Zeitschrift für Zellforschung 86, 393—400 (1968)

Cytochemistry of 5-Hydroxytryptamine at the Electron Microscope Level

II. Localization in the Autonomic Nerves of the Rat Pineal Gland*

GUILLERMO JAIM ETCHEVERRY** and LUIS M. ZIEHER

Instituto de Anatomía General y Embriología (Director: Prof. E. DE ROBERTIS)
Facultad de Medicina, Buenos Aires (Argentina)

Received December 5, 1967

Summary. The nerves of rat pineal gland are known to contain norepinephrine and 5-hydroxytryptamine. With the glutaraldehyde-dichromate reaction for the cytochemical localization of unsubstituted catechol- and indoleamines, dense reactive granules could be demonstrated in such endings. A similar reaction was observed in the adrenergic nerves supplying the vas deferens and storing exclusively norepinephrine. Formaldehyde fixation, prior to the glutaraldehyde-dichromate treatment, interferes with the reaction given by catecholamines not affecting the indolic reactive sites. After this combined procedure pineal nerves still exhibited the dense reactive granules, while these were not found in the nerves of the vas deferens. Following bilateral cervical sympathectomy reactive granules disappeared from the perivascular processes of the pineal gland. No reaction could be observed in the cytoplasm of parenchymal cells neither in their perivascular processes.

These cytochemical results suggest that both catecholamines and 5-hydroxytryptamine are contained within the granulated vesicles of pineal nerves.

Knowledge of the subcellular localization of monoamines is of great importance to interpret their physiological role in the nervous system. Electron microscopic studies of adrenergic sympathetic nerve fibers have established the presence of a mixed population of vesicles, composed of clear ones resembling synaptic vesicles (DE ROBERTIS and BENNETT, 1954) and of granulated vesicles of the same size but having an electron dense core within the limiting membrane (DE ROBERTIS and PELLEGRINO DE IRALDI, 1961a, b; RICHARDSON, 1962, 1964). DE ROBERTIS and PELLEGRINO DE IRALDI (1961a) postulated that such granulated vesicles are the specific site of storage of monoamines in autonomic nerves. This assumption has been confirmed in recent years using different experimental approaches (PELLEGRINO DE IRALDI and DE ROBERTIS, 1961; WOLFE et al., 1962; PELLEGRINO DE IRALDI and DE ROBERTIS, 1963; PELLEGRINO DE IRALDI et al., 1963, 1965; CLEMENTI, 1965; BONDAREFF and GORDON, 1966; VAN ORDEN et al., 1966; BLOOM and BARRNETT, 1966; HÖKFELT, 1967).

Work has also been devoted to the development of cytochemical techniques for the demonstration of monoamines at a fine structural level in the adrenal medulla (WOOD and BARRNETT, 1964; COUPLAND et al., 1964; TRAMEZZANI et al.,

* This work has been supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina and U.S.Air Force (AF-AFOSR 963-66).

** Fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina. We want to express our gratitude to Prof. E. DE ROBERTIS for his constant help and encouragement; and to Miss NÉLIDA FERNÁNDEZ ORANGES, Mr. RAÚL CASTELLI and Mr. ALBERTO SÁENZ for their skillful technical assistance.

1964). The reactions so far developed are based on the fact that an insoluble complex is formed between aldehydes and amines and that, upon exposure to a metal-containing oxidizing agent, a dense precipitate identifiable under the electron microscope is formed. One of these reactions has been applied to the study of the autonomic nerve endings of the vas deferens and dense reactive granules within these endings were demonstrated (BLOOM and BARRNETT, 1966). Such granules, identical in size and shape to the osmiophilic core of the granulated vesicles characteristic of these fibers (RICHARDSON, 1962), were identified as the site of storage of norepinephrine (NE). However the application of this technique involving dichromate oxidation to other structures containing biogenic amines was made difficult because "in vivo" and "in vitro" experiments demonstrated that not only catecholamines, NE and dopamine (DA), but also 5-hydroxytryptamine (5-HT) gave a positive reaction (WOOD, 1965, 1966). In an attempt to find more selective cytochemical tests for the demonstration of monoamines, formaldehyde fixation, prior to the dichromate reaction, was found to render negative, at the light microscopic level, the reaction given by catecholamines and to leave unaffected indolic reactive sites (see PEARSE, 1960; WOOD, 1967). This reaction provides an efficient tool for the localization of 5-HT at a fine structural level and recently we have demonstrated that it is highly specific in a pharmacological and ultrastructural study in isolated blood platelets (JAIM ETCHEVERRY and ZIEHER, 1967).

