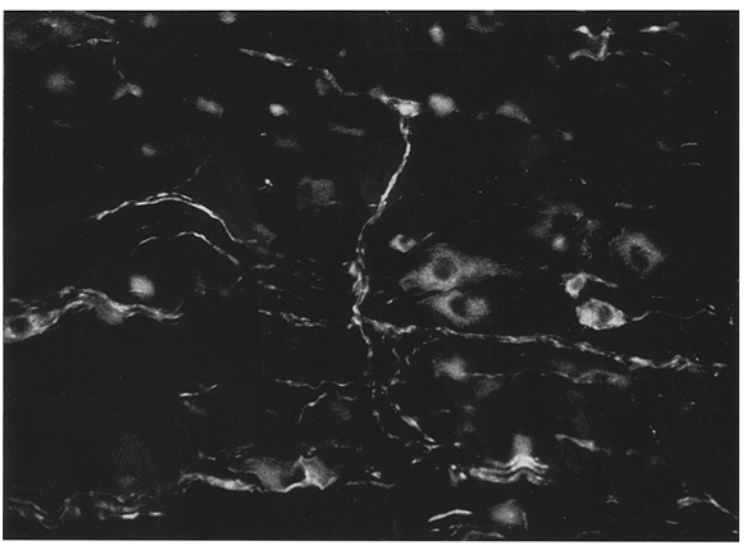


Fig. 6

Figs. 5 and 6. Wide-meshed subserosal indoleamine plexus of lamprey hindgut. 5) small and medium 5-HT perikarya, 6) medium and large, faintly yellow fluorescent indoleamine perikarya. ×280

the physiochemical properties of the yellow fluorophore suggested that it was caused by an indoleamine, probably serotonin, and that the green fluorophore was due to a catecholamine (see "Microspectrofluorometry").

The yellow fluorescent substance was exclusively contained in nerves and ganglion cells (Figs. 3–6). All indoleamine-containing nervous elements were confined to the broad subepithelial connective tissue layer of the gut, which contained ran-

dom smooth muscle cells, fibroblasts, blood vessels, and masses of collagen, verified in toluidine-blue pyronine stained plast sections and Goldner stained freeze-dried paraffin sections.

Indoleamine-containing nerve cell perikarya of at least two varieties occurred in the subepithelial connective tissue layer: 1. large multipolar cells that fluoresced with low to high intensity and had fairly thick, strongly fluorescent processes, some of which could be followed over long distances (cf. Fig. 4c); 2. small to medium size, generally highly fluorescent cells with two or more processes (Figs. 3, 4b, 5,6).

Processes of both types of indoleamine perikarya formed a rather dense plexus of mainly longitudinally arranged fibre bundles underneath the epithelium (basiepithelial plexus; Fig. 4a, b, c). All along the gut, this plexus contained ganglion cells of the second variety. In more peripheral parts of the connective tissue layer and underneath the serosal epithelium, a much less intricate network of small fibre bundles was observed (subserosal plexus; Figs. 5, 6). Small, medium, and large yellow fluorescent ganglion cells contributed with their processes to this wide-meshed fibre network. It was only in this subserosal plexus that single axons could be seen to separate from the small bundles and to develop small irregular and elongated varicosities, suggestive of terminals.

Axons inside the big fascicles (Fig. 4a, c) of the basiepithelial plexus appeared less varicose and extremely twisted, suggesting relatively higher numbers of non-terminal axons.

Nialamide pretreatment (200 mg/kg i.p., 6 hrs) did not notably enhance the fluorescence intensity nor did it alter the morphology of the indoleamine nerves. Reserpine (10 mg/kg i.p., 24 hrs) caused only slight reduction in fluorescence intensity. 24 hrs after the i.p. application of 45 mg/kg 5,7-DHT, many yellow fibres appeared distended and showed enhanced fluorescence, as did the cell bodies (cf. Fig. 4c). No alteration in fluorescence morphology was detected in the green fluorescent nerves after pretreatment with the same dose of 5,7-DHT (24 hrs).

Green fluorescent nerve cells of medium size were comparatively rare and intermingled with the 5-HT neurons in the foregut and the midgut, but absent in the terminal portion of the hindgut. These cells had processes that were difficult to follow and appeared partly smoothly contoured and partly varicose.

In addition, small groups of brightly green fluorescent cells were observed in relation to blood vessels near the serosal surface of the gut. These cells lacked processes and might be catecholamine-containing chromaffin-like cells.

Microspectrofluorometry. Three types of fluorescent structures were analyzed: the yellow-brownish autofluorescent granules in mucosal epithelial cells, and the formaldehyde-induced yellow and green fluorescent intrinsic neurons of the gut. The autofluorescence had spectral characteristics entirely different from the formaldehyde-induced fluorophores, the emission being very broad with the maximum at about 600–650 nm. The yellow-fluorescent neurons had excitation/emission maxima (390–410/520–530 nm) corresponding to those of the formaldehyde-induced serotonin fluorophore. The spectral characteristics of the green-fluorescent neurons were those of the catecholamine fluorophores (exc./em. max. 320 and 410/475–480 nm). Upon HCl treatment of the formaldehyde-reacted sections according to the method of Björklund *et al.* (1968), spectral changes

