CHEMICAL AND ULTRASTRUCTURAL IDENTIFICATION OF 5-HYDROXYTRYPTAMINE IN AN IDENTIFIED NEURON

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ABSTRACT

The two largest cells in a typical ganglion of the leech (*Hirudo medicinalis*) nervous system are the colossal cells of Retzius. These cells show a positive chromaffin reaction, and it has been suggested that they contain 5-hydroxytryptamine (5-HT). In this study, the presence of 5-HT in the colossal cells was confirmed by *microspectrofluorometry* and by *thin-layer chromatography* and *spectrofluorometry* of extracts of individually dissected and pooled colossal cell bodies. A single colossal cell body was found to contain, on the average, 3.8×10^{-10} g (6 mM) 5-HT. Electron microscopy shows that the colossal cells are distinguished by the presence of 1000 A granules with irregular, electron-opaque cores. Since the granules are distributed in the same pattern as the 5-HT fluorescence, we have suggested that they contain 5-HT. Furthermore, a chromaffin reaction modified for the electron microscope provides evidence that 5-HT is present in the granule cores. These data can now serve as a basis for further studies on the metabolism, distribution, and function of 5-HT in these identified neurons.

INTRODUCTION

Much of the interest in 5-hydroxytryptamine (5-HT, serotonin, enteramine) centers on the possibility that this compound may be a neurotransmitter. There is evidence for this suggestion (e.g. Welsh, 1953; Gerschenfeld and Stefani, 1966), but these previous studies are not concerned with individually identified cells. In order to obtain more precise information on the ultrastructural localization and function of 5-HT, it would be desirable to use a system in which 5-HT can be studied at the level of the single identified cell. The simple nervous system of the leech is a favorable preparation for this type of work.

The earliest report of chemical specialization in leech neurons was made in 1903 when Poll and Sommer noted that some neurons reduced chromium salts and were thus analogous to the chromaffin cells of the vertebrate adrenal medulla. Gaskell (1914) extended these findings by showing six precisely located chromaffin cells in each ventral ganglion. Of particular interest for the present study is the fact that two of the chromaffin cells in each ganglion are very large and easily recognized, these being the two "*kolossale Ganglienzellen*" or colossal cells first described by Retzius (1891). Biedl (1910) and Gaskell (1919) both claimed to show adrenaline (epinephrine) in the ganglia by bioassays, and on this basis Gaskell (1914; 1919) speculated that adrenaline was the chromaffin substance in the six chromaffin cells.

The location of the six chromaffin cells has been confirmed (Vialli, 1934; Perez, 1942), but the statement that these cells contain adrenaline has been challenged. Vialli, for example, in a perceptive study, noted that the chromaffin substance did not behave like adrenaline but seemed to be a closely related compound. The first indication that another biogenic amine might be involved came from the finding that leech ganglia contain 6.9 μg of 5-HT/g of tissue (Welsh and Moorhead, 1960). Kerkut et al. (1966) confirmed the presence of 5-HT by paper chromatography and were unable, by the same method, to demonstrate catecholamines (CA) (Kerkut et al., 1966).

In the early 1960's, a sensitive and relatively specific fluorescence histochemical technique was developed for biogenic amines (e.g. Falck, 1962; Falck and Owman, 1965). Myhrberg (1965) used this method on leech ganglia and found cells with the yellow fluorescence that is usually associated with 5-HT. Kerkut et al. (1967) repeated these observations and showed six yellow fluorescent cells in the same positions as the six chromaffin cells first described by Gaskell (1914). Therefore, since this nervous system contains 5-HT and no demonstrable CA and also since the chromaffin cells have a fluorescence usually associated with 5-HT, Kerkut et al. (1967) speculated that the six chromaffin cells contain 5-HT rather than adrenaline.

Unfortunately, the evidence that localizes 5-HT to the six chromaffin cells is not conclusive. First, the chromaffin reaction does not distinguish between the biogenic amines. Second, the presence of 5-HT and apparent absence of CA does not localize these compounds to individual cells, especially when small amounts of a CA could go undetected by paper chromatography. Finally, the subjective estimation of the color of the fluorophores of 5-HT and CA is not a dependable criterion by which to distinguish these compounds, for both 5-HT and the CA can have a yellow fluorescence after the formaldehyde histochemical technique for monoamines (Norberg, Ritzén, and Ungerstedt, 1966; Corrodi and Jonsson, 1967).

Because of the large size and easy visualization of colossal cells in fixed and living ganglia, it seemed logical to use these cells as subjects for a correlation of ultrastructure with chemical content in individually identified cells. Before proceeding, however, it was necessary to analyze these cells further in order to characterize the material which is responsible for their formaldehyde-induced fluorescence and chromaffinity. First, therefore, microspectrofluorometric analyses were performed on both individually dissected colossal cell bodies and on colossal cells in histological sections. These data ruled out the presence of CA in these cells and provided strong evidence for the presence of 5-HT. Second, the presence of 5-HT was confirmed by thin-layer chromatography of material extracted from individually dissected colossal cells. Third, individual colossal cells were dissected, pooled, extracted for 5-HT, and the extracts were analyzed spectrofluorometrically. This analysis further confirmed the presence of 5-HT in these cells and gave a quantitative estimate of the amount of 5-HT per cell body. After completion of the above analyses for 5-HT, the fine structure of both individually dissected and *in situ* colossal cells was investigated and a characteristic granule was found to be associated with the presence of 5-HT. Finally, a chromaffin reaction modified for the electron microscope (Wood, 1967) was applied to see what part of the granule was responsible for the chromaffin reaction.

MATERIALS AND METHODS

For all analyses, leeches (*Hirudo medicinalis* L.) were anesthetized in 10% ethanol and pinned to corkboards or paraffin slabs ventral side up. The ventral body wall was opened, and the nervous system was exposed by incising the pigmented wall of the vascular sinus in which the nervous system is found. The ventral ganglia[1] (Fig. 1) were then removed from the animal by transecting the connectives and roots. For those procedures involving the study of cells in histological sections, the ganglia were first treated appropriately (see below) and then embedded,

[1] The nervous system of *Hirudo* consists of 34 ganglia. 13 of these are fused to form the brain and the subpharyngeal and anal ganglionic masses, while 21 are unfused and form a chain of ventral ganglia located between the subpharyngeal and anal ganglionic masses (Mann, 1953). Each ventral ganglion consists of six packets or follicles that surround an interior neuropil. Each packet contains approximately 65 neuronal cell bodies, one giant glial cell, and many small glial cells (e.g. Apathy, 1897; Coggeshall and Fawcett, 1964). The packets are labeled by their positions, and thus there are two anterior lateral, two posterior lateral, one anterior ventral, and one posterior ventral packets.

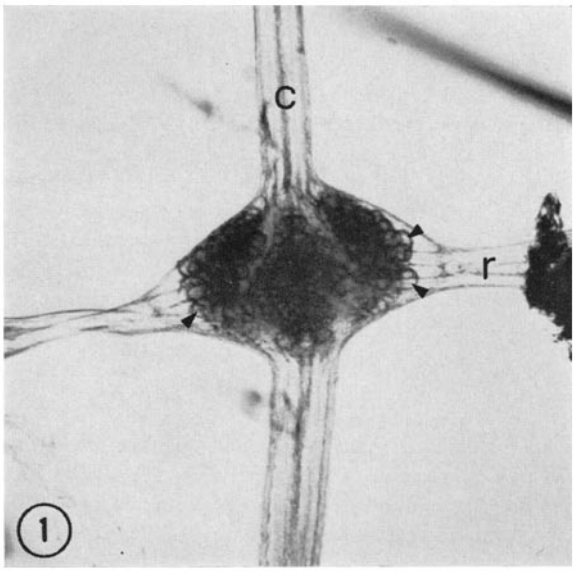


FIGURE 1 A photomicrograph of a living ventral ganglion. The peripheral nerves (*r*) and connectives (*c*) can be seen. Note the large neuronal perikarya (arrowheads). × 50.

sectioned, and examined. For those procedures requiring the analysis of dissected neuronal cell bodies, the ganglia were pinned ventral side up in a disposable petri dish containing a layer of polymerized Sylgard 184 silicone resin (Dow Corning Corp., Midland, Mich.) and filled with cold leech saline (Kuffler and Potter, 1964). Under a dissecting microscope, the colossal and *control* neuron cell bodies were easily recognized. The colossal cell bodies are situated on the ventral surface of the anterior ventral packet,[1] and they are the largest cells in that packet (Fig. 2). The *control* cell bodies refer to any of the neuronal cell bodies in the anterior lateral packets[1] (Fig. 2) because none of these cells reduce chromium salts or react with formaldehyde vapors to form fluorescent compounds indicative of monoamines. The anterior ventral and anterior lateral packets were torn open by a pair of fine tweezers. This exposed the neurons in the packet. Individual colossal or groups of *control* cell bodies were then plucked out of the packet with fine forceps and allowed to fall to the bottom of the dish. Colossal or *control* cell bodies were then sucked into a small pipette (Otsuka et al., 1967) and transferred (*1*) onto a glass slide (for histochemistry and microspectrofluorometry), (*2*) into acetone (for thin-layer chromatography), (*3*) into perchloric acid (for extraction), or (*4*) into fixing fluid (for light and electron microscopy).

Histochemistry and Microspectrofluorometry

These analyses were performed on sections of whole ganglia as well as on individually dissected cells. For histological sections, whole ganglia were frozen in isopentane cooled to −160°C with liquid nitrogen and were freeze-dried in a Pearse freeze-dryer (Edwards High Vacuum, Ltd., Grand Island, N.Y.) equipped with a molecular sieve foreline trap (Varian Associates, Palo Alto, Calif.). For individually dissected cells, colossal or *control* cell bodies were removed, placed on slides, and then dried overnight in a vacuum desiccator over P_2O_5.

Frozen-dried ganglia or slides containing dried cells were placed with paraformaldehyde which had been kept at relative humidities of 0, 15, 30, or 50% in a sealed jar. The jar was then heated to 80°C for 1–3 hr, according to the formaldehyde condensation technique for monoamines (Falck and Owman, 1965). To determine what structures were autofluorescent, frozen-dried ganglia or dried cells were not exposed to formaldehyde vapor.

For histological sections, frozen-dried ganglia were embedded directly in Maraglas (Polysciences, Inc., Rydal, Pa.). Sections of this material as well as the slides with the dried cell bodies were examined first and photographed in a Reichert fluorescence microscope (Reichert Instruments, W. Caldwell, N.J.) equipped with an HBO 200 mercury lamp source and the usual heat-absorbing, excitation, and barrier filters (Dahlström and Fuxe, 1964; Falck and Owman, 1965). Sometimes, a different set of filters was employed (Angelakos, 1964; Rude, in preparation). These filters served to increase the color difference between CA and 5-HT so that CA fluoresced blue or blue-green instead of green or yellow-green while 5-HT continued to fluoresce yellow.