The pineal gland of the rat contains a remarkable high concentration of 5-HT (QUAY and HALEVY, 1962) as also of NE, DA and histamine (see PELLEGRINO DE IRALDI et al., 1965). The available evidence indicates that part of pineal 5-HT is localized in nerve fibers, the rest being found in pinealocytes or parenchymal cells (BERTLER et al., 1963; PELLEGRINO DE IRALDI et al., 1963, 1965). These findings led us to attempt the localization of 5-HT in the nerve endings of the pineal gland at the electron microscope level using the above mentioned reaction. Comparative observations were also made on the nerve fibers of the vas deferens known to contain NE but not 5-HT.

Material and Methods

To minimize differences due to cyclic variations in amine content, the pineal gland and the vas deferens from adult male rats were fixed between 1 and 3 p.m. These organs were processed according to the following schedules: A. 3% glutaraldehyde in 0.2 M cacodylate buffer pH 7.4 during 4 to 24 h, followed by a 2.5% solution of potassium dichromate plus 1% sodium sulphate in 0.2 M acetate buffer pH 4.0 for 4 to 16 h. This procedure will be referred to as glutaraldehyde-dichromate or GD technique. B. 8% formaldehyde 24 h, prior to glutaraldehyde-dichromate treatment as in A. This procedure will be referred to as formaldehyde-glutaraldehyde-dichromate or FGD technique. C. Some blocks processed as in A and B were postfixed for 20 min in 1.5% buffered osmium tetroxide after the dichromate treatment. All procedures were carried at 4° C. The blocks were dehydrated in graded ethanol, being finally embedded in Epon 812 (LUFT, 1961). Thin sections, without further staining were examined under a Siemens Elmiskop I electron microscope.

In a group of rats, the superior cervical ganglia were excised bilaterally under light ether anesthesia. The pineal glands were removed 6 days after gangliectomy and subsequently processed for electron microscopy as the normal ones. Criteria for successful gangliectomy were the rapid development of bilateral ptosis of the superior eyelid and the demonstration of ganglionic tissue in the excised pieces by light microscopy.

Observations

Fig. 1 shows a group of nerve fibers and endings located in the perivascular space of the normal pineal gland, as it is revealed by the glutaraldehyde-dichromate (GD) technique followed by a brief osmication. Such endings contain a rather homogenous population of vesicles of 400—600 Å in diameter with