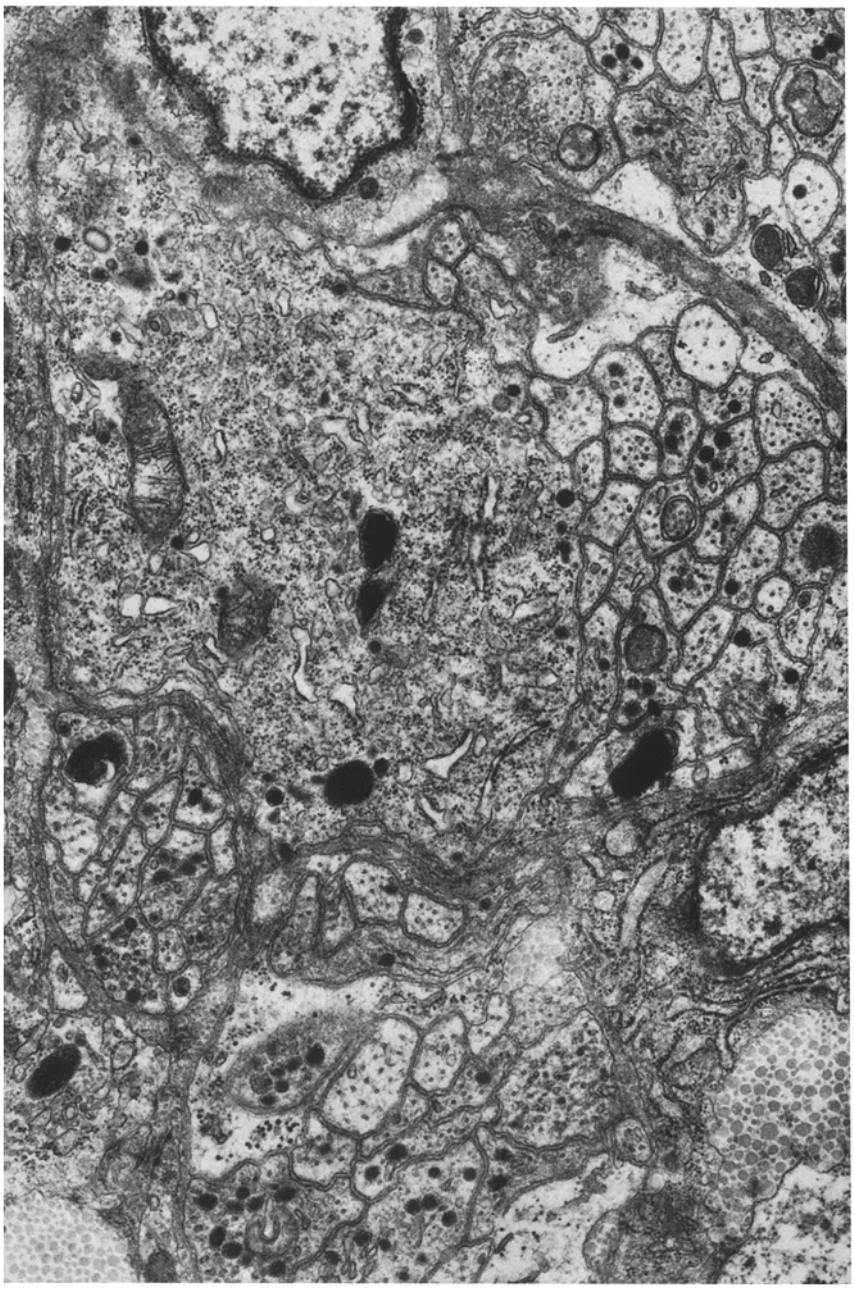


Fig. 7. Plexus neuropil of *Lampetra* hindgut from control specimen. Note close apposition of narrow and distended, varicose portions of axons, many of them filled with varying proportions of small empty and/or medium to large granular vesicles. Fractions of the neuropil are delimited from each other by extensions of connective tissue septula, either filled with filamentous, electron opaque basement membrane material or collagen fibrils (bottom of the micrograph). Note perikaryon of an indoleamine nerve cell with many free and membrane-attached ribosome complexes, dense bodies and granular vesicles. ×18000

typical for either the noradrenaline or the dopamine fluorophores were registered. In some cases, the emission curve of the green-fluorescent cells was broader, occasionally resulting in a slight displacement of the emission maximum towards higher wave lengths.

Electron Microscopy. Bundles of nerve fibres were easily identified in the connective tissue layer interposed between the intestinal columnar epithelium and the serosal mesothelium. Most bundles were very large, composed of multiaxonal Schwann cell units. In the vicinity of ganglion cells, aggregates of several multiaxonal units constituted a vast plexus-like neuropil (Fig. 7). Nerve cell processes were recognized by rough-surfaced endoplasmic reticulum, many free ribosomes, and granular vesicles (750–1 600 Å); these processes, probably dendrites, intermingled with swellings of axonal profiles. The extracellular space between the tigthly packed neural elements measured about 200 Å. The axodendritic contacts could be called synaptoid as membrane thickenings have not been detected at either pre- or postsynaptic structures. Ganglion cell containing plexus neuropil (Fig. 7) comprised two types of axonal swellings; one type was characterized by varying proportions of small empty (400–650 Å) and large granular vesicles (750–1 600 Å) (Fig. 8), outnumbering the rare, second type, which comprised many small empty vesicles. Ganglion cell perikarya from hindgut plexus showed ordinary features of nerve cells (Fig. 7): A large, light nucleus with rather evenly dispersed chromatin material, many rosette like free ribosomes complexes, and cisternes of rough endoplasmic reticulum, mitochondria with a rather dense intercristal matrix, Golgi areas, and peripherally located large granular vesicles.

Plexus neuropil and axons in nerve bundles were supported by glial cell processes. Glial cell nuclei (Fig. 8) showed aggregates of coarse granular chromatin, condensed at the inner nuclear envelope; they thus differed in their morphology from nuclei of ganglion cells. They are directly comparable to Schwann cells in peripheral nervous tissue of mammals. Processes and perikarya of supporting cells in nerve bundles of the lamprey gut contained scattered tubules and occasional bundles of filaments.

Contrary to the compact structure of plexus neuropil in, e.g., Auerbach's plexus of the mammalian gut, which is practically devoid of intervening connective tissue spaces and elements, and thus resembles central nervous tissue, portions of the plexus neuropil in Lampetra were penetrated by connective tissue septula filled with filamentous basement membrane material or islands of collagen fibrils (Fig. 7).

In transverse sections, nerve trunks revealed small, medium and large axon portions intermingled. Generally, the small parts of axons (0.03–0.1 μ; cf. Fig. 7) contained tubules, single mitochondria, and few granular vesicles. Medium and large portions of axons (0.1–8 μ; cf. Figs. 7, 8) were crowded with vesicles, in addition to mitochondria, glycogen granules, and neurotubules. This suggests that axons might show narrow and distended segments alternating with each other, giving the individual fibre a varicose appearance. Each Schwann unit was surrounded by a continuous basal lamina, which was not amorphous but seemed to contain many tiny filaments. The peripherally located vesicle-filled axons were partly exposed from their Schwann cell covering but generally retained their basement membrane. Schwann cell denuded axonal swellings in

