HISTOCHEMISTRY OF NERVOUS TISSUES: CATECHOLAMINES AND CHOLINESTERASES[1,2,3]

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Histochemical methods have made it possible to explore the chemical mechanisms of nervous transmissions at the cellular level. The introduction by Koelle (130) of a reliable method for histochemical demonstration of cholinesterases greatly facilitated the study of the cholinergic mechanisms and led to a new concept of cholinergic transmission (133). Histochemical methods for the demonstration of catecholamines were improved only a few years ago to a degree permitting the study of these amines in the nervous tissue.

A large number of publications has accumulated on both subjects. Within the space allotted to this review it is impossible to refer to all of them, but the studies cited provide further references, and these virtually cover the whole field. A special effort has been made to give fair balance to the recent observations, which are truly stimulating and give promise of a rapid further development.

Catecholamines

Light microscopic demonstration.—Eränkö (55, 57) observed about ten years ago that following the exposure of sections of the adrenal medulla to formaldehyde solution, a fluorescence developed in some cells, which were shown with specific chemical methods to be norepinephrine-containing. Since it was also found that norepinephrine forms a fluorescent compound with formaldehyde, treatment with this compound was proposed to be a histochemical method for the demonstration of norepinephrine. Although the fluorescence was stronger in sections dried after exposure to formaldehyde (58), the method was not sufficiently sensitive for localizing catecholamines in adrenergic nerve fibers. However, it was later observed that a very intense fluorescence appeared when monoamines in freeze-dried tissues were exposed to formaldehyde vapor (64, 86, 140). A successful modification based on this principle was described in the publications of Falck, Hillarp and associates (80, 82). In this connection it is of interest to note that the adrenergic nerve net of the iris can be equally well demonstrated by treatment in a solution of formaldehyde, made in Ringer's fluid so as to avoid osmotic damage, as by exposure to formaldehyde vapor (74).

[1] The survey of the literature pertaining to this review was concluded in June 1966.

[2] The following abbreviations will be used: 5-HT (5-hydroxytryptamine); ACh (acetylcholine).

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Reaction mechanism and specificity.—Corrodi and Hillarp observed that DOPAmine and formaldehyde condense with the formation of 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, which is then dehydrogenated into an intensely fluorescent compound, 6,7-dihydroxy-3,4-dihydroisoquinoline (27, 28). Dihydroxyphenylalanine, norepinephrine, and epinephrine are similarly converted into fluorescent compounds. 5-Hydroxytryptamine (5-HT) condenses into 6-hydroxy-3,4-dihydro-β-carboline, which is also intensely fluorescent (31).

Disappearance of the formaldehyde-induced fluorescence after treatment with sodium borohydride (29), which reduces the dihydroisoquinolines back into nonfluorescent tetrahydroisoquinolines, is highly suggestive of monoamines, especially if the fluorescence can be regenerated by repeated exposure to formaldehyde. However, specific chemical analysis is always valuable, and should be carried out if there is any doubt of the nature of the fluorogenic substance.

Discrimination between amines.—Isoquinoline derivatives from catecholamines generate a green fluorescence with an emission maximum at 4800 Å, while the fluorescent compound obtained from 5-HT is yellow with an emission maximum at 5300 Å (31). These spectral differences make it possible to discriminate between catecholamines and 5-HT.

Epinephrine, being a secondary amine, is more slowly condensed with formaldehyde than DOPAmine or norepinephrine and can thus be discriminated from these (61, 80). Corrodi & Jonsson (30) found that the hydroxy-group at the position 4 in the 3,4-dihydroisoquinoline derivative from norepinephrine can be split off by treatment with thionyl chloride with formation of the fully aromatic 6,7-dihydroxyisoquinoline. This compound retains its fluorescence after treatment with sodium borohydride, in contrast to 6,7-dihydroxy-3,4-dihydroisoquinoline formed by formaldehyde condensation from DOPAmine, which is reduced by thionyl chloride into nonfluorescent 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (30).

Practical procedure.—In practice, the method is simple to perform but all the steps are somewhat critical. Methodological aspects have recently been dealt with in several papers (40, 61, 80, 82, 84, 107, 125). Four steps are involved: (*a*) freeze-drying, (*b*) exposure to formaldehyde, (*c*) embedding, sectioning, and mounting, and (*d*) fluorescence microscopy.

Freezing must be rapid to avoid ice-crystal artifacts (56, 164). It is usually carried out by immersion in isopentane cooled with liquid nitrogen (164), but metal surfaces precooled in liquid nitrogen provide more rapid cooling (56, 116). Drying must occur at a temperature below −35° C, otherwise diffusion of the amine to be demonstrated destroys the localization [for methods see (164, 188)].

The tissues have usually been embedded in paraffin wax but sections embedded in an epoxy resin can be cut thinner, which improves the localizing power of the method (119).

Formaldehyde vapor is generated from paraformaldehyde powder, whose

water content must first be standardized by storage in a desiccator over sulfuric acid to create an atmosphere of suitable relative humidity (114). Besides the relative humidity, the temperature and the length of exposure also influence the reaction; a rule of thumb is that the sum of humidity and temperature (e.g., 70 per cent plus 40° C) must be approximately 110 (63).

A good microscope lamp with a high-pressure mercury burner such as HBO 200 is essential for fluorescence microscopy but, against a widespread misconception, quartz optics are not necessary or even desirable.

Amine concentration and fluorescence intensity.—While the intensity of the fluorescence induced by treatment with formaldehyde solution bears a linear relationship with the catecholamine concentration (71), recent observations indicate that the same does not apply to the intensity of the fluorescence induced by formaldehyde vapor, the intensity remaining the same or even decreasing above certain amine concentrations (172).