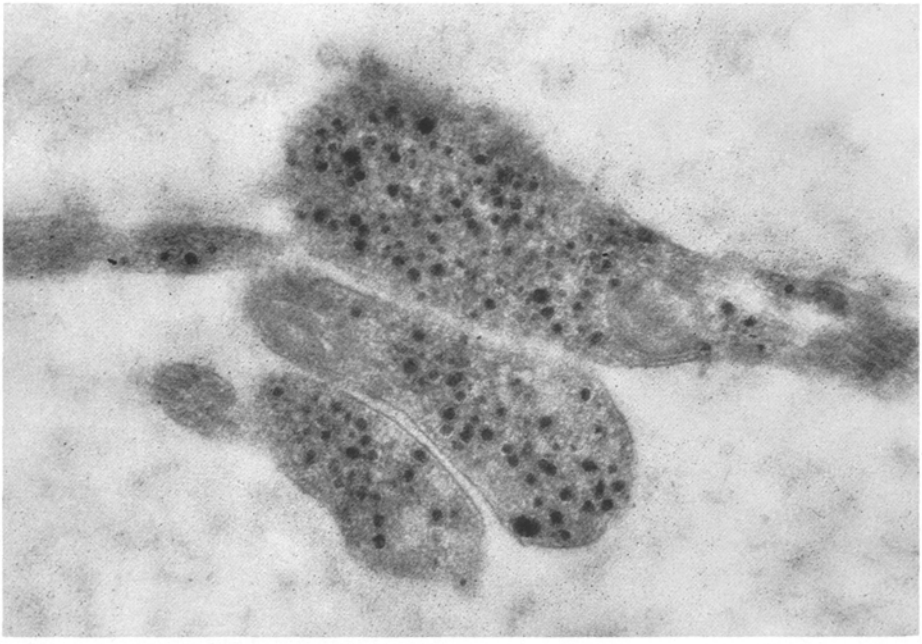


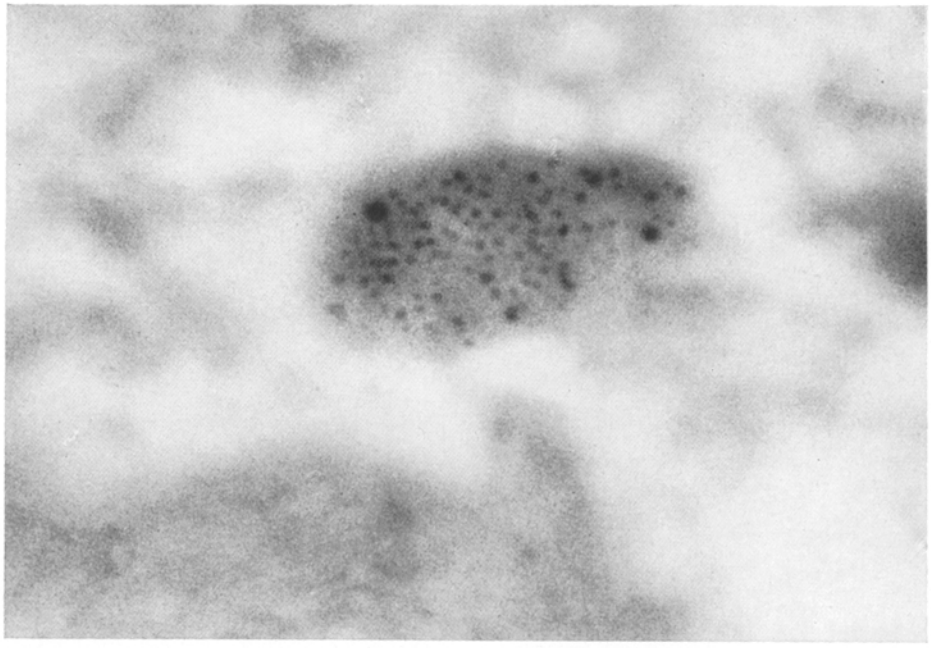
Fig. 1. A group of nerve fibers lying in the perivascular space of the pineal gland of the normal rat. When processed with glutaraldehyde-dichromate technique followed by a brief osmication, granulated vesicles of various types are observed within the nerve endings. × 50,000

a dense core inside. Electron lucent or "clear vesicles" previously described in these endings are only occasionally observed. Few larger granulated vesicles of 800—1,000 Å as well as mitochondria are also seen.

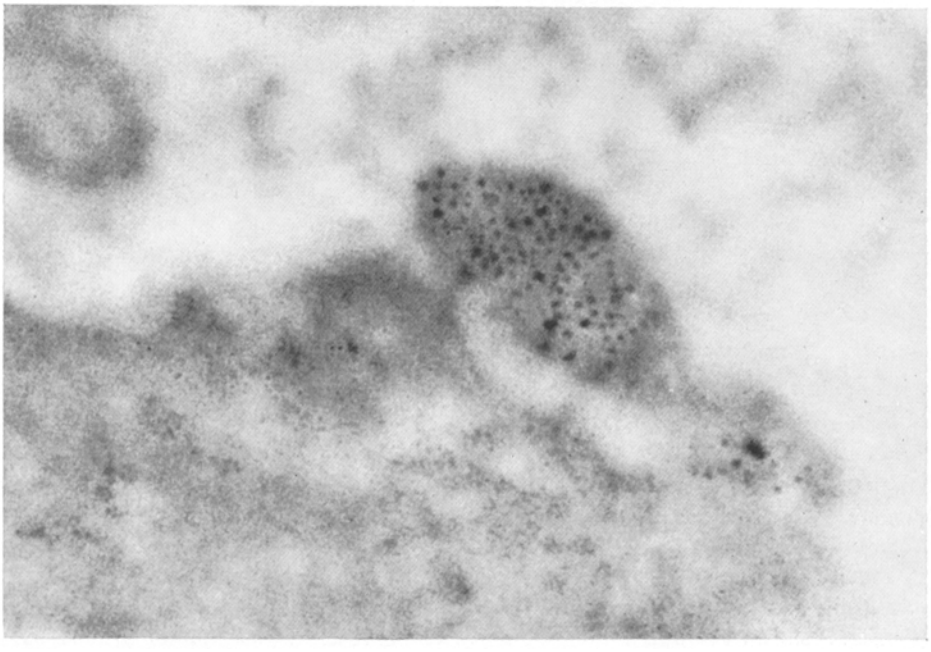
If in material processed with the GD technique the osmium tetroxide fixation is omitted, the membranous structures are not visible but the dense reactive granules are still observed in the perivascular endings (Fig. 2). Such granules are similar in size and shape to the dense core of the granulated vesicles observed in Fig. 1. Processes devoid of reactive material, corresponding to the club-shaped expansions of pinealocytes, were also seen lying in the perivascular space.