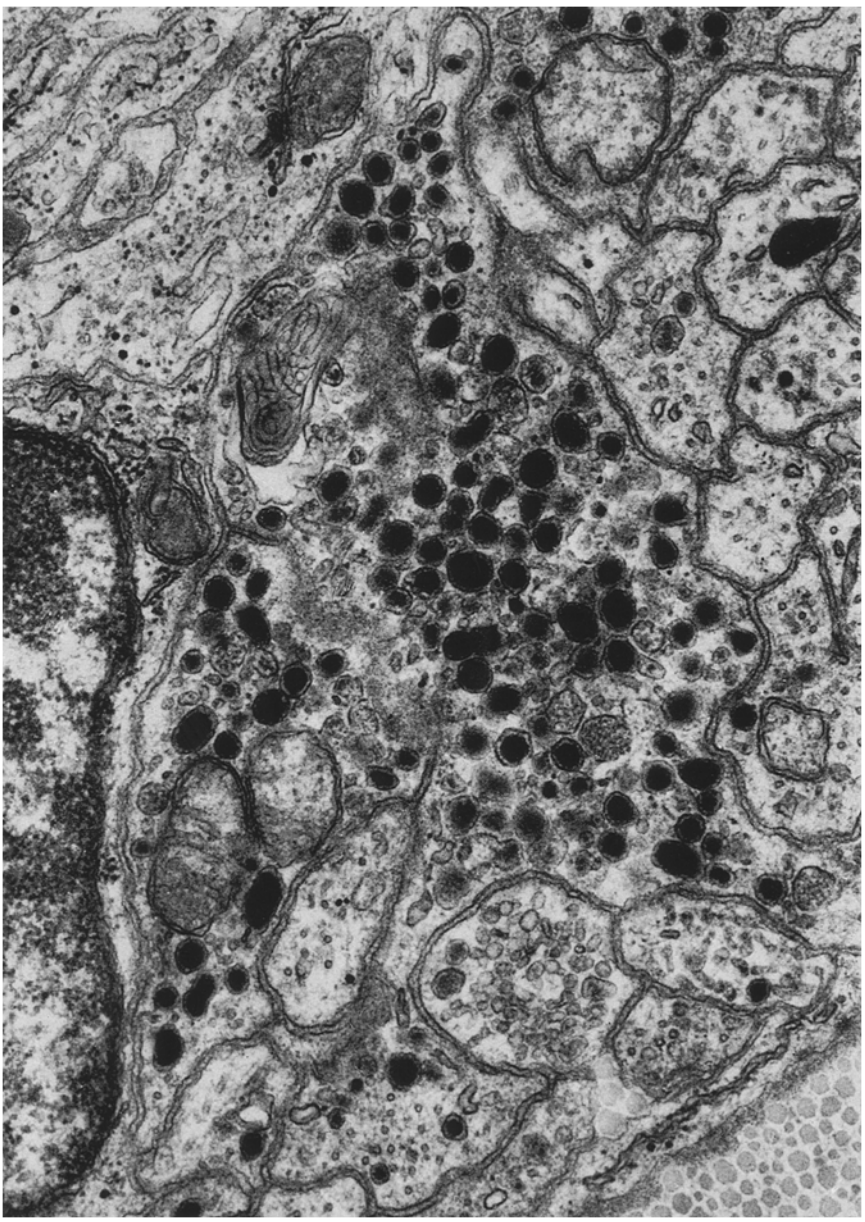


Fig. 8. High power view of dense core granules in axonal profiles of the lamprey hindgut. Note differences in size and electron density of individual granular vesicles. Control specimen. ×40000

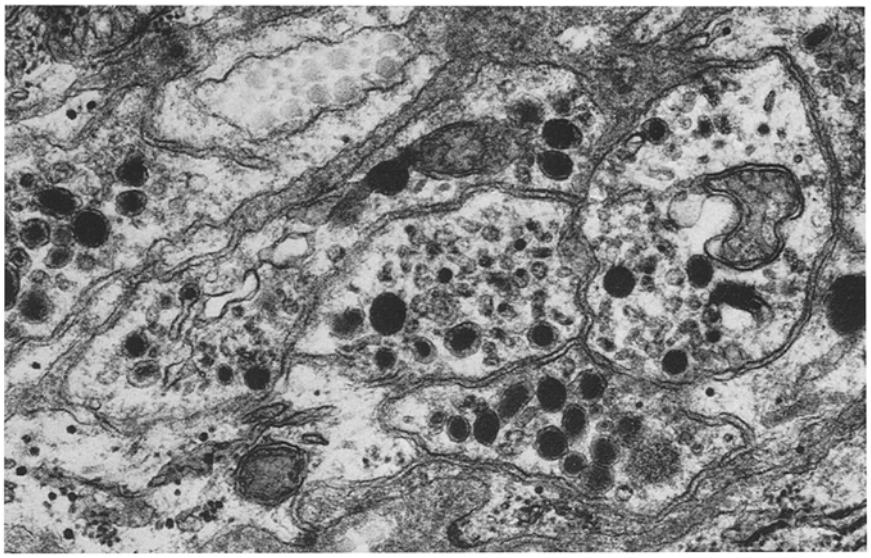
most instances faced the collagen-filled intercellular space, but on rare occasions approached true smooth muscle cells and even processes of fibrocyte-like cells which were devoid of a limiting basal lamina, showed parallel arrays of rough endoplasmic reticulum, a prominent golgi field, large membrane-bounded fat droplets,

and conspicuous cytoplasmic filaments; dense patches at the inner aspect of the cell membrane, as seen in the few smooth muscle cells and known as areas of filament insertion, were lacking. These cells might be interpreted as contractile cells and intermediate forms between fibrocytes and smooth muscle cells. Some axons ran contiguous to the basal surface of mucosal epithelial cells. The closest approach of nerves to any potential effector tissue (smooth muscle cells, fibrocyte-like cells and epithelial cells) was in the range of 500 Å separation.