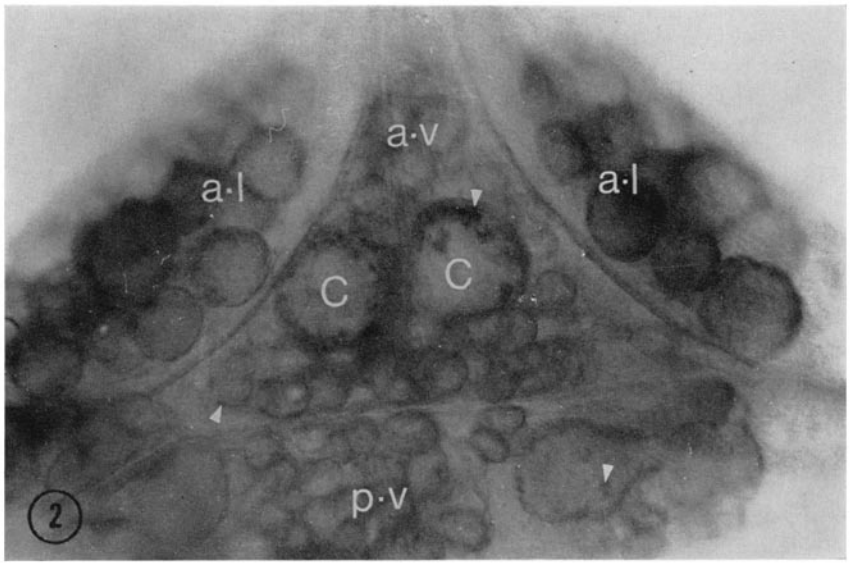


FIGURE 2 A photomicrograph of the ventral surface of a ganglion showing portions of the single anterior ventral (*a-v*) and posterior ventral (*p-v*) packets and the bilateral anterior lateral packets (*a-l*). The colossal cells (*C*) can be seen in the anterior ventral packet and the *control* cells can be seen in the anterior lateral packets. The dark spots which can be seen on many of the neurons are the pigment-laden small glial cells (arrowheads). Nomarski optics. × 150.

For microspectrofluorometry, similar histological sections and slides with dried cells were used. Fluorescence characteristics of colossal and *control* neurons were compared with model systems composed of 10 mM 5-HT or norepinephrine (NE, noradrenaline) in a 1% albumin solution which were dried as microdroplets on slides and treated with formaldehyde vapors in the same way as the cellular material. Fluorescence excitation and emission spectra were obtained with a Leitz Orthoplan microscope. The light source was a 1000-watt xenon lamp (Hanovia 976-C-1) in a Schoeffel LH-151-N lamp house with a Schoeffel QPM-30 quartz prism monochromator (Schoeffel Instruments, Westwood, N.J.). With the usual slit width of 0.2–0.4 mm, the half-intensity band width of excitation light was approximately 6–12 nm at 400 nm peak wavelength. Portions of cell bodies or other structures were examined with dark-field illumination through a 54x fluorite oil immersion objective. A Leitz MPV microphotometric attachment was used to control the area of specimen examined, and apertures corresponding to 5–20 μ in the object plane were employed. A second Schoeffel QPM-30 monochromator was attached to the Leitz MPV microphotometer. The half-intensity width of emitted fluorescent light passed by this monochromator, with slit widths of 0.2–0.4 mm, was approximately 12–25 nm at 500 nm peak wavelength. An EMI 9558-B photomultiplier tube in a Schoeffel D-502 housing was attached to the emission monochromator. The photomultiplier tube was operated at 900 v d-c, and its output was amplified by a Schoeffel M-600 photometer, and recorded on a Sanborn 7701-A direct writing oscillographic recorder. The emission spectra were corrected for spectral response by calibration with a tungsten lamp of known relative spectral energy distribution operated at 2848°K. The excitation spectra were not corrected.

Chromatography

Individual colossal cell bodies, individual *control* cell bodies, or known quantities of standard 5-HT were placed in test tubes containing 1 ml of acetone. After 48 hr, the acetone was decanted and the tubes were washed with another aliquot of acetone. The acetone extract was then evaporated to dryness and the residue was dissolved in a small quantity of 0.05 N hydrochloric acid (Erspamer, 1940; 1966).

Extracts of colossal cells, *control* cells, and exogenous 5-HT were spotted along with standard 5-HT and 5-hydroxytryptophan (5-HTP) on silica-gel thin-layer chromatogram sheets (Eastman Kodak Co., Rochester, N.Y.) and run in a solvent system, composed of methyl acetate:isopropanol:25% ammonia (9:7:4). Double-distilled water containing 0.01% Versene (disodium ethylenediaminetetra-

acetic acid) was used to make up the ammonia solution. The developed chromatograms were dried, sprayed with ethylenediamine-potassium ferricyanide solution (Schneider and Gillis, 1966), heated for 3–5 min at 60°C, and examined and photographed under ultraviolet light. Microspectrofluorometric readings were made of the fluorescent spots on the reagent-treated chromatogram.

Extraction and Spectrofluorometric Determination of 5-Hydroxytryptamine

Individual colossal cell bodies, individual *control* cell bodies, or known quantities of standard 5-HT were placed in test tubes containing 1 ml of ice-cold perchloric acid (0.4 M) with 0.2% ascorbic acid.

Perchloric acid extracts of colossal and *control* cell bodies and standard 5-HT were then treated by the method of Snyder et al. (1965) modified for use with small amounts of material.[2] The single milliliter of perchloric acid extract was carefully transferred to a glass-stoppered centrifuge tube and adjusted to pH 10 with 5 N NaOH. 0.5 ml of 0.5 N sodium borate buffer was added, and the pH was readjusted to 10. 6 ml of *n*-butanol and excess NaCl were added, and the tube was shaken and centrifuged. A 4-ml aliquot of the butanol layer was transferred to another glass-stoppered centrifuge tube. 6 ml of *n*-heptane and 0.3 ml of 0.05 M pH 7.0 phosphate buffer were added, and the tube was shaken and centrifuged. The organic layer was aspirated off, 0.1 ml of 0.1 M ninhydrin solution was added to the phosphate buffer remaining in the test tube, and the mixture was heated for 60 min at 60°C. Fluorescence excitation and emission spectra were recorded from standard 5-HT carried through the extraction procedure and from the material extracted from colossal or *control* cells. A 50–60% recovery of 5-HT was obtained.

Electron Microscopy

Leech ganglia were fixed in one of five different fixing fluids: (*1*) 1.33% osmium tetroxide buffered by collidine (Bennett and Luft, 1959), (*2*) 3% glutaraldehyde with 0.1 M phosphate buffer (Sabatini et al., 1963) followed by osmium tetroxide and collidine, (*3*) 2% formaldehyde made from paraformaldehyde (polyoxymethylene) in 0.1 M cacodylate buffer followed by osmium tetroxide and collidine, (*4*) a 1% potassium permanganate in distilled water (Luft, 1956), and (*5*) a 3% potassium permanganate solution in 0.1 M phosphate buffer (Richardson, 1966). Fixed ganglia were dehydrated in ethanol and embedded in Araldite. Serial 0.5-μ sections were cut for the light microscope and, when the colossal or *control* cells were reached, thin sections were cut with glass or diamond knives. The sections were stained with lead citrate (Venable and Coggeshall, 1964) or a saturated aqueous solution of uranyl acetate, followed by lead citrate. The sections were then examined in an RCA EMU 3G electron microscope.

For studying individual colossal and *control* cells, cell bodies were dissected and placed directly into the glutaraldehyde phosphate mixture (fixation *2* above). After 1–2 hr the cells were gently centrifuged, the fixing fluid was poured off, a brief rinse was applied, and osmium tetroxide with collidine buffer was added to the tube. The cells were again gently centrifuged and the osmium tetroxide solution was replaced by ethanol and then propylene oxide. The cells were then picked up in a disposable pipette and placed along with a small amount of propylene oxide in a BEEM capsule full of warm Araldite. The capsules were centrifuged, which drove the cells to the bottom of the capsule, the Araldite was hardened, and the blocks were then processed as described above.

A chromaffin reaction modified for the electron microscope, as detailed by Wood (1967), was applied to ventral ganglia. Individual ventral ganglia were fixed in glutaraldehyde-phosphate, then rinsed in acetate buffer at pH 4.1, and placed in a mixture of 1% sodium sulfate and 2.5% potassium dichromate in acetate buffer at pH 4.1 for 16–24 hr. The blocks were then dehydrated and embedded in the usual manner. In order to ensure that the darkened granule cores found in colossal cells resulted from this treatment and not from intrinsic electron opacity, ganglia were fixed in glutaraldehyde-phosphate, rinsed in acetate buffer, embedded, and sectioned.

RESULTS

Histochemistry and Microspectrofluorometry

FLUORESCENCE HISTOCHEMISTRY: In histological sections of frozen-dried ganglia not exposed to formaldehyde vapor, a yellowish autofluorescent pigment was seen in neurons and glia. In colossal cell bodies, the autofluorescent regions were located in two concentric layers, one just around the nucleus, the other in the more peripheral cytoplasm (Fig. 8). Spokes of yellowish autofluorescent material connected the two layers. *Control* neuron cell bodies also possessed yellowish layers, bands, or clumps of autofluorescent material (Fig. 8). The remainder of the

[2] This method is similar to that of Snyder et al. (1965) except that the perchloric acid sample is reduced to 1 ml and the other reagents are scaled down accordingly.

cytoplasm and axon hillock of both colossal and *control* cells had a very dim dark green to dark blue appearance depending on the filter system used. Small glial cells filled with a brilliant yellow autofluorescent pigment were found at the periphery of colossal and *control* cell bodies.

When histological sections of frozen-dried ganglia that had been exposed to formaldehyde vapor (for 1½ hr at 80°C) were examined, the *control* neurons and glial cells showed no change in their fluorescence. The colossal cells, however, showed a large increase in the intensity of fluorescence in the regions containing the autofluorescent pigment (Figs. 9, 10). Furthermore, a light yellow haze was seen in regions previously having only a dim background fluorescence, namely those portions of the cell body that contain no autofluorescent pigment and the axon hillock (Figs. 9, 10). In no instance did the nucleus of any cell show fluorescence.

In the past, a yellow fluorescence induced by formaldehyde vapors has been equated with the presence of 5-HT. However, more recent evidence has indicated that CA will also fluoresce yellow when the concentration of water vapor is too high or the length of time of exposure to formaldehyde vapors is too long (e.g. Corrodi and Jonsson, 1967). The concentration of water vapor was varied so as to determine if such a variation would cause a change in the color of the formaldehyde-induced fluorescence in colossal perikarya. In ganglia treated for 1 hr with formaldehyde vapors kept at relative humidities of 0, 15, 30, and 50%, the colossal perikarya always fluoresced yellow. Only the intensity of the yellow fluorescence changed, the intensity of fluorescence increasing with an increase in the amount of water vapor present.