Fig. 3 shows the appearance of a pineal nerve ending as demonstrated with the formaldehyde fixation followed by the glutaraldehyde-dichromate treatment. A similar content of reactive granules as in Fig. 2 is observed.

In pineal glands of rats 6 days after bilateral extirpation of the superior cervical ganglia fixed with osmium tetroxide and stained with uranyl acetate and lead citrate, the nerve endings containing granulated vesicles had disappeared.



2



3

Fig. 2. A nerve ending of the perivascular space of the pineal gland of the normal rat. If osmium tetroxide fixation is omitted in material processed with the glutaraldehyde-dichromate reaction, only dense reactive granules remain in the endings, corresponding to the dense core of the granulated vesicles. × 50,000

Fig. 3. The prefixation with formaldehyde, followed by glutaraldehyde-dichromate treatment, shows an essentially similar image in a pineal ending to that observed in Fig. 2. × 50,000

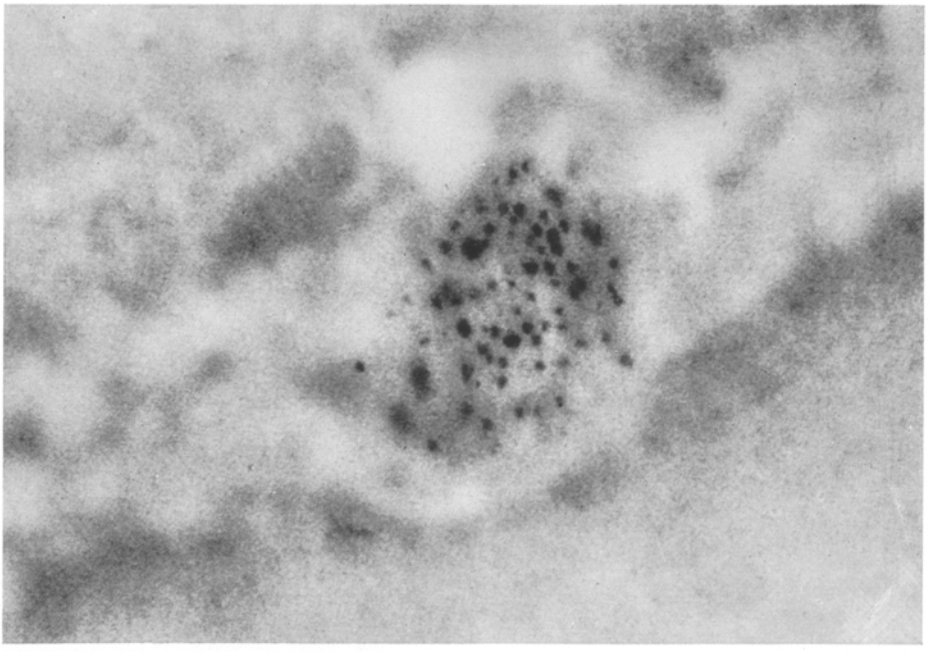


Fig. 4. A nerve ending located between adjacent smooth muscle cells of the inner layer of the rat vas deferens. Reactive granules of different diameters are observed within the endings. Glutaraldehyde-dichromate reaction. × 50,000

The lack of granular material reacting with both cytochemical techniques was noticed after ganglionectomy.