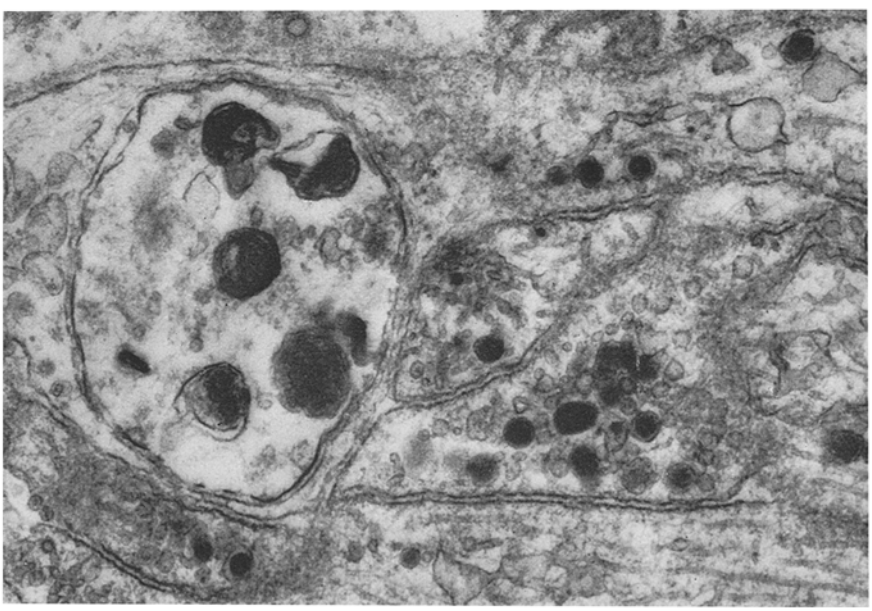
An attempt to prove the monoaminergic nature of the neuronal profiles equipped with large granular vesicles was made by the application of the dihydroxyindoles 5,6- or 5,7-dihydroxytryptamine (5,6-DHT,5,7-DHT), known to provoke toxic damage to CNS indoleamine neurons in mammals (Baumgarten and Lachenmayer, 1972a, b). Two hrs after a single injection of 15 mg/kg 5,6-DHT the cores of many large granular vesicles appeared more electron dense than in control specimens, and osmiophilic material was incorporated into the clear interspace between the limiting membrane of the vesicle and the granular core. Also, many small vesicles—most of them electron lucent in controls—now contained a small, sometimes excentrically placed, dense core (Fig. 9), suggesting uptake and firm retention of 5,6-DHT to the matrix of the vesicular core. Although many profiles exhibited no obvious impairment of their ultrastructure by 20 hrs after 5,6-DHT, some were found to be involved in degenerative processes (Fig. 10). Damage was indicated by enlargement and lysis of granular vesicles, progressive loss of electron dense material from the distorted vesicles, impregnations of the axoplasm with granular osmiophilic material, and grotesque swelling of tubules, most probably belonging to extensions of smooth surfaced endoplasmic reticulum (Fig. 10). Other axonal profiles reacted by clumping of vesicles and hypertrophy of tubules, a disruption and loss of structural integrity of the axoplasm, and frequent transformation of mitochondria to myelin-like bodies (Fig. 10). 5,6-DHT (45 mg/kg) provoked similar signs of ultrastructure impairment in the supposed serotonin-containing nerves and in addition caused axons to undergo tremendous swelling (Fig. 11). The classical picture of dark degeneration of axonal profiles was also revealed after 5,7-DHT treatment (Fig. 12). Already at 24 hrs heavily distended, stump-like axons were detected filled with hypertrophied, dark tubules and masses of dense bodies (transformed mitochondria; cf. Fig. 11).

Discussion

The present study conclusively proves the existence of peripheral serotonin-containing neurons in a vertebrate, the cyclostome Lampetra fluviatilis. That the compound stored in these nerves is indeed serotonin (5-HT) is supported by several findings. *First*, in the fluorometric determinations, extremely high serotonin concentrations were detected in the gut, and less high but significant amounts of 5-HT in the kidney, gills, skin, and even striated muscle. The method used for the determination of tissue serotonin (Bertler, 1961), however, is not entirely specific for 5-HT (see Andén and Magnusson, 1967), although it is particularly sensitive for this indoleamine. *Second*, more specific evidence that the yellow fluorophore is identical to serotonin was gained by thin layer chromatography which gave a yellow spot (using the formalin spray reagent) with an Rf



9



10

Fig. 9. Increase in electron density of granular vesicles in indoleamine axons of the lamprey hindgut after pretreatment with 5,6-DHT (2 hrs). Granular cores are now seen also in small vesicles. ×40000

Fig. 10. Clumping of vesicles, enlargement of tubular profiles, loss of structural integrity of the axoplasm, and formation of dense bodies in 5-HT axons of the lamprey hindgut, 24 hrs after a single injection of 15 mg/kg 5,6-DHT. ×40000

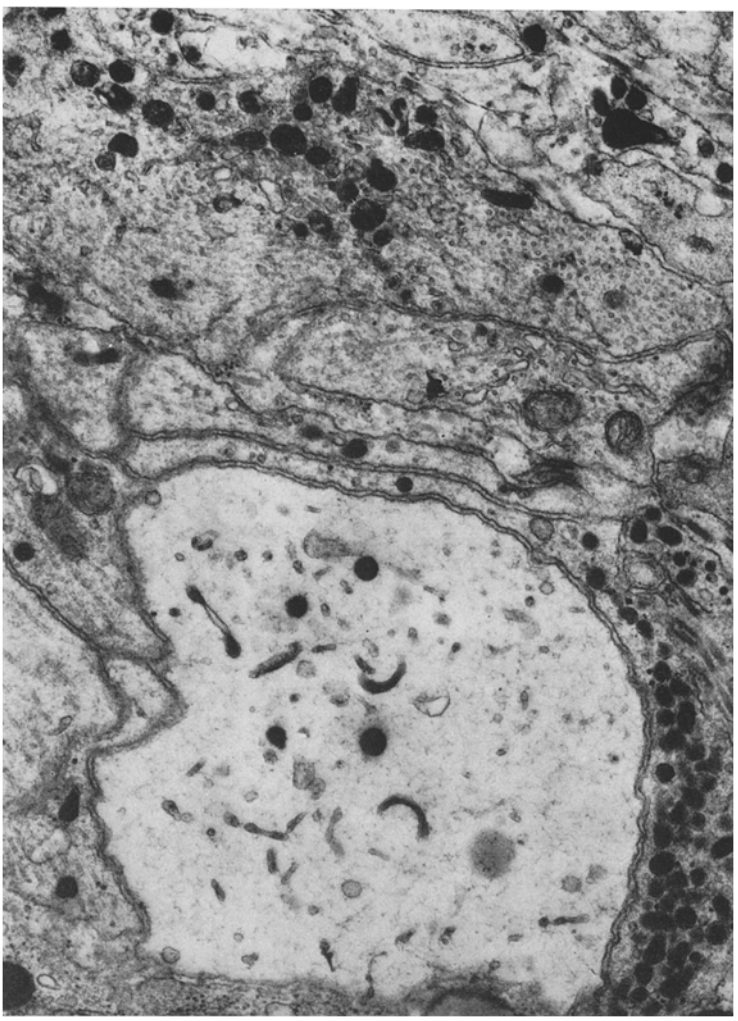


Fig. 11. Reactive (upper part of the micrograph) and degenerative (lower part) alterations in 5-HT axons of the lamprey hindgut after administration of 45 mg/kg 5,7-DHT. Note massive increase in the number of tubular profiles in the reactive axon concomitant with piling-up of many myelin-like bodies (transformed mitochondria). The degenerating axon reveals tremendous swelling, disruption of the structural integrity of the axoplasm, and remnants of dense granules as well as impregnated tubular profiles. ×20000

value and a fluorescence colour corresponding to that of true serotonin. Similar spots, isographic with authentic serotonin, were also found in the extracts from lamprey kidney and gills. *Third,* microspectrofluorimetric analysis of the formaldehyde-induced UV-labile yellow fluorophore in ganglion cells and their processes of the intestine revealed spectra identical to those of the 5-HT fluorophore in histochemical models and in tissues (Björklund, Falck and Stenevi, 1971). Furthermore, the fading properties of the yellow indoleamine fluorophore conformed