Examination of individually dissected cells confirmed the observations made on histological sections. If individual colossal and *control* cell bodies were not exposed to formaldehyde vapor, a yellowish autofluorescent pigment was seen, and the bright yellow small glial cells were seen clinging to the surface of the neuronal cell bodies (Figs. 11, 13). After exposure to formaldehyde vapor, the fluorescence of *control* and glial cells was unchanged (Fig. 14), whereas the fluorescence of the colossal cells was greatly increased (Fig. 12). The only difference between individual neuronal cell bodies and the same cells seen in histological sections was that the fluorescent cytoplasmic

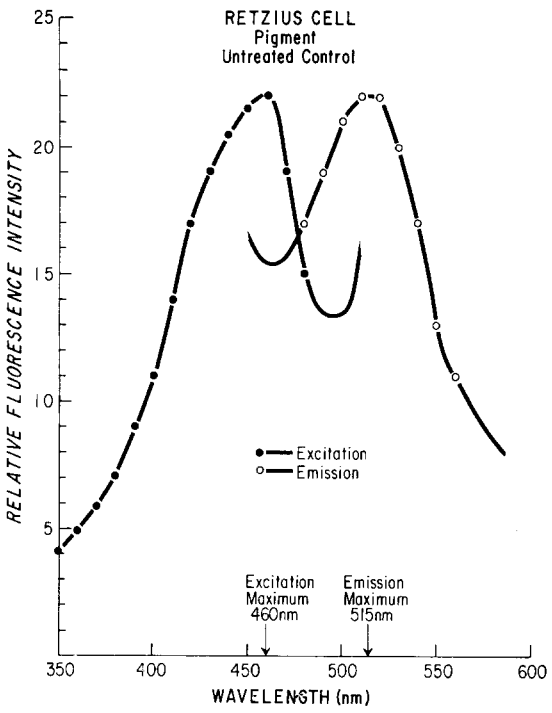


FIGURE 3 Fluorescence excitation and emission spectra from the pigmented region of a whole, air-dried, colossal (Retzius) cell body not exposed to formaldehyde vapor. These spectra are identical to the spectra obtained from *control* neurons and from the small glial cells. Excitation monochromator slit 0.4 mm produced a half-intensity band width of 16 nm at the excitation maximum; emission monochromator slit 0.4 mm produced a half-intensity band width of 24 nm at the emission maximum. Ordinate: arbitrary units of fluorescence intensity, not to the same scale as other figures.

banding was not so clear in individual cells as in sections because the bands were obscured by the necessity of examining the whole thickness of the cell at once.

MICROSPECTROFLUOROMETRY: In histological sections of ganglia not exposed to formaldehyde vapor, the autofluorescent pigment in colossal cells (Fig. 3), *control* cells, and glial cells had an excitation maximum of 460–470 nm and an emission maximum of 510–520 nm (Table I). After formaldehyde exposure, these values were unchanged in *control* and glial cells but there was a change in the colossal cell spectra. In the pigmented regions of the cell, the excitation spectrum became bimodal, with one maximum at 410–430 nm and the other at 460-470 nm, but the

TABLE I

Microspectrofluorometric Analyses

Structure		Formaldehyde vapor treatment	Excitation maximum	Emission maximum
			(*nm*)	(*nm*)
Colossal neuron, pigment area	Section	−	460	515
Colossal neuron	Whole cell	−	470	510
Colossal neuron, pigment area	Section	+	410–430	510–520
			460–470	510–520
Colossal neuron	Whole cell	+	430, 465	510
Colossal neuron, axon hillock	Section	+	430	520
Control neuron	Section	+	460–470	510
Control neuron	Section	−	460–470	510
Small glial cell	Section	+	460	520
Small glial cell	Section	−	460	520
Neuropil (green or blue-green fibers)	Section	+	400–410	480–490
5-Hydroxytryptamine in dried albumin film		+	425	520
Norepinephrine in dried albumin film		+	430	480

emission maximum remained at 510–520 nm (Fig. 4, Table I). Since the colossal cells are large, it was also possible to make microspectrofluorometric readings on the axon hillock, a region of the cell which has no autofluorescent pigment. The formaldehyde-induced fluorescence in this region had an excitation maximum of 430 nm and an emission maximum of 510–520 nm (Fig. 5, Table I).

Model systems of formaldehyde-treated 5-HT or NE in albumen were analyzed at the same time. For 5-HT, the excitation maximum was 425 nm and the emission maximum was 520 nm. For NE, the two maxima were 430 and 480 nm, respectively (Table I).

Examination of individually dissected cells confirmed the observations made on histological sections, except that it was not possible to separate the autofluorescent pigmented regions from the rest of the cell body. Thus, the *control* and small glial cells showed the same spectra (excitation maximum 460–470 nm; emission maximum 510 nm) whether they were exposed to formaldehyde vapor or not. Colossal cell bodies not exposed to formaldehyde showed the same excitation and emission spectra as the *control* and small glial cells. However, after formaldehyde treatment they showed a bimodal excitation spectrum with peaks at 430 and 465 nm and an unchanged emission spectrum (Table I).

Because of the decay in the intensity of the formaldehyde-induced fluorescence with exposure to ultraviolet light and also because there may be some variation in the amount of fluorescent material from one colossal cell to the next, it is difficult to get a precise estimate of the increase in intensity of fluorescence in colossal cells treated with formaldehyde vapor. Nevertheless, a rough idea of the increase can be obtained. Several measurements were made from the same parts of colossal cells treated and not treated with formaldehyde vapors. With one exception, the fluorescence intensity in formaldehyde-treated cells was four to five times that of untreated cells. The exception was a case in which the exposed cell showed a 20-fold increase over the untreated cells.

Finally, although these data will be reported more completely in a later publication, several large green or blue-green processes (depending on the filter system) were seen in the neuropil of formaldehyde-treated ganglia. Microspectrofluorometry of these fibers revealed spectra with an excitation maximum at 400–410 nm and an emission maximum at 480–490 nm (Table I). These spectra are similar to those obtained for NE and quite different from the spectra obtained from 5-HT.

Chromatography

Individual colossal or *control* cell bodies were dissected, extracted in acetone, and the extracts

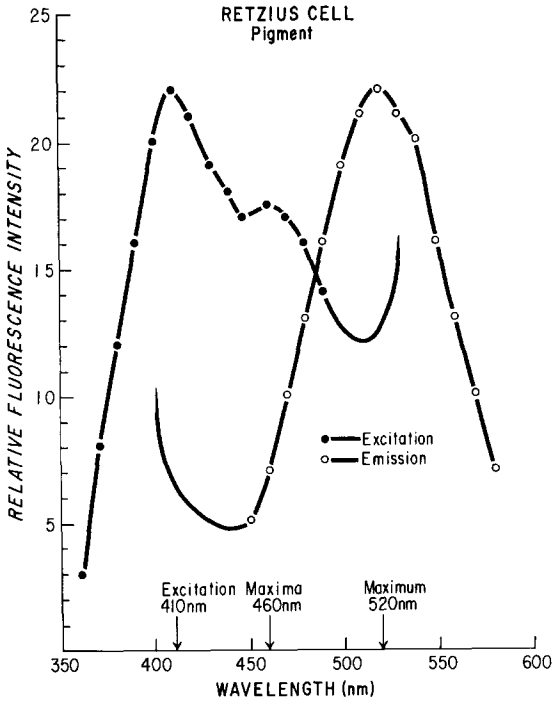


FIGURE 4 Fluorescence excitation and emisson spectra from the pigmented region of a colossal (Retzius) cell body exposed to formaldehyde vapor. The cell was located in a 2-μ-thick Maraglas section of a freeze-dried ganglion treated with formaldehyde vapor. Note that the emission spectrum is unchanged from that in Fig. 3, whereas the excitation spectrum is now bimodal. One of the excitation maxima (460 nm) is identical with the maximum of the autofluorescent pigment (460 nm), and the other (410 nm) is near the excitation maximum of 5-HT. Instrument parameters same as Fig. 3. Ordinate: arbitrary units of fluorescence, not to the same scale as other figures.

were analyzed by thin-layer chromatography. In order to obtain enough material for chromatography, it was necessary to dissect and pool 600 colossal cells. Chromatography of lesser numbers of cells did not give unequivocal results. Chromatography of the acetone extract of 600 colossal cells resulted in two spots. One of these spots did not move from the origin, while the other migrated to the same level as standard 5-HT (Fig. 6). Chromatography of the acetone extract of 1800 *control* cells resulted in one spot which did not move from the origin (Fig. 6). The material in both colossal and *control* cell extracts which did not migrate from the origin with the solvent system used was fluorescent both before and after treatment with ethylenediamine-potassium ferricyanide spray.

Microspectrofluorometric readings were made of the fluorescent spots on the reagent-treated chromatogram. That portion of the colossal cell extract which migrated to the same level as standard 5-HT had the same excitation and emission spectra as 5-HT (excitation maximum, 430 nm; emission maximum, 490 nm). Also, the nonmigratory material present in both the colossal and *control* cells had identical excitation and emission spectra (excitation maximum, 460 nm; emission maximum, 520 nm). The excitation and emission spectra of the nonmigratory material extracted from the cells were the same as the spectra obtained from the autofluorescent pig-

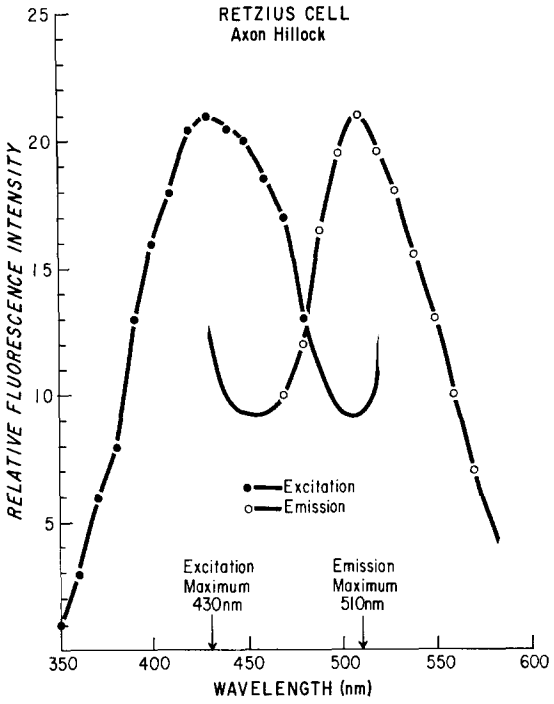


FIGURE 5 Fluorescence excitation and emission spectra from the axon hillock of the same colossal cell as in Fig. 4, with the same instrument parameters. The fluorescence intensity of this area was one-fourth that of the pigment region. Note that the excitation and emission spectra are unimodal and the same as for 5-HT. This region of the cell is not autofluorescent and only fluoresces after exposure to formaldehyde vapor. Ordinate: arbitrary units of fluorescence intensity, not to the same scale as other figures.