No reactive sites could be found in parenchymal cells with either cytochemical procedures.

In the vas deferens the GD technique demonstrates dense reactive granules within nerve fibers located between smooth muscle cells of the inner muscle layer (Fig. 4). They correspond to the adrenergic fibers of the vas deferens characterized by their content of numerous dense-cored vesicles. Most of the granules exhibiting a positive reaction are of 200—500 Å in diameter but occasionally larger reactive structures of 700—1,000 Å are present. After prefixation with formaldehyde and subsequent treatment with glutaraldehyde and dichromate, no reactive sites could be identified between muscle cells of the vas deferens.

Discussion

The observation with the GD technique of dense reactive granules in the nerve endings found in the perivascular space of the pineal gland, is indicative of the presence of a catechol- or an indoleamine. The observation of a similar reactive pattern after formaldehyde fixation, known to interfere with the reaction given by catecholamines, implies that the reactive granules of such endings contain an indoleamine. This assumption is supported by the fact that sympathetic endings of the vas deferens, containing exclusively NE, give a positive reaction

only with GD, but not with the FGD technique. The absence of reactive products following superior cervical gangliectomy with both cytochemical techniques, clearly indicates that such nerve endings containing reactive granules are of sympathetic origin (Ariëns Kappers, 1960).

With the present cytochemical approach, no reactive sites could be demonstrated in parenchymal cells in spite of results obtained with the fluorescence histochemical method indicating that they contain 5-HT (Bertler et al., 1963). Differences in 5-HT metabolism, known to proceed at different rates between neural and parenchymal compartments (Pellegrino de Iraldi et al., 1963) may result in a storage form not morphologically identifiable in pinealocytes.

Since the FGD technique reacts with indoleamines, the possibility that melatonin, an indole derivative concentrated in pineal gland (Lerner et al., 1958), could give a positive reaction should be considered. "In vitro" test tube tests have failed to demonstrate any precipitate of melatonin with the FGD procedure under conditions similar to those in which 5-HT gives a strong precipitate (Zieher and Jaim Etcheverry, unpublished observations).

The interpretation of Bertler et al. (1963) and Pellegrino de Iraldi, Zieher and De Robertis (1963) that rat pineal autonomic nerves are both adrenergic and tryptaminergic seems now fully confirmed. Strong evidences have been provided by results obtained from comparative studies on the action of drugs affecting monoamine metabolism in normal and denervated glands, using biochemical procedures for amine assay (Bertler et al., 1963; Pellegrino de Iraldi et al. 1963, 1965), electron microscopy (Pellegrino de Iraldi and De Robertis, 1961, 1963; Pellegrino de Iraldi et al., 1965) and the fluorescence histochemical method of Falck and Hillarp for the cellular demonstration of monoamines (Bertler et al., 1963, 1964; Owman, 1964a, b).

The subcellular localization of NE in granulated vesicles postulated by De Robertis and Pellegrino de Iraldi (1961a) has been demonstrated in pineal as well as in other autonomic nerves. On the contrary attempts to localize 5-HT stores in pineal nerves with electron microscopy have previously failed (Pellegrino de Iraldi et al., 1965; Bondareff and Gordon, 1966). Recent studies with p-chlorophenylalanine, a potent inhibitor of tryptophan hydroxylase, have succeeded in localizing 5-HT in granulated vesicles of pineal nerves by demonstrating a significant diminution of such vesicles together with a marked drop in pineal 5-HT content (Bloom and Giarman, 1967).

With the GD technique, followed by osmium tetroxide, virtually all vesicles present in pineal nerve endings exhibit a dense core. The absence of clear vesicles formerly described in such endings, may be explained on the basis of variations in the fixation procedures which are known to affect the amount of osmiophilic material present in the granulated vesicles (Richardson, 1966; Tranzer and Thoenen, 1967). Therefore, in pineal autonomic nerves, as well as in those of cat's iris (Tranzer and Thoenen, 1967), there is no justification to consider the presence of clear vesicles as a morphological evidence of the existence of a cholinergic link in postganglionic sympathetic transmission (Burn and Rand, 1965). Reactive sites of larger size were occasionally observed in the nerve endings of the pineal gland with both cytochemical reactions, and in the vas deferens with the GD technique. They presumably correspond to the few vesicles of the "intermediate type", according to the classification of Pellegrino de Iraldi and De Robertis (1964) observed in such endings, whose significance is still

in a controversial stage (BONDAREFF, 1965; BONDAREFF and GORDON, 1966; BLOOM and BARRNETT, 1966; HÖKFELT, 1966). This observation suggest that they may be also related to the process of amine storage.