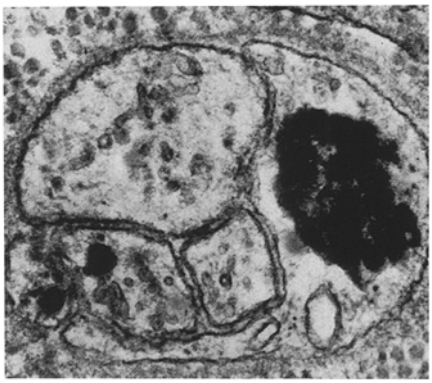


Fig. 12. Dark degeneration of indoleamine axon from terminal hindgut. × 40000

to that of authentic 5-HT. Together, these findings justify the conclusion that the presence of serotonin is responsible for most of the yellow fluorescence that develops in many organs of the lamprey upon exposure of freeze-dried tissue to formaldehyde.

In contrast, the green fluorophore stored in another set of intrinsic neurons exhibited the spectral characteristics and also the slow photodecomposition rate typical for catecholamines (NA, DA, A). The microspectrofluorometric analysis also revealed both dopamine- and noradrenaline-containing intrinsic neurons, which agrees with the significant levels of these catecholamines detected fluorometrically and chromatographically. The finding of green fluorescent cells with a broader emission spectrum might indicate a mixture of a catecholamine and serotonin in these cells, similar to that described in sympathetic nerve terminals of the pineal (Owman, 1964) and in the guinea-pig pancreas (Cegrell, 1968). But it cannot be excluded that this compound spectrum is purely an artefact, due to diffusion of serotonin from adjacent structures into the catecholamine-containing cells during the histotechnical procedure.

Interestingly, the chromatographic investigations on acid extracts from homogenized gut, kidney, and gill samples showed not only the serotonin spot but also another yellowish spot that was isographic with NN-dimethyltryptamine or possibly N-acetyl-5-methoxytryptamine (melatonin). It remains to be clarified whether any of these compounds are present in peripheral tissues of the lamprey. In view of the previous reports of melatonin in peripheral mammalian nerves (Lerner *et al.*, 1959; Barchas and Lerner, 1964), the possible presence of melatonin in peripheral nerves of Lampetra, which is so far only speculation, deserves further investigation.

For a safe electron microscopical identification of the serotonin-containing nerves in the lamprey gut, it was important that much fewer catecholamine-containing nerves occurred among many serotonin ones. This is also reflected by the high serotonin and comparatively low catecholamine levels revealed in the chemical determinations. The regional pattern of distribution of catechol- and indoleamine-containing nerves, moreover, showed that at least one portion

of the intestine, the terminal hindgut, was largely devoid of any green fluorescent structures. This advantageous situation was used for the ultrastructural characterization of the serotonin-containing nerves and their differentiation from non-fluorescent ones.

Classification according to vesicle populations revealed two separate sets of neuronal processes: 1. a frequent type characterized by small empty (350–600 Å), and very rare small granular vesicles, and by abundant large granular (700 to 1600 Å) vesicles; and 2. a rare type with mainly small empty vesicles (cholinergic neurons?). The occurrence of occasional small granular vesicles in the first type of axon strongly suggests that it is monoamine-containing, as it is now well established that, in glutaraldehyde/osmium fixed material, small granular vesicles reflect the presence of monoamines, mainly catecholamines, in peripheral sympathetic nerves (cf. review by Bloom, 1971), but also serotonin (e.g., the sympathetic terminals of the rat pineal, cf. review by Jaim-Etcheverry and Zieher, 1973). The presence of granular material in vesicles of the larger variety (700–1600 Å) cannot, as such, be taken as evidence for the monoaminergic nature of the neurons involved, because the electron opacity in large vesicles is resistant to many pharmacological manipulations known to influence intraneuronal monoamine content (e.g., reserpine, monoamine oxidase inhibition, monoamine synthesis inhibition) when glutaraldehyde/osmium is applied as the fixative. Large granular vesicles of, e.g., adrenergic neurons, however, do react to at least one type of treatment, i.e., to the application of certain false transmitters that form electron-dense precipitates after incubation with glutaraldehyde and osmium (e.g., 5-hydroxydopamine, 6-hydroxydopamine, or 5,6-dihydroxytryptamine; cf. Thoenen 1969; Wartenberg and Baumgarten 1969; Baumgarten, Göthert, Holstein and Schlossberger, 1972). The increase in electron density of large granular vesicles upon administration of these drugs can therefore be used to identify monoamine-containing axons.

Identification of subcellular storage sites of serotonin in neurons is nowadays possible, at least in some cases, thanks to the discovery that 5,6-dihydroxytryptamine is incorporated into synaptic vesicles (Baumgarten, Björklund, Holstein and Nobin, 1972) and causes enhancement of their electron contrast (see also this study). Because of the unspecific uptake and storage of 5,6-DHT in catecholamine-containing nerves (see Baumgarten, Göthert, Holstein and Schlossberger, 1972), this drug gives valuable information on the identity of serotonergic neurons only if intermingled catecholamine-containing structures are sparse in the analyzed tissue, or if the interfering catecholamine neurons have been removed from the tissue before treatment with 5,6-DHT, e.g., through their destruction with 6-hydroxydopamine.

In the present study it was possible to increase the electron density, the size, and the number of cores in vesicles of the 5-HT neurons in the lamprey by pretreatment with 5,6-DHT. This suggests that they represent a possible storage compartment (small and large granular vesicles) for monoamines, in this case probably serotonin, as the terminal hindgut contains almost solely yellow fluorescent neurons.