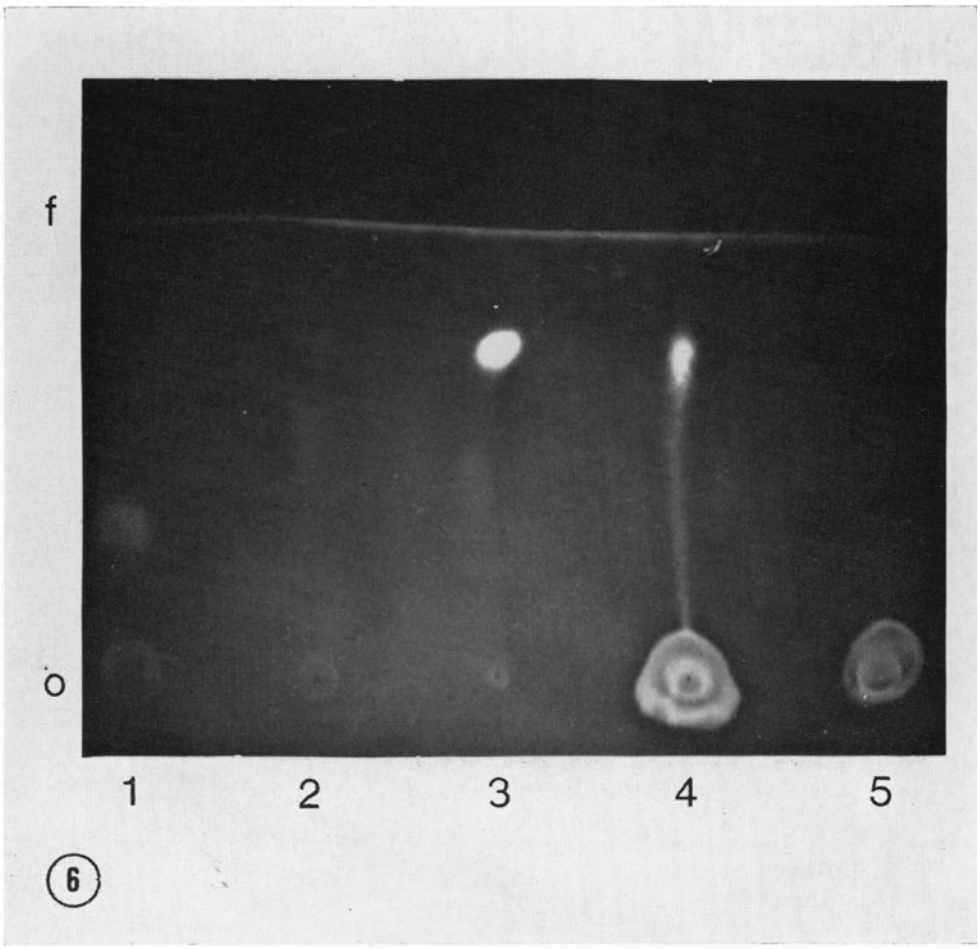


FIGURE 6 A thin-layer chromatogram showing the behavior of an acetone extract of colossal and *control* neuron cell bodies as compared with the behavior of standard 5-HT and 5-HTP. *1*, 0.6 μg standard 5-HTP; *2*), acetone extract of 0.4 μg standard 5-HT; *3*), 0.6 μg standard 5-HT; *4*, acetone extract of 600 colossal neuron cell bodies; *5*, acetone extract of 1800 *control* neuron cell bodies. *f* = solvent front; *o* = origin. Solvent system: methylacetate: isopropanol: 25% ammonia (9:7:4). Chromatogram reagent: ethylenediamine-potassium ferricyanide. The photograph was taken under ultraviolet light.

ment in the cells, both before and after treatment with formaldehyde vapor.

Extraction and Spectrofluorometric Determination of 5-HT in Individual Cells

Individual colossal or *control* cell bodies were dissected, extracted for 5-HT, and analyzed spectrofluorometrically. In order to obtain enough material to be registered in the assay, 35–49 colossal cells were pooled. The results of eight determinations on a total of 335 cells showed an average of 3.8×10^{-10} g of a material presumed to be 5-HT per colossal cell body (Table II). In order to confirm that the material extracted from colossal cells was 5-HT, excitation and emission spectra were determined for the extracted material; these spectra were identical with the spectra obtained from standard 5-HT (Fig. 7). The average diameter of a living colossal cell body is 90 μ, and thus the volume is approximately 3.8×10^{-7} cm^3. Therefore, the average concentration of 5-HT per cell body is approximately 6×10^{-3} molar (or 1 mg of 5-HT per g of colossal cell bodies). The reasons for the almost 10 fold range

TABLE II

Spectrofluorometric Analyses: Quantitative Estimation of 5-HT in Colossal and Control Neuron Cell Bodies

Neuron type	No. of neurons	5-HT (g/cell body $\times 10^{-10}$)
Colossal	41	1.4
Control	100	0
Col	47	1.5
C	107	0
Col	49	2.0
C	145	0
Col	46	2.0
C	146	0
Col	49	2.8
C	104	0
Col	35	3.6
C	100	0
Col	44	7.4
C	135	0
Col	44	9.8
C	109	0

Single colossal and bunches of *control* cell bodies were dissected from leech ganglia, pooled, and extracted for 5-HT. The amount of 5-HT in the extracts was determined spectrofluorometrically after treatment with ninhydrin. Spectra from the ninhydrin-treated colossal cell extracts were the same as the spectra obtained from ninhydrin-treated standard 5-HT (see Fig. 7).

in values of 5-HT per cell body are not clear (Table II), but the two most obvious explanations are: first, that the range represents a real variation in the amount of 5-HT from cell to cell; or second, that it represents varying amounts of damage to the cells during their dissection.

Dissection of a group of colossal cell bodies was always accompanied by a dissection of *control* neuron cell bodies. Since these cells are smaller, the eight *control* samples consisted of greater numbers of pooled cells, ranging from 100 to 146 cell bodies each. Spectrofluorometric readings of the extracts from these samples were negative. Thus, colossal cell bodies contain a considerable amount of a substance that assays as 5-HT whereas *control* cells do not.[3]

Fine Structure

FINE STRUCTURE OF COLOSSAL AND CONTROL PERIKARYA *in situ*: The cytoplasmic organelles in leech neuronal perikarya are segregated (e.g. Apathy, 1897; Ito, 1936) (Fig. 15). With the electron microscope, it can be seen that the periphery of the cell body is filled with the granular endoplasmic reticulum whereas the other cytoplasmic organelles, namely pigment bodies that resemble lysosomes, Golgi complexes, mitochondria, neurofibrillar bundles (Grey and Guillery, 1963), and neurotubules, are concentrated around the nucleus (Coggeshall and Fawcett, 1964). This organization characterizes mainly small and medium-sized cells, but in large neurons a pigment-filled organelle-rich zone is also found in the peripheral cytoplasm and is thus demarcated on both sides by granular endoplasmic reticulum (Fig. 15). Where the cell body gives way to the axon, there is an abrupt transition to axoplasm that contains mainly smooth-surfaced tubules and vesicles, neurofibrillar bundles, neurotubules, and an occasional mitochondrion.

In addition to the above organelles, colossal perikarya contain small granules whose appearance varies with the fixation (Figs. 16–19). They are most prominent after glutaraldehyde fixation and, with this procedure, they average 900–1000 A in diameter (Figs. 16, 18, and 19). The cores of these granules are very electron-opaque and irregular, often being confined to one quadrant of the granule (Figs. 18 and 19). The granules are most abundant in the pigment-rich zones, but are also numerous in the zones containing the granular endoplasmic reticulum and in the axon itself.

These granules seem to be formed in the Golgi complexes, as evidenced by micrographs showing

[3] 5-HT could not be measured in *control* cell bodies. However, amounts of 5-HT below 1×10^{-9} g per sample would not be detected with this method. Since the smallest *control* sample contained 100 cell bodies, the maximum amount of 5-HT in a single *control* cell body would be 1×10^{-11} g. Thus, there is *at least* 38 times as much 5-HT in colossal cell bodies as in *control* cell bodies. *Control* cell bodies have an average diameter of about 60 μ and thus a volume of approximately 1.1×10^{-7} cm^3. Thus, on a molar basis, the colossal cell body contains *at least* 11 times as much 5-HT as the average *control* cell body.

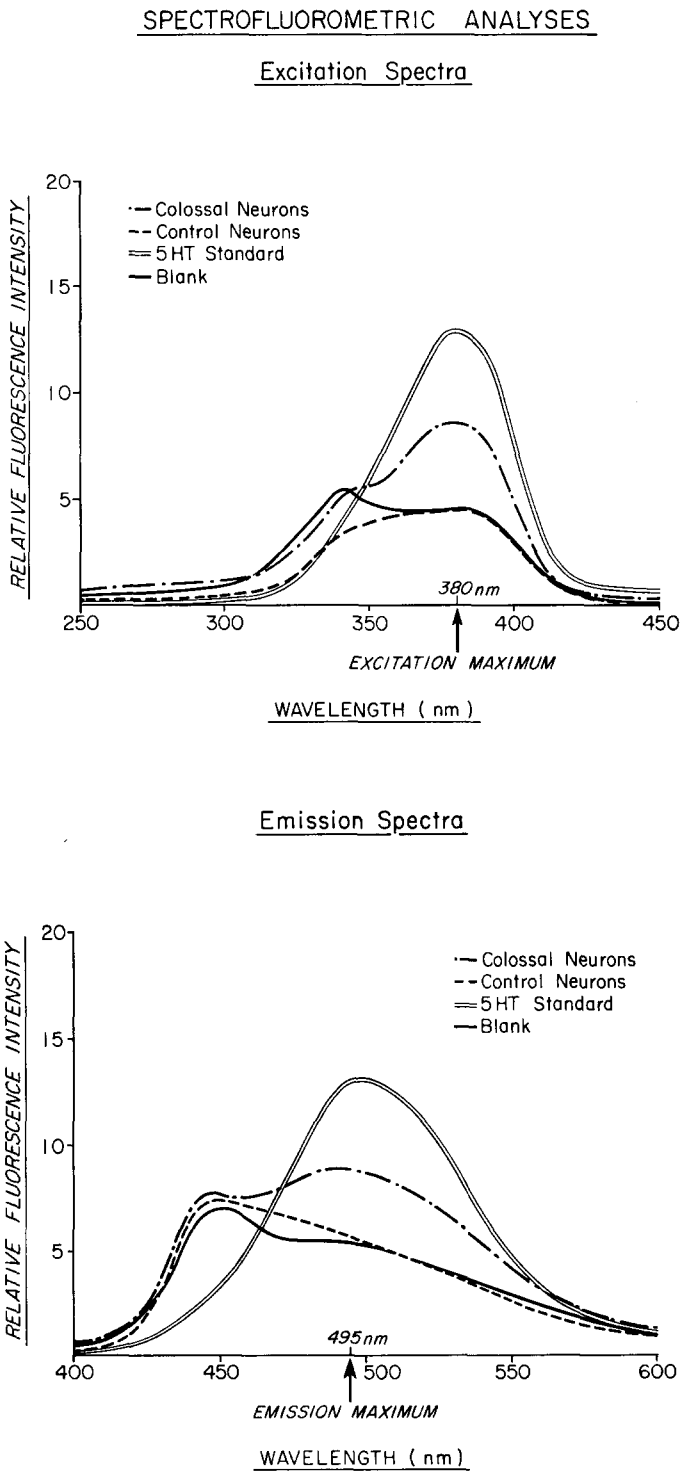


FIGURE 7 The fluorescence excitation and emission spectra obtained from ninhydrin-treated extracts of colossal and *control* neuron cell bodies are compared with the spectra obtained from standard 5-HT also carried through the extraction procedure and treated with ninhydrin. (The fluorescence excitation and emission spectra of ninhydrin-treated blanks are also shown.)