The almost similar distribution of reactive granules with both cytochemical techniques in pineal nerves does not permit to differentiate the localization of 5-HT and NE in separated entities within the nerve terminal. Thus the possibility that both amines may be stored in the same organelle should be seriously considered. That an interchange of amines may occur in some systems (BERTLER et al., 1960) is supported by the fact that the NE incorporated "in vitro" to blood platelets is histochemically demonstrated in a storage site apparently identical to that normally containing 5-HT (JAIM ETCHEVERRY and ZIEHER, 1967). However, regional differences exist in amine pools that must be considered when attempting these generalizations.

The cytochemical approach used makes possible the recognition of 5-HT in pineal nerve fibers and endings. The amine is stored in granulated vesicles, morphologically similar and most probably identical, to those previously shown to contain NE in such endings. Combined pharmacological and cytochemical studies are being made to investigate the mechanisms of amine storage in different autonomic nerve endings.

References

ARIËNS KAPPERS, J.: The development, topographical relations and innervation of the epiphysis cerebri in the albino rat. Z. Zellforsch. **52**, 163—215 (1960).

BERTLER, Å., B. FALCK, and C. OWMAN: Cellular localization of 5-hydroxytryptamine in the rat pineal gland. Kungl. Fysiogr. Sällsk. Lund Förh. **33**, 13—16 (1963).

— — — Studies on 5-hydroxytryptamine stores in pineal gland of the rat. Acta physiol. scand. **63**, Suppl. 239 (1964).

— A. M. ROSENGREN, and E. ROSENGREN: In vivo uptake of dopamine and 5-hydroxytryptamine by adrenal medullary grains. Experientia (Basel) **16**, 418—419 (1960).

BLOOM, F. E., and R. J. BARRNETT: Fine structural localization of noradrenaline in vesicles of autonomic nerve endings. Nature (Lond.) **210**, 599—601 (1966).

—, and N. J. GIARMAN: Fine structure of granular vesicles in pineal autonomic nerve endings after serotonin depletion. Anat. Rec. **157**, 351 (1967).

BONDAREFF, W.: Submicroscopic morphology of granular vesicles in sympathetic nerves of rat pineal body. Z. Zellforsch. **67**, 211—218 (1965).

—, and B. GORDON: Submicroscopic localization of norepinephrine in sympathetic nerves of rat pineal. J. Pharmacol. exp. Ther. **133**, 42—47 (1966).

BURN, J. H., and M. J. RAND: Acetylcholine in adrenergic transmission. Ann. Rev. Pharmacol. **5**, 163—182 (1965).

CLEMENTI, F.: Modifications ultrastructurelles provoqués par quelques medicaments sur les terminaisons nerveuses adrenergiques et sur la médullaire surrénale. Experientia (Basel) **21**, 171—176 (1965).

COUPLAND, R., A. PYPER, and D. HOPWOOD: A method for differentiating between noradrenalin and adrenalin storing cells in the light and electron microscope. Nature (Lond.) **201**, 1240—1242 (1964).

DE ROBERTIS, E., and H. S. BENNETT: Submicroscopic vesicular component in synapses. Fed. Proc. **13**, 35 (1954).

—, and A. PELLEGRINO DE IRALDI: A plurivesicular component in adrenergic nerve endings. Anat. Rec. **139**, 299 (1961 a).

— — Plurivesicular secretory processes and nerve endings in the pineal gland of the rat. J. biophys. biochem. Cytol. **10**, 361—372 (1961 b).

HÖKFELT, T.: The effect of reserpine in the intraneuronal vesicles of the rat vas deferens. Experientia (Basel) **22**, 56 (1966).

— Ultrastructural studies on adrenergic nerve terminals in the albino rat iris after pharmacological and experimental treatment. Acta physiol. scand. **69**, 125—126 (1967).