The diameter scale of large granular vesicles in peripheral adrenergic axons (700–1100 Å) of mammals, birds, lizards, and amphibians (Bloom, 1972) differs

from those in the supposed serotonin-containing axons of the lamprey gut (700 to 1600 Å). The diameter range of large granular vesicles in the intestinal serotonin neurons of the lamprey more closely corresponds to that of dense core vesicles in non-cholinergic, non-adrenergic intrinsic (probably inhibitory) neurons of the mammalian gut (Baumgarten, Holstein and Owman, 1970) and the amphibian gut (Robinson, McLean and Burnstock, 1971). This induces the possibility of erroneous classification of neurons in the lamprey gut of control specimens, in case the axonal profiles lack small granular vesicles. Additional positive evidence that the neuronal profiles in the lamprey gut equipped with mainly large granular vesicles are serotonin-containing was presented by showing that the neurons in question degenerated upon application of 5,6- or 5,7-dihydroxytryptamine, compounds known to more or less selectively damage axons and terminals of central serotonin neurons in the rat (Baumgarten and Lachenmayer, 1972a, b; Baumgarten, Lachenmayer and Schlossberger, 1972; Baumgarten, Björklund, Holstein and Nobin, 1972; Baumgarten, Björklund, Lachenmayer, Nobin and Stenevi, 1971; Björklund, Nobin and Stenevi, 1973; Nobin, Baumgarten, Björklund, Lachenmayer and Stenevi, 1973).

Whereas 5,6-DHT was found to induce staining and consecutive degeneration of terminal and preterminal serotonin axons in rat brain, 5,7-DHT caused damage to both serotonin and noradrenaline axons, although to a much a lesser extent the noradrenaline ones. The dose of 5,6-DHT given to lampreys was kept low (15 mg/kg i.p.) to avoid simultaneous lesioning of any possible catecholamine-containing axons. In rats, it has been established that single doses of 5,6-DHT up to 45 mg/kg cause only a temporary release of noradrenaline from sympathetic nerve terminals, without any indication of permanent ultrastructure impairment (Baumgarten, Göthert, Holstein and Schlossberger, 1972). With a dose as low as 15 mg/kg applied to the lamprey, detectable alterations in the catecholamine axons would not be expected. 15 mg/kg 5,6-DHT or 45 mg/kg 5,7-DHT 20 and 24 hrs after their application, respectively, provoked dramatic signs of axonal damage in the proposed serotonin neurons of the lamprey gut. The identification of these axons—equipped with a typical population of large dense core vesicles—as serotonin-containing thus seems most probable.

Although there does not seem to exist any comparable monoamine-containing peripheral neuron type in mammals or birds, there is evidence for a similar set of 5-HT neurons provided with large granular vesicles (800–2000 Å) in, e.g., molluscs (hindgut of snails: Burnstock and Robinson, 1967; Campbell and Burnstock, 1968). Furthermore, central and peripheral neurons in the earthworm (Lumbricus terrestris), shown in fluorescence microscopical investigations to store 5-HT, have been found to exhibit numerous small (300–500 Å) dense core vesicles upon the application of alpha-methylnoradrenaline in permanganate fixed specimens (Myhrberg, 1972), besides occasional larger electron opaque vesicles (600–900 Å). This suggests that small granular vesicles are the main storage organelles for serotonin in neurons of certain forms of invertebrates, very similar to the situation in mammals (cf. Hökfelt, 1968). The only known mammalian non-neuronal cells equipped with a population of 5-HT-storing large granular vesicles comparable to those in the 5-HT nerves of the lamprey gut

are mucosal enterochromaffin cells of the intestine (Schofield and Silva, 1968). It is noteworthy in this context that the lamprey gut completely lacks enterochromaffin cells. The lamprey thus deviates also in this respect from other forms of vertebrates that have been shown to store most, or all, of their gut serotonin in enterochromaffin cells (see review by Vialli, 1966).

Most, if not all, of the serotonin axons in the lamprey gut seem to originate from intramural ganglion cells, again in harmony with the condition in the snail (Burnstock and Robinson, 1967). At least, as far as the serotonergic component of the hindgut innervation is concerned, there seems no need for an additional extrinsic serotonergic inflow, because 5-HT ganglion cells occur in the hindgut itself, and because the number of 5-HT axons in this terminal part of the intestine is not higher than in more cranial portions. Thus, if a separate posterior spinal innervation of the terminal hindgut exists in the lamprey, as stated by Johnels (1956), this innervation is probably not serotoninergic. Johnels reached the conclusion of a spinal hindgut innervation in the lamprey by conventional staining methods only; it is thus not known whether these nerves are afferent or efferent, nor has the transmitter of these nerves so far been characterized. The non-vertebrate principle of peripheral autonomic innervation in the lamprey (which is probably valid also for other visceral organs than the gut) is not only substantiated by the existence of intramural 5-HT neurons, but also by the finding that the lamprey lacks sympathetic chain ganglia typical for practically all forms of vertebrates, except the cyclostomes (see Burnstock, 1969).

A further difference in innervation pattern of the cyclostome gut compared with that in higher forms of vertebrates is the discovery of intramural dopamine- and noradrenaline-containing neurons in the lamprey intestine. Intrinsic dopaminergic neurons do not seem to exist in the gut of vertebrate species higher than cyclostomes, whereas they have been demonstrated in, e.g., the snail hindgut (Burnstock and Robinson, 1967). Dopamine has been found to be the dominant catecholamine in the peripheral and central nervous system of many forms of invertebrates (see Juorio and Killick, 1972; review by Welsh, 1972) and this feature is also characteristic for the nervous systems of the lamprey. Although the sympathetic noradrenergic innervation of the gut in all forms of vertebrates higher than cyclostomes is by way of extrinsic neurons, there are a few examples of additional intrinsic, possibly noradrenaline-containing, perikarya in the intestine of lizards, birds (see review by Burnstock, 1969; Bennett and Malmfors, 1970), and even the guinea-pig colon (Furness and Costa, 1971).

A peculiar feature of the 5-HT-containing plexus in the lamprey is its paucity in varicose terminals and richness in non-terminal axons, which suggests that few axons establish synaptic (or synaptoid) contacts. Electron microscopically, there are only few examples of rather close approach (about 500 Å minimum distance) of 5-HT axons to smooth muscle cells, fibrocyte-like cells, and even less frequently to epithelial cells. This poses the question of the functional significance of the serotonin nerves in the cyclostome gut. The potential role of 5-HT as an initiator of peristalsis in the lamprey gut was established by Johnels and Oestlund (1958), who demonstrated that the isolated hindgut of Lampetra fluviatilis could be made to forcefully contract in a peristaltic fashion by the application of 5-HT ($^1/_2 \times 10^{-5}$ M), whereas there was hardly any effect on the middle

or anterior portion of the intestine. The poor effect of 5-HT on all parts of the lamprey gut except the terminal hindqut (which has a reasonably thick layer of circularly arranged smooth muscle cells) is most likely connected with the atrophy of the intestinal smooth musculature, common in adult river lampreys. This might be responsible for the low number of varicose terminals and the paucity of synaptoid contacts of the 5-HT-containing axons, as revealed in the fluorescence and electron microscope. This would mean that nerves deprived of their potential effector cells might show regressive signs leading to an involution of their terminals. Investigations on ammocoetes forms of river lampreys with an intact alimentary canal should help to clarify this interesting question.