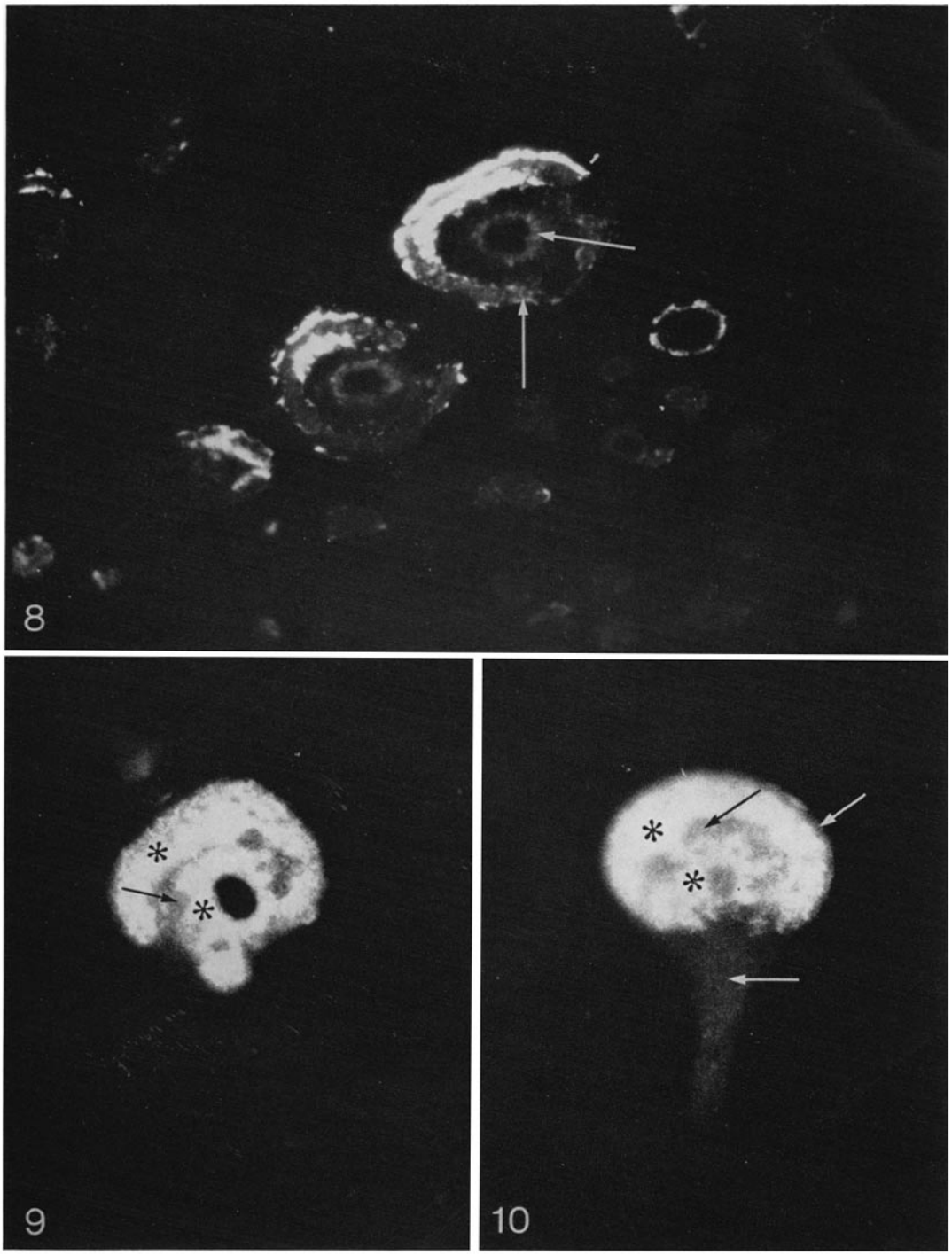
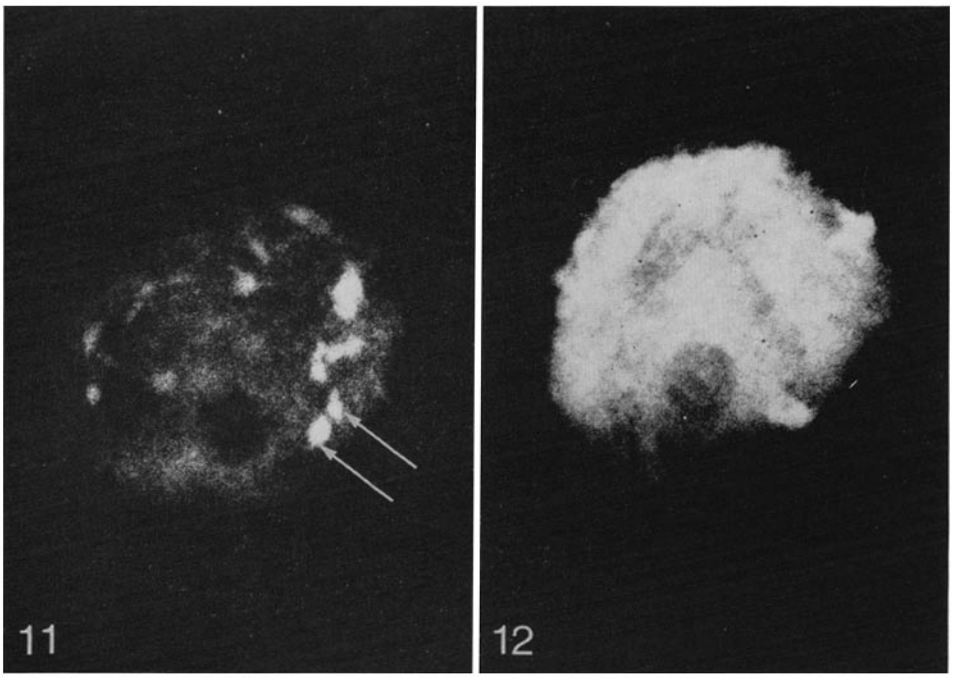


FIGURE 8 Photomicrograph of an 8 μ section through the anterior ventral packet of a freeze-dried ganglion. Autofluorescent pigment is present in the colossal cell bodies and adjacent neurons. The autofluorescent pigment in the colossal cell bodies is arranged in concentric layers (arrows). × 450.

FIGURES 9 AND 10 Photomicrographs of 8 μ sections through the anterior ventral packet of a freeze-dried ganglion treated with formaldehyde vapor, showing the colossal cells. The brilliant formaldehyde-induced fluorescence in the colossal cells is present in two concentric layers (*) and several bands which interconnect the two layers. A dim formaldehyde-induced fluorescence is present in those regions of the neuron which do not possess a brilliant fluorescence (arrows). × 450. Fig. 9, A dim yellow fluorescence can be seen in the area between the two bright yellow fluorescent layers (arrow). Fig. 10, A dim yellow fluorescence can be seen in the area between the two bright yellow fluorescent layers, outside the outermost bright yellow fluorescent layer and in the axon hillock region (arrows).



FIGURES 11–14 Photomicrographs of whole nerve cell bodies dissected from living ganglia and dried on slides. Exposure times were the same for each photograph. × 600.

FIGURE 11 An untreated colossal cell body showing its weak autofluorescent cytoplasm and the bright autofluorescent small glial cells (arrows) attached to its surface.

FIGURE 12 A colossal cell body treated with formaldehyde vapor, showing its bright formaldehyde-induced fluorescence. The fluorescence is so bright in this whole cell that it obscures the small, bright autofluorescent glial cells attached to its surface.

the close association of the granules with some Golgi complexes and the presence of plaques of material that resemble the granule cores in the cisternae of these Golgi complexes (Fig. 19). They are not observed in all Golgi complexes, however, and it may be that the manufacture of these granules is relatively slow in the leeches used in this study.

With osmium tetroxide fixation, the granules are smaller, 600–700 A in diameter, and the cores, although still electron-opaque, are smaller and round (Fig. 17). Their distribution is the same as after glutaraldehyde fixation. Formaldehyde or permanganate fixatives do not preserve the granule core. It is interesting that the structure of other granules that presumably contain a biogenic amine also depends on fixation (Richardson, 1966; Duncan and Yates, 1967).

The colossal cells are not the only cells that contain granules, but the granules found in *control* cells are always larger, 1500–2500 A in diameter, round, and contain large round cores, regular in outline, and not very electron-opaque. In summary, if appropriate fixatives are used, colossal cells can be distinguished from *control* cells by their content of characteristic small granules.

FINE STRUCTURE OF INDIVIDUALLY DISSECTED COLOSSAL AND CONTROL PERIKARYA: Colossal and *control* cells were individually dissected and placed into the glutaraldehyde fixing fluid. After postfixation, embedding, and sectioning, it could be seen that the cytoplasmic and nuclear structure of these cells was not greatly distorted (Figs. 20, 21). Some swelling of the granular endoplasmic reticulum was evident in the periphery of the cells, and some "empty"

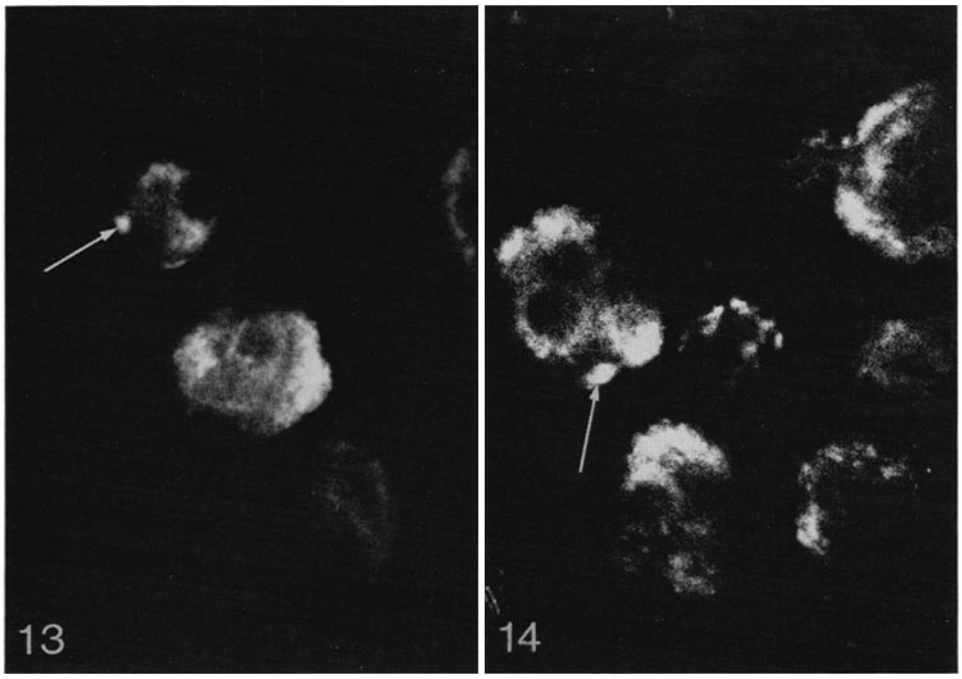


FIGURE 13 *Control* cell bodies heated in the absence of formaldehyde vapor. Note their weak autofluorescent cytoplasm and the bright autofluorescent small glial cells (arrow) attached to their surfaces.

FIGURE 14 *Control* cell bodies treated with formaldehyde vapor to show that the appearance of the control cells is the same before and after formaldehyde treatment. A small glial cell is marked by an arrow.