Jaim Etcheverry, G., and L. M. Zieher: Cytochemistry of 5-hydroxytryptamine at the electron microscope level. I. Study of the specificity of the reaction in isolated blood platelets. J. Histochem. Cytochem., accepted for publication (1967).

Lerner, A. B., J. D. Case, Y. Takahashi, T. H. Lee, and W. Mori: Isolation of melatonin, the pineal gland factor that lightens melanocytes. J. Amer. chem. Soc. **80**, 2587 (1958).

Luft, J. H.: Improvements in epoxy resin embedding methods. J. biophys. biochem. Cytol. **9**, 409—414 (1961).

Orden, L. S. van, F. E. Bloom, R. J. Barrnett, and N. J. Giarman: Histochemical and functional relationships of catecholamines in adrenergic nerve endings. I. Participation of granular vesicles. J. Pharmacol. exp. Ther. **154**, 185—199 (1966).

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

— New aspects of the mammalian pineal gland. Acta physiol. scand. **63**, Suppl. 240 (1964).

Pearse, A. G. E.: Histochemistry. Theoretical and applied. London: J. & W. Churchill 1960.

Pellegrino de Iraldi, A., and E. De Robertis: Action of reserpine on the submicroscopic morphology of the pineal gland. Experientia (Basel) **17**, 122—123 (1961).

— — Action of reserpine, iproniazid and pyrogallol on nerve endings of the pineal gland. Int. J. Neuropharmacol. **2**, 231—239 (1963).

— — Ultrastructure and function of catecholamine containing systems. In: Proc. 2nd Internat. Congr. Endocrinology, London. Excerpta Med. Internat. Congr. Series No 83, 355—363 (1964).

— L. M. Zieher, and E. De Robertis: The 5-hydroxytryptamine content and synthesis of normal and denervated pineal gland. Life Sci. **1**, 691—696 (1963).

— — — Ultrastructure and pharmacological studies of nerve endings in the pineal gland. In: Structure and function of the Epiphysis cerebri (J. Ariëns Kappers and J. P. Schadé, eds.), Progress in brain research, vol. 10, p. 389—421. Amsterdam: Elsevier 1965.

Quay, W. B., and A. Halevy: Experimental modification of the rat pineal content of serotonin and related indolamines. Physiol. Zool. **35**, 1—7 (1962).

Richardson, K. C.: The fine structure of autonomic nerve endings in smooth muscle of the rat vas deferens. J. Anat. (Lond.) **96**, 427—442 (1962).

— The fine structure of the albino rabbit iris with special reference to the identification of adrenergic and cholinergic nerves and nerve endings in its intrinsic muscles. Amer. J. Anat. **114**, 173—206 (1964).

— Electron microscopic identification of autonomic nerve endings. Nature (Lond.) **210**, 756 (1966).

Tramezzani, J., S. Chiocchio, and G. Wassermann: A technique for the light and electron microscopic identification of adrenalin- and noradrenalin-storing cells. J. Histochem. Cytochem. **12**, 890—899 (1964).

Tranzer, J. P., and H. Thoenen: Significance of "empty vesicles" in post ganglionic sympathetic nerve terminals. Experientia (Basel) **23**, 123—124 (1967).

Wolfe, D. E., L. T. Potter, K. C. Richardson, and J. Axelrod: Localizing tritiated norepinephrine in sympathetic axons by electron microscopic autoradiography. Science **138**, 440—441 (1962).

Wood, J.: Electron microscopic localization of 5-hydroxytryptamine (5-HT). Tex. Rep. Biol. Med. **23**, 828—837 (1965).

— Electron microscopic localization of amines in central nervous tissue. Nature (Lond.) **209**, 1131—1133 (1966).

— Cytochemical localization of 5-hydroxytryptamine (5-HT) in the central nervous system (CNS). Anat. Rec. **157**, 343 (1967).

—, and R. J. Barrnett: Histochemical demonstration of norepinephrine at a fine structural level. J. Histochem. Cytochem. **12**, 197—209 (1964).

Dr. Guillermo Jaim Etcheverry
Instituto de Anatomía General y Embriologia
Facultad de Medicina
Paraguay 2155
Buenos Aires (Argentina)