In conclusion, peripheral, intramural, serotonin-containing, dopamine-containing, and noradrenaline-containing neuron systems have been detected and characterized in a primitive vertebrate. The innervation by intrinsic serotonin-containing neurons is in all probability a remnant of an autonomic innervation principle common in invertebrates, but presumably not retained in higher forms of vertebrates.

Literature

Andén, N. E., Magnusson, T.: An improved method for the fluorimetric determination of 5-hydroxytryptamine in tissues. Acta physiol. scand. **69**, 87–94 (1967).

Barchas, J. D., Lerner, A. B.: Localization of melatonin in the nervous system. J. Neurochem. **11**, 489–491 (1964).

Baumgarten, H. G.: Über die Verteilung von Catecholaminen im Darm des Menschen. Z. Zellforsch. **83**, 133—146 (1967).

Baumgarten, H. G.: Biogenic monoamines in the cyclostome and lower vertebrate brain. Progr. Histochem. Cytochem. Vol. **4**, No. 1 (1972).

Baumgarten, H. G., Björklund, A., Holstein, A. F., Nobin, A.: Chemical degeneration of indolamine axons in rat brain by 5,6-dihydroxytryptamine. Z. Zellforsch. **129**, 256–271 (1972).

Baumgarten, H. G., Björklund, A., Lachenmayer, L., Nobin, A., Stenevi, U.: Long-lasting selective depletion of brain serotonin by 5,6-dihydroxytryptamine. Acta physiol. scand., Suppl. **373** (1971).

Baumgarten, H. G., Evetts, K. D., Holman, R. B., Iversen, L. L., Vogt, M., Wilson, G.: Effects of 5,6-dihydroxytryptamine on monoaminergic neurones in the central nervous system of the rat. J. Neurochem. **19**, 1587–1597 (1972).

Baumgarten, H. G., Göthert, M., Holstein, A. F., Schlossberger, H. G.: Chemical sympathectomy induced by 5,6-dihydroxytryptamine. Z. Zellforsch. **128**, 115–134 (1972).

Baumgarten,.H. G., Holstein, A. F., Owman, Ch.: Auerbach's plexus of mammals and man: Electron microscopic identification of three different types of neuronal processes in myenteric ganglia of the large intestine from rhesus monkeys, guinea-pigs and man. Z. Zellforsch. **106**, 376–397 (1970).

Baumgarten, H. G., Lachenmayer, L.: Chemically induced degeneration of indoleamine-containing nerve terminals in rat brain. Brain Res. **38**, 228–232 (1972a).

Baumgarten, H. G., Lachenmayer, L.: 5,7-Dihydroxytryptamine: Improvement in chemical lesioning of indoleamine neurons in the mammalian brain. Z. Zellforsch. **135**, 399–414 (1972b).

Baumgarten, H. G., Lachenmayer, L., Schlossberger, H. G.: Evidence for a degeneration of indoleamine-containing nerve terminals in rat brain induced by 5,6-dihydroxytryptamine. Z. Zellforsch. **125**, 553–569 (1972).

Bennett, T., Malmfors, T.: The adrenergic nervous system of the domestic fowl (*Gallus domesticus*, L.). Z. Zellforsch. **106**, 22–50 (1970).

Bertler, Å.: Effect of reserpine on the storage of catecholamines in brain and other tissues. Acta physiol. scand. **51**, 75–83 (1861).

Bertler, Å., Carlsson, A., Rosengren, E., Waldeck, B.: A method for the fluorimetric determination of adrenaline, noradrenaline and dopamine in tissues. Kgl. Fysiogr. Sällsk. Lund Förh. **28**, 121–123 (1958).

Björklund, A., Ehinger, B., Falck, B.: A method for differentiating dopamine from noradrenaline in tissue sections by microspectrofluorometry. J. Histochem. Cytochem. **16**, 263–270 (1968).

Björklund, A., Falck, B., Håkanson, R.: Microspectrofluorometric characterization of catecholamines and indoleamines on silica gel thin layer after formaldehyde condensation. Anal. Biochem. **35**, 264–276 (1970).

Björklund, A., Falck, B., Owman, Ch.: Fluorescence microscopic and microspectrofluorimetric techniques for the cellular localization and characterization of biogenic amines. In: S. Berson (ed.), Methods of investigative and diagnostic endocrinology, vol. 1, J. E. Rall, I. J. Kopin (eds.), The thyroid and biogenic amines, pp. 318–368. Amsterdam: North-Holland Publ. Co., 1972.

Björklund, A., Falck, B., Stenevi, U.: Microspectrofluorimetric characterization of monoamines in the central nervous system: Evidence for a new neuronal monoamine-like compound. In: O. Eränkö (ed.), Progr. Brain Res., vol. 34, Histochemistry of nervous transmission, pp. 63–73. Amsterdam: Elsevier Publ. Co. 1971.

Björklund, A., Nobin, A., Stenevi, U.: Effects of 5,6-dihydroxytryptamine on nerve terminal serotonin and serotonin uptake in the rat brain. Brain Res. **53**, 117–127 (1973).

Bloom, F. E.: The fine structural localization of biogenic monoamines in nervous tissue. Int. Rev. Neurobiol. **13**, 27–66 (1970).

Bloom, F. E.: Electron microscopy of catecholamine-containing structures. In: Catecholamines, H. Blaschko, E. Muscholl (eds.), pp. 46–78. In: Handb. der experimentellen Pharmakologie, Bd. XXXIII. Berlin-Heidelberg-New York: Springer 1972.

Bülbring, E., Gershon, M. D.: 5-Hydroxytryptamine participation in the vagal inhibitory innervation of the stomach. J. Physiol. (Lond.) **192**, 823–846 (1967).

Burnstock, G.: Evolution of the autonomic innervation of visceral and cardiovascular systems in vertebrates. Pharmacol. Rev. **21**, 247–324 (1969).

Burnstock, G.: Purinergic nerves. Pharmacol. Rev. **24**, 509–581 (1972).

Burnstock, G., Robinson, P. M.: Localization of catecholamines and acetylcholinesterase in autonomic nerves. Suppl. III to Circulat. Res. 20 and 21, 43–55 (1967).

Campbell, G. Burnstock, G.: Comparative physiology of gastrointestinal motility. In: Handbook of physiology, vol. IV, 6, pp. 2213–2266, Ch. F. Code (ed). Washington, D. C.: American Physiological Society 1968.