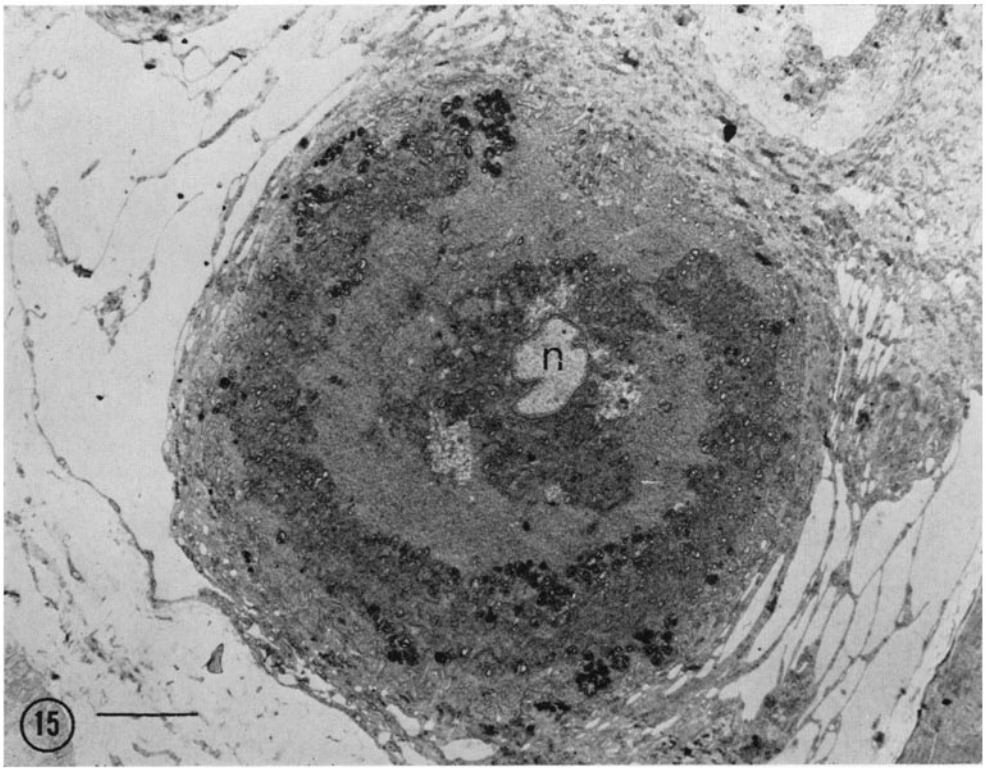


FIGURE 15 A low-power electron micrograph of an OsO_4-fixed colossal cell. The two dark pigment bands, one around the nucleus (*n*), the other in the more peripheral cytoplasm, can be clearly seen. The pigment bands consist of mitochondria, pigment bodies, Golgi complexes, neurofilaments, and neurotubules, whereas the lighter regions of the cytoplasm consist mainly of granular endoplasmic reticulum. × 1400. Scale line indicates 10 μ.

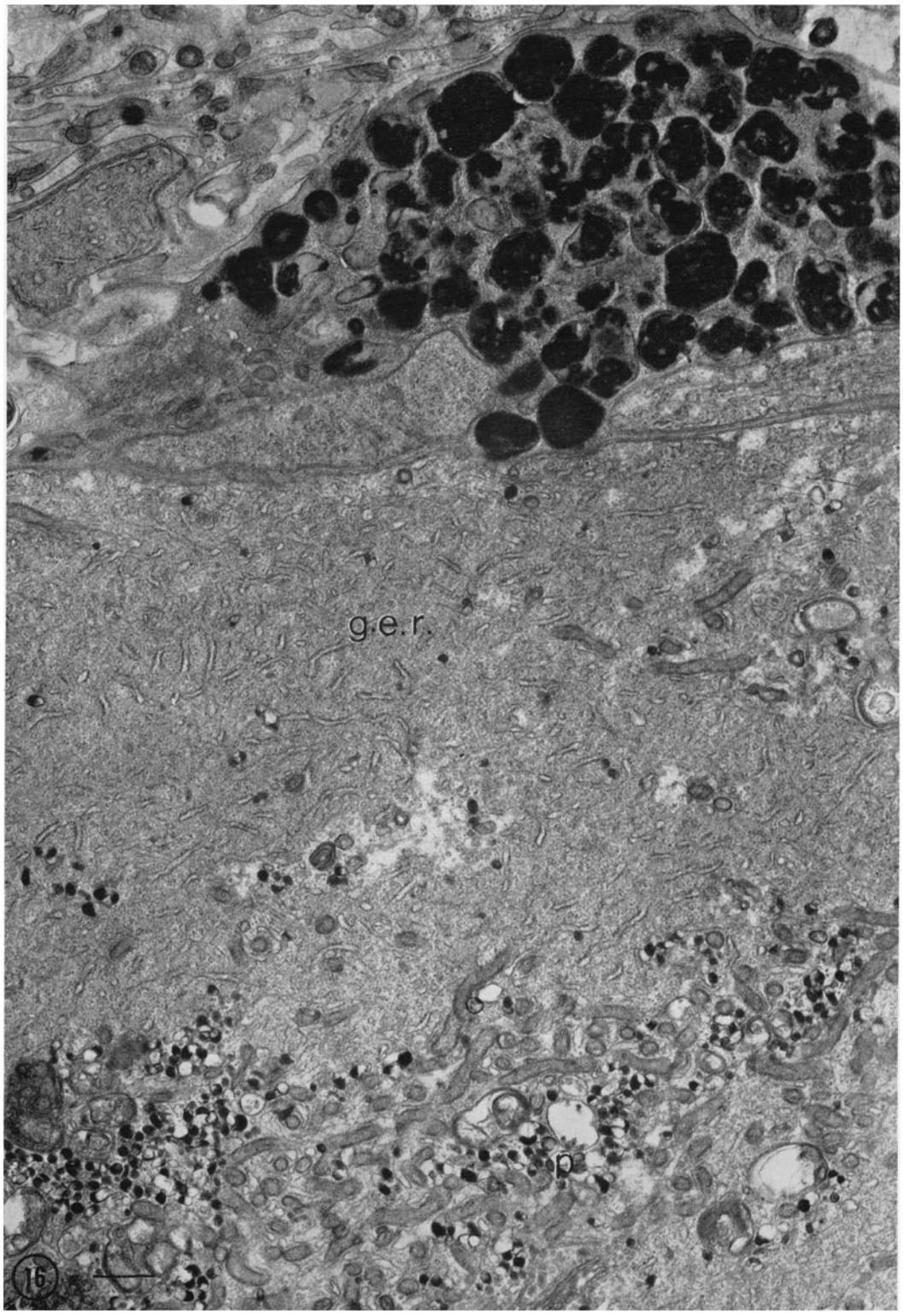
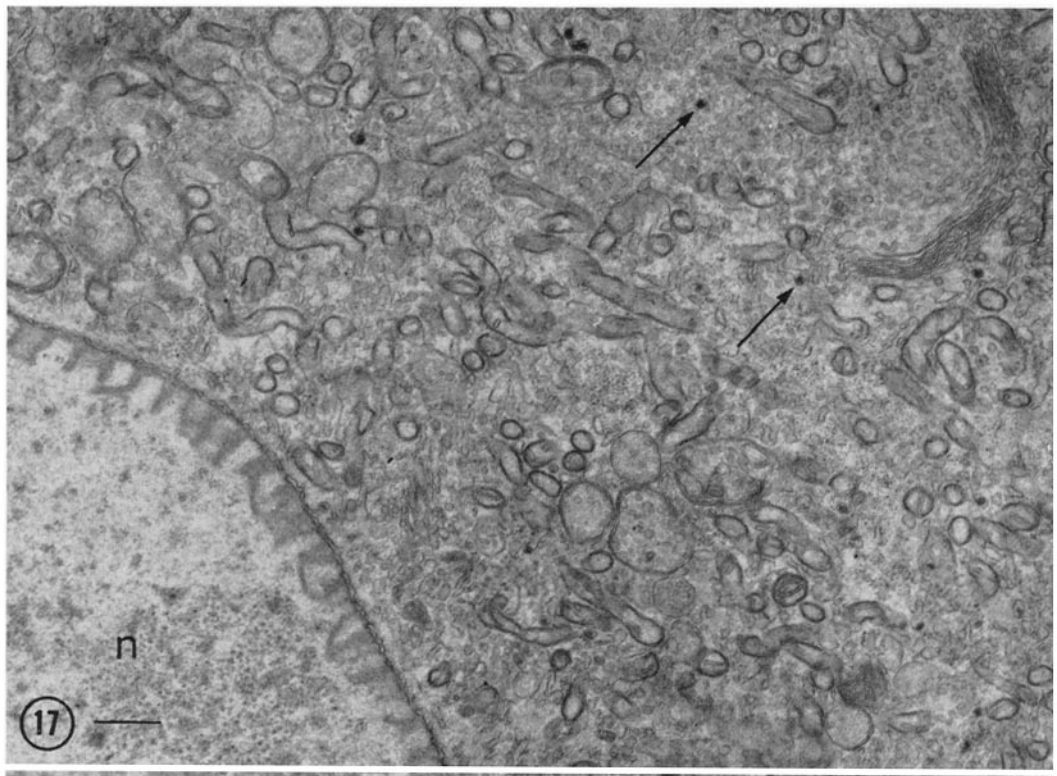


FIGURE 16 A small glial cell laden with pigment granules that resemble lysosomes is located in the upper part of the micrograph. This cell has a brilliant yellow autofluorescence. The edge of a colossal cell occupies the lower two-thirds of the micrograph. Note that the periphery of the cell consists mainly of granular endoplasmic reticulum (*g.e.r.*) with a few granules interspersed. The pigment layer (*p*) consists of myriad granules, mitochondria, Golgi complexes, and pigment bodies. × 12,000. Scale line, 0.75 μ.



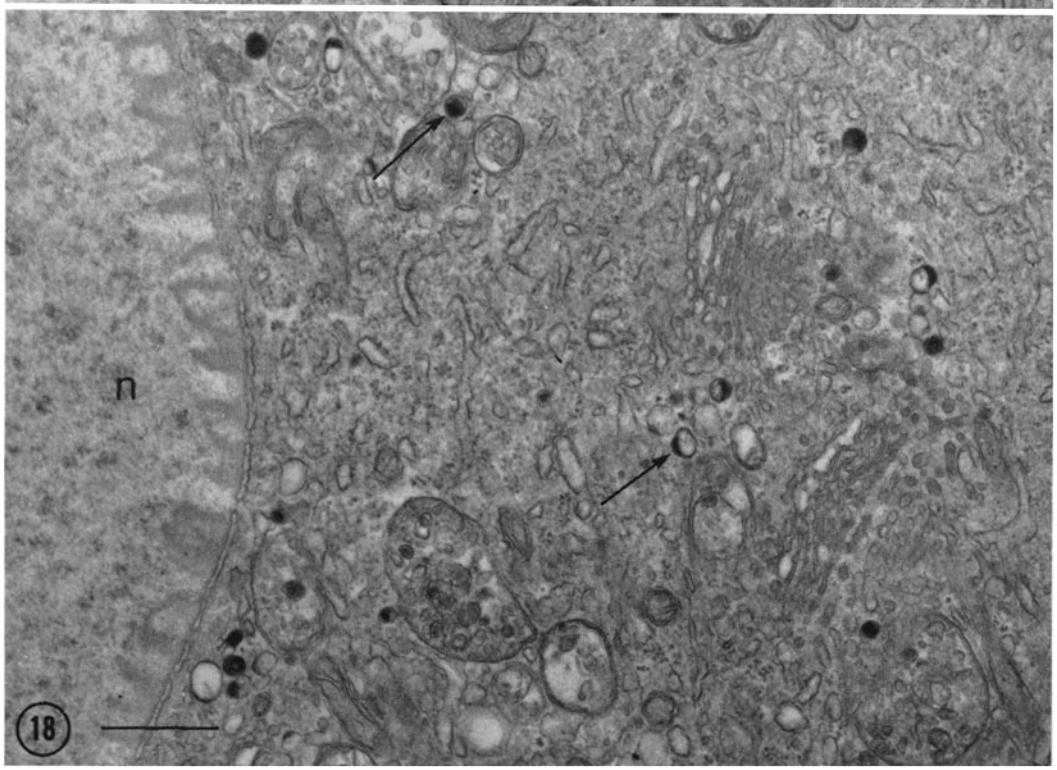


FIGURE 17 The perinuclear region of an OsO_2-fixed colossal cell. The granules that characterize this cell (arrows) are 600–800 A in diameter and have a round electron-opaque core. *n*, nucleus. × 34,000. Scale line, 0.2 μ.

FIGURE 18 The perinuclear region of a glutaraldehyde-fixed colossal cell. The granules that characterize this cell (arrows) are 1000 A in diameter and have an irregular electron-opaque core. *n*, nucleus. × 42,000. Scale line, 0.4 μ.

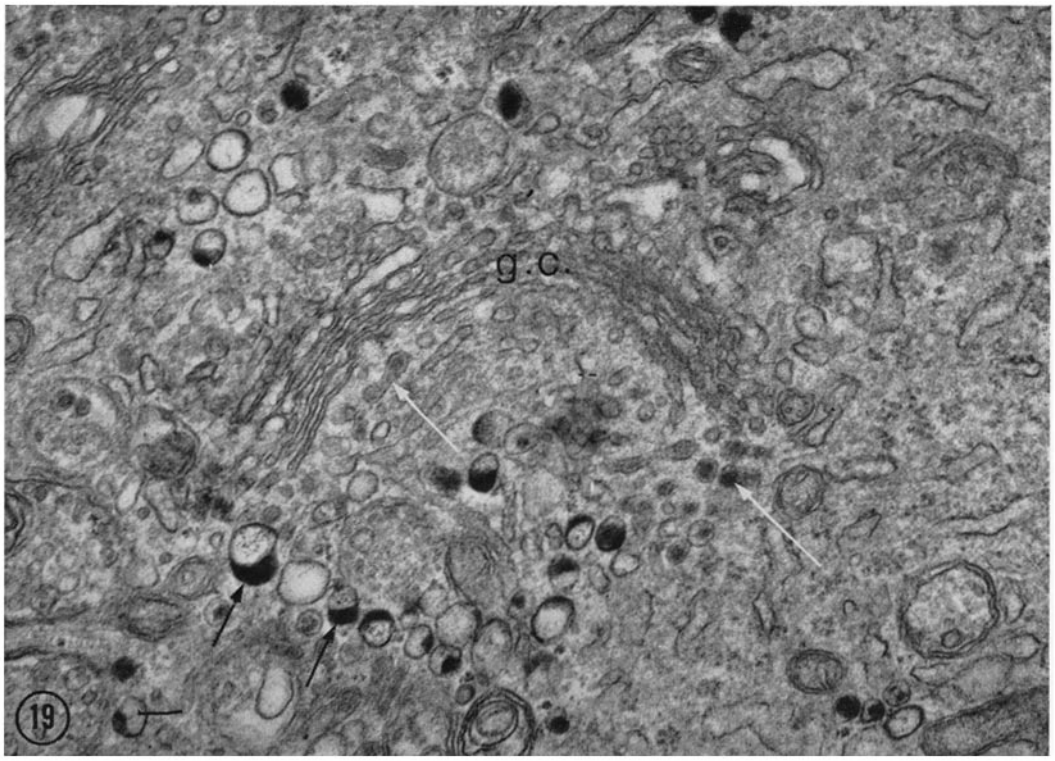


FIGURE 19 A Golgi complex (*g.c.*) of a colossal cell. Note that many granules (black arrows) are in close association with the complex, but that the granules that are closest to the Golgi lamellae (right hand, white arrow) are slightly smaller and have less electron-opaque cores. Also there are several structures (left hand, white arrow) which appear to be short segments of a Golgi lamella that contain a moderately electron-opaque material. × 60,000. Scale line, 0.1 μ.

spaces could be found in the perinuclear regions (Fig. 21), but otherwise the cells seemed to be intact. Colossal and *control* perikarya were always surrounded by a layer of packet glial cell cytoplasm (Fig. 20). This layer is usually 1–2 μ thick. The pigment-laden, small glial cells are often located between the neuron and adhering cytoplasm of the giant packet glial cell.

When dissected collossal cells were compared with colossal cells seen in histological section, the two appeared identical except for the relatively mild cytoplasmic changes described above for the dissected cells. In particular, the characteristic granules were seen in the same distribution in both preparations (Fig. 21). *Control* cells did not show these granules (Fig. 20).

THE WOOD REACTION, A CHROMAFFIN REACTION MODIFIED FOR THE ELECTRON MICROSCOPE: If ganglia are fixed in glutaraldehyde and then passed through the solutions that make up the Wood reaction, the colossal cells take on a distinct brown coloration, which can easily be seen under the dissecting microscope. This is thus a confirmation of Gaskell's observations made in 1914. If thin sections are taken in order to see what structures underlie the gross brown coloration, it can be seen that the only structures that are darkened are the cores of the granules that characterize the colossal cells (Fig. 22). If ganglia are fixed and washed in this manner, but not passed through the dichromate solution, the granule cores do not stain, indicating that there is a substance in the granule cores that interacts with the dichromate mixture to produce an electron-opaque product.

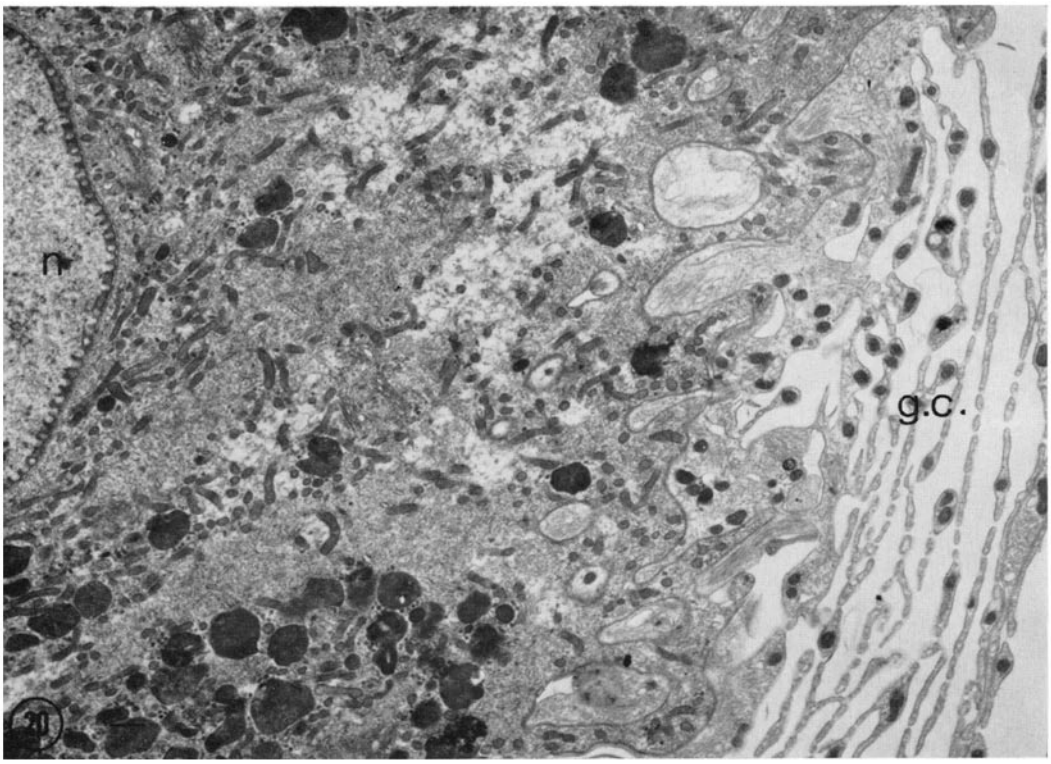


FIGURE 20 A dissected *control* cell. Note that the nucleus (*n*) and cytoplasm of this cell do not appear abnormal. A layer of glial cytoplasm (*g.c.*) can be seen clinging to the side of the neuron. This is the cytoplasm of the giant packet glial cell. × 8,000. Scale line, 0.625 μ.

DISCUSSION

The Analysis for 5-HT in Colossal Cells

In this study, three different assays for 5-HT were performed on the colossal cells of Retzius. The first procedure was a microspectrofluorometric analysis of colossal and *control* cells in histological sections and of individually dissected perikarya. Before formaldehyde treatment, the colossal cell bodies, as well as the *control* cell bodies and small glial cells, show an autofluorescent pigment. This pigment has an excitation maximum of 460–470 nm and an emission maximum of 510–520 nm. After formaldehyde treatment, the *control* cell bodies and glial cells show no change in the intensity or spectral characteristics of their fluorescence. There is, however, a marked change in both the intensity and spectral characteristics of the fluorescence of the colossal cells. The regions of the cell which already contain autofluorescent pigment become much brighter and, in addition to an excitation maximum of 460–470 nm, the excitation spectrum shows a second maximum at 410–430 nm. Furthermore, after formaldehyde exposure, those regions of the cell which do not contain autofluorescent pigment take on a weak yellow fluorescence with an excitation maximum at 430 nm and an emission maximum at 520 nm. The fluorescence induced by formaldehyde treatment in the pigment regions and throughout the nonpigmented regions of the colossal cell has the same spectral characteristics as the fluorescence induced in formaldehyde-treated 5-HT in model systems.