Cegrell, L.: The occurrence of biogenic monoamines in the mammalian endocrine pancreas. Acta physiol. scand., Suppl. 314 (1968).

Costa, M., Furness, J. B.: Storage, uptake and synthesis of catecholamines in the intrinsic adrenergic neurons in the proximal colon of the guinea-pig. Z. Zellforsch. **120**, 364–385 (1971).

Ehinger, B., Falck, B., Myhrberg, H. E.: Biogenic monoamines in *Hirudo medicinalis*. Histochemie **15**, 140–149 (1968).

Ehinger, B., Myhrberg, H. E.: Neuronal localization of dopamine, noradrenaline, and 5-hydroxytryptamine in the central and peripheral nervous system of *Lumbricus terrestris* (L.). Histochemie **28**, 265–275 (1971).

Furness, J. B., Costa, M.: Morphology and distribution of intrinsic adrenergic neurons in the proximal colon of the guinea-pig. Z. Zellforsch. **120**, 346–363 (1971).

Furness, J. B., Costa, M.: The nervous release and the action of substances which affect intestinal muscle through neither adrenoreceptors nor cholinoreceptors. Phil. Trans. roy. Soc. Lond. B (1973), in press.

Häggendal, J.: An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissues. Acta physiol. scand. **59**, 242–254 (1963).

Hökfelt, T.: In vitro studies on central and peripheral monoamine neurons at the ultrastructural level. Z. Zellforsch. **91**, 1–74 (1968).

Jacobowitz, D.: Histochemical studies of the autonomic innervation of the gut. J. Pharmacol. exp. Ther. **149**, 358–364 (1965).

Jaim-Etcheverry, G., Zieher, L. M.: Localizing serotonin in central end peripheral nerves. Intensive Study Program NRP, Boulder, 1973, in press.

Johnels, A. G.: On the peripheral autonomic nervous system of the trunk region of *Lampetra planeri*. Acta zool. **37**, 251–286 (1956).

Johnels, A. G., Oestlund, E.: Anatomical and physiological studies on the enteron of *Lampetra fluviatilis* (L.). Acta zool. **39**, 9–12 (1958).

Juorio, A. V., Killick, S. W.: Monoamines and their metabolism in some molluscs. Comp. gen. Pharmacol. **3**, 283–295 (1972).

Lerner, A. B., Case, J. D., Mori, W., Wright, M. R.: Melatonin in the peripheral nerve. Nature (Lond.) **27**, 1821 (1959).

Molinoff, P. B., Axelrod, J.: Biochemistry of catecholamines. Ann. Rev. Biochem. **40**, 465–500 (1971).

Myhrberg, H. E.: Ultrastructural localization of monoamines in the central nervous system of *Lumbricus terrestris* (L.) with remarks on neurosecretory vesicles. Z. Zellforsch. **126**, 348–362 (1972).

Nobin, A., Baumgarten, H. G., Björklund, A., Lachenmayer, L., Stenevi, U.: Axonal degeneration and regeneration of the bulbospinal indoleamine neuron systems after 5,6-dihydroxytryptamine treatment. Brain Res. **56**, 1–24 (1973).

Norberg, K. A.: Adrenergic innervation of the intestinal wall studied by fluorescence microscopy. Int. J. Neuropharmacol. **3**, 379–382 (1964).

Owman, Ch.: Sympathetic nerves probably storing two types of monoamines in the rat pineal gland. Int. J. Neuropharmacol. **2**, 105–112 (1964).

Pletscher, A., Gey, K. F., Burkard, W. P.: Inhibitors of monoamine oxidase and decarboxylase of aromatic amino acids. In: Handbuch der experimentellen Pharmakologie, Bd. XIX, 5-Hydroxytryptamine and related indolealkylamines, V. Erspamer (ed.), pp. 593–735. Berlin-Heidelberg-New York: Springer 1966.

Robinson, R. G., Gershon, M. D.: Synthesis and uptake of 5-hydroxytryptamine by the myenteric plexus of the guinea-pig ileum: A histochemical study. J. Pharmacol. exp. Ther. **178**, 311–324 (1971).

Robinson, P. M., McLean, J. R., Burnstock, G.: Ultrastructural identification of non-adrenergic inhibitory nerve fibres. J. Pharmacol. exp. Ther. **197**, 149–160 (1971).

Ross, L. L., Gershon, M. D.: Electron microscopic radioautographic and fluorescence localization of the site of 5-hydroxytryptamine (5-HT) uptake in the myenteric plexus of the guinea-pig ileum. J. Cell Biol. **55**, 220a (1972).

Schofield, G. C., Silva, D. G.: The fine structure of enterochromaffin cells in the mouse colon. J. Anat. (Lond.) **103**, 1–13 (1968).

Seiler, N., Wiechmann, H.: Die fluorimetrische Bestimmung des Meskalins und einiger β-Phenyläthylamine. Hoppe-Seylers Z. physiol. Chem. **337**, 229–240 (1964).

Shaskan, E. G., Snyder, S. H.: Kinetics of serotonin accumulation into slices from rat brain: relationship to catecholamine uptake. J. Pharmacol. exp. Ther. **175**, 404–418 (1970).

Thoenen, H.: Bildung und funktionelle Bedeutung adrenerger Ersatztransmitter. Experimentelle Medizin, Pathologie und Klinik, Bd. 27, pp. 1–85. Berlin-Heidelberg-New York: Springer 1969.

Vialli, M.: Histology of the enterochromaffin cell system. In: Handb. exper. Pharmak. Vol. XIX, 5-Hydroxytryptamine and related indolealkylamines, V. Erspamer (ed.), pp. 1–65. Berlin-Heidelberg-New York: Springer 1966.

Wartenberg, H., Baumgarten, H. G.: Über die elektronenmikroskopische Identifizierung von noradrenergen Nervenfasern durch 5-Hydroxydopamin und 5-Hydroxydopa im Pinealorgan der Eidechse *(Lacerta muralis)*. Z. Zellforsch. **94**, 252–260 (1969).

Welsh, J. H.: Catecholamines in invertebrates. In: Catecholamines, H. Blaschko, E. Muscholl (eds.), Handbuch der experimentellen Pharmakologie, Bd. XXXIII, pp. 79–109. Berlin-Heidelberg-New York: Springer 1972.

Priv.-Doz. Dr. H. G. Baumgarten
Department of Neuroanatomy
University of Hamburg
D-2000 Hamburg 20, Martinistr. 52
Federal Republic of Germany