We interpret the microspectrofluorometric data in the following way. We believe that the colossal cells contain both an autofluorescent pigment and a 5-HT–like material. Furthermore, the autofluorescent material is located in specific regions in the perikaryon of the colossal cell, whereas the 5-HT–like material is present in all regions of the perikaryon and the axon hillock, but concen-

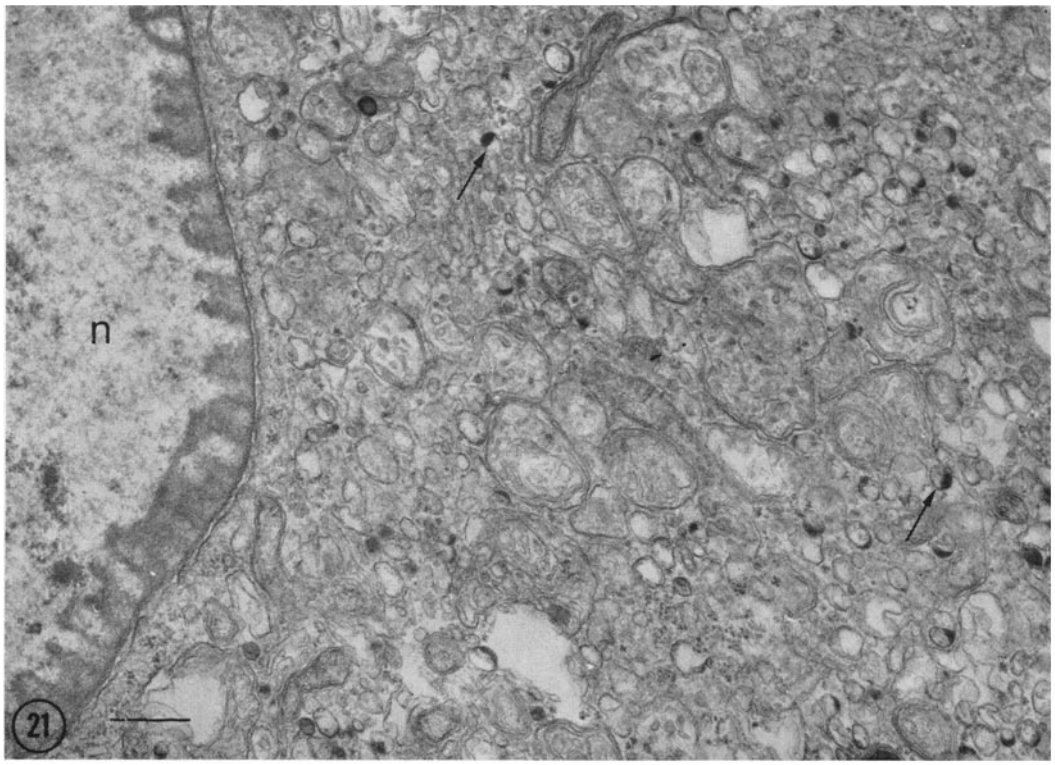


FIGURE 21 The perinuclear region of a dissected colossal cell. The nucleus (*n*) appears normal, but there are a few "empty spaces" in the cytoplasm. These "empty spaces" are the only morphological signs of damage. Note that the characteristic colossal cell granules (arrows) have the same appearance as they did in sections of colossal cells *in situ*. × 54,000. Scale line, 0.2 μ.

trated in the regions that also contain the autofluorescent pigment.

The second assay was a chromatographic analysis of acetone-extracted material obtained from individually dissected colossal cells and *control* cell bodies. The colossal cells contain a material that migrates exactly as 5-HT and possesses the same excitation and emission spectra as 5-HT. The *control* cells do not contain such a substance.

The third assay was a spectrofluorometric analysis for 5-HT of extracts of individually dissected colossal and *control* cell bodies. A substance that has the same excitation and emission spectra as 5-HT is found in a 6 mM concentration in colossal cells, but not in *control* cells.

Thus, three different assays indicate that the colossal cells contain a substance indistinguishable from 5-HT. On this basis, we believe we have established beyond any reasonable doubt that the colossal cells contain 5-HT. Furthermore, we have provided quantitative data indicating that the average concentration of 5-HT is 6 mM (approximately 3.8×10^{-10} g/cell body or 1 mg/g of cell body).

The Presence of Catecholamines in the Leech Nervous System

As mentioned above, Biedl (1910) and Gaskell (1919) found a substance that bioassayed as adrenaline in this nervous system, and Gaskell suggested that the six chromaffin cells contained adrenaline. Perez (1942) agreed with this suggestion for the collosal cells, and recently Bianchi (1967) suggested that the six chromaffin cells contained CA. By contrast, Kerkut et al (1966) could find no evidence for CA when they tested ganglionic extracts by paper chromatography. We believe that this nervous system contains CA for we have found large green or blue-green

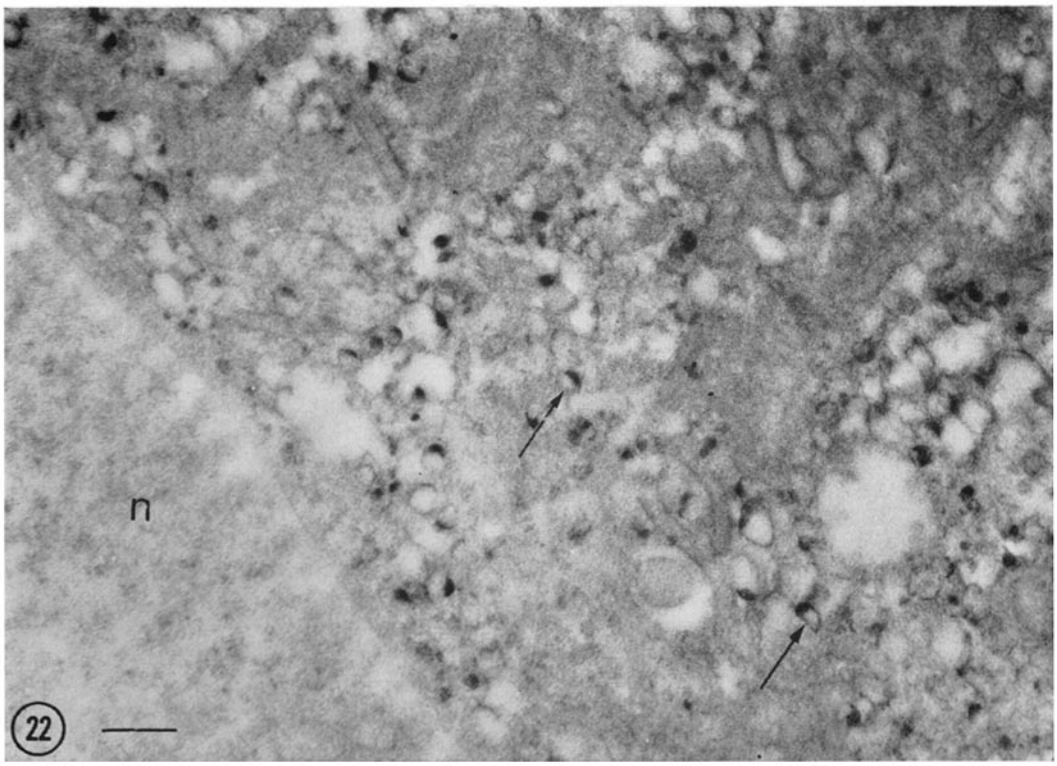


FIGURE 22 The perinuclear region of a colossal cell as it appears after the Wood reaction. This cell appears brown under the dissecting microscope. Note that the only darkened structures are the granule cores (arrows). *n*, nucleus. × 40,000. Scale line, 0.25 μ.

fluorescent processes in the neuropil that have the same spectral characteristics as NE. We do not believe, however, that there is a CA in the colossal cells, for there is no microspectrofluorometric or chromatographic evidence for this material here.

The Correlation of Fine Structure and Fluorescence Microscopy

PIGMENT GRANULES AND AUTOFLUORESCENCE: All neuron cell bodies and many of the small satellite glial cells contain a yellowish autofluorescent material. In the neuronal cell bodies, the autofluorescence is distributed in layers, bands, or clumps, whereas the entire glial cytoplasm seems to fluoresce. Electron microscopy shows that the cytoplasm of the small glial cells is filled with pigment granules. The pigment granules are also a very prominent component of the autofluorescent areas in the neurons and do not appear to be present in regions which show no autofluorescence, e.g. the axon hillock. Thus, since the pigment granules are the only organelles distributed in the same pattern as the autofluorescence, we believe that the pigment granules are the autofluorescent organelles in these cells.

It is interesting that the small glial cells that flank the nerve cell bodies are laden with pigment granules, although those glial cells not located next to the neuronal cell bodies contain none or a very few pigment granules (Coggeshall and Fawcett, 1964; and unpublished data). Apathy (1897) was the first to suggest that these ubiquitous small cells were the migratory scavengers of this nervous system, a view to which Coggeshall and Fawcett (1964) also subscribed. There has been no previous suggestion, however, that a distinction could be made between those small cells located at the edge of the neuronal cell bodies and those located elsewhere in this nervous system. The functional implications of this morphological distinction are not clear.

SMALL GRANULES AND FORMALDEHYDE-INDUCED FLUORESCENCE: In addition to being larger, the colossal cells differ from their neighbors by (*1*) the presence of small granules, and (*2*) a high concentration of 5-HT. Since the small granules are the only structures not found in neighboring cells that lack 5-HT, we assume that the granules are correlated with the presence of, and probably contain, 5-HT. The case for this correlation can be considerably strengthened if we consider the distribution of the fluorescence and small granules within the colossal cells themselves. We have shown that, exclusive of the nucleus, the cell body and axon hillock develop a yellow formaldehyde-induced fluorescence, and that this fluorescence is brightest in the pigment layers. The only organelles common to these regions, most abundant in the pigment layers, and not present in neighboring cells, are the small granules. Thus, we conclude that the small granules contain the fluorescent material, which by several assays has been shown to be 5-HT.

Further evidence for the assertion that the granules contain the 5-HT comes from the chromaffin reaction used in this study. If the reaction is applied to leech ganglia, the colossal cells take on a brown color and electron microscopic examination reveals that the only darkened structures in the colossal cells are the cores of the characteristic granules. This shows that the cores of the granules contain the chromaffin substance. Thus, since there is good evidence that the chromaffin substance is 5-HT, 5-HT must be located in the granule cores.

A COMPARISON OF COLOSSAL CELLS AND OTHER 5-HT-CONTAINING CELLS: Perhaps the two best studied 5-HT–containing cell types are the platelet and the enterochromaffin cell. Both cell types are characterized by cytoplasmic granules, and, as with the colossal cells, the 5-HT has been localized in the granule cores. The granules in the enterochromaffin cells are very different from those in the colossal cells, however, in that they are larger, 1500–2500 A in diameter (e.g. Ito and Winchester, 1963), and their cores are less electron opaque. The granules in platelets resemble the colossal cell granules in that they are the same size and seem to have cores of the same electron opacity (Etcheverry and Zieher, 1968; Tranzer et al., 1968). They differ in that the cores in platelet granules are not preserved by osmium tetroxide alone whereas the cores in colossal cell granules are, and also the cores in the colossal cell granules are often pressed against the granule membrane whereas the platelet granule cores tend to be round. At the present time, it seems reasonable to state that intracellular 5-HT is contained in granules, but unfortunately there does not seem to be a single morphological form of granule that can be used to identify the presence of 5-HT.

We thank Dr. J. H. Welsh in whose laboratory much of the work in this study was done. Dr. E. A. Kravitz taught us the technique of dissecting single cells. Dr. R. J. Wurtman kindly showed us the extraction and assay procedure for 5-HT. We also thank Drs. D. W. Fawcett, J. H. Welsh, and R. J. Wurtman for their critical readings of the manuscript. Finally, Dr. J. McMahan supplied Fig. 2 for us.

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Note Added in Proof

Recently Ehinger et al. (*Histochemie.* 1968. **15**:140) published the results of a spectrofluorometric examination of the leech nervous system treated according to the fluorescence histochemical technique for monoamines. Our results are in basic agreement with theirs. We did, however, note that the autofluorescent pigment had an excitation maximum around 460 nm. This differs considerably from the excitation maximum of 5-HT (425 nm).

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