Neuronal distribution.—Distribution of the amines has been extensively studied with this method in the different parts of the aminergic neurons. As could be expected from earlier chemical observations by Von Euler [see (77)], the nerve cell bodies have been found to exhibit a moderate fluorescence, a weak fluorescence is seen in the preterminal axons, while the terminal nerve fibers and, especially, the synapses exhibit an intense fluorescence [e.g. (40, 149, 158)].

Sympathetic ganglia.—A diffuse fluorescence of variable intensity was observed by Eränkö & Härkönen (65) in the cytoplasm of all ganglion cells of the superior cervical ganglion of the rat. However, brilliantly fluorescent small granules were observed in the cytoplasm of many cells and in the fibers between the cells. The observations were understood to suggest that, in addition to a diffuse soluble pool, there is a pool of catecholamines concentrated in granules, which migrate from the perikaryon to the axon. This view has been strongly opposed by Norberg & Hamberger (158) who maintain that the cytoplasmic amine fluorescence is always diffuse; the granules, if any, cannot be resolved by light microscopy. The matter should be further examined, possibly by the conjoint use of fluorescence and electron microscopes.

Norberg & Hamberger (108, 112, 157) made the highly interesting observation that there are large numbers of intensely fluorescent synapses in many sympathetic ganglia, apparently in direct axosomatic contact with fluorescent ganglion cell bodies. Because neither preganglionic nor postganglionic nerve division had any effect on these terminals, they were concluded to originate from cell bodies present in the same ganglion, either from intraganglionic interneurons or from collaterals of the postganglionic fibers (108). The former alternative is especially attractive in view of the previously observed adrenergic inhibition of synaptic transmission in the sympathetic ganglia (32).

Small cells which exhibit a much more intense fluorescence than ganglion cells were first reported by Eränkö & Härkönen (65, 68) in the superior cervical ganglion of the rat. Since the cells were nonchromaffin, looked electron

microscopically like adrenal medullary cells, and their fluorescence appeared yellow, it was tentatively concluded that they perhaps contained 5-HT. It now seems more likely that DOPAmine is responsible, because the fluorescence spectrum of these cells is typical of a catecholamine (173). Similar small, intensely fluorescent cells have also been reported in other ganglia and nerve bundles (158, 162, 183).

Peripheral innervation apparatus.—As described first in Falck's (80) paper, the peripheral end of the sympathetic neuron has been observed to consist of a fine, fluorescent nerve fiber which ramifies and forms, with branches of other fibers, a network of terminal axons. Along the 0.2 μ thick terminal axons there are at regular intervals varicosities, 0.5–1 μ thick and 1–2 μ long, which exhibit an intense formaldehyde-induced fluorescence. Several varicosities are seen along each fiber, and they seem to be synapses of the "boutons de passage" type. The amine histochemistry of the peripheral neurons has recently been reviewed by Falck & Owman (83). The iris has been a favorite organ for studying the peripheral nerve net, because the amine fluorescence of its nerve fibers can be easily studied in fresh stretch preparations. Adrenergic terminals have been repeatedly found not only in the dilator (87) but also in the sphincter muscle (149).

Ramification of peripheral axons.—Crushing the superior cervical ganglion with tweezers, so as to leave but a few ganglion cells intact, has been ingeniously performed by Malmfors & Sachs (150) to study the mode of ramification of the adrenergic fibers in the peripheral innervation apparatus of the iris. It was reported that a single adrenergic preterminal axon may ramify and send richly branched terminal fibers into (*a*) the ciliary body, (*b*) an arteriole, (*c*) the dilator, and (*d*) the sphincter muscles of the iris. This is a most interesting observation, but it cannot be quite excluded that what appears in the light microscope as a single axon, may in fact be a group of axons running closely together but each separately innervating one of the four tissues. Electron microscopic examination of the preterminal fiber after fluorescence microscopy could settle this problem.

Innervation of different organs.—An extensive study on the iris has been published by Malmfors (149), and other ocular and orbital structures have been reviewed by Ehinger (51). The presence of DOPAminergic neurons and fibers in the inner plexiform layer of the retina (52, 104) is of special interest.

Both the atrial and the ventricular muscles of the heart and, especially, its atrioventricular node are supplied with adrenergic networks (7, 8, 81). On the other hand, the adrenergic fibers of the skeletal muscle are limited to blood vessels (95). Serous acini of the submandibular and parotid glands are richly innervated by adrenergic fibers, while the mucous acini of the sublingual gland contain none (4, 159).

The pineal body has been thoroughly studied by Owman and co-workers (14, 105, 160, 161). The pineal parenchymal cells contain histochemically demonstrable 5-HT, and they are innervated by noradrenergic fibers originating from the superior cervical ganglion. Curiously, these fibers contain not

only norepinephrine but also 5-HT, apparently taken up from the parenchymal cells.

A rich adrenergic innervation has been found in almost all organs which contain smooth muscle: the iris (see above); the arteries, the arterioles, and the veins (95), which are especially densely innervated in the erectile tissues of the nasal mucosa (43) and the penis (70, 166); hair arrector muscles (85, 93); the nictitating membrane (125); the bronchial muscle (45); the trabecular network of the spleen (47, 99); the vas deferens, the seminal vesicle, and the prostate (125, 162, 183); as well as the oviduct and the uterus (17–19, 125).

However, the smooth muscle is not everywhere supplied with monoamine-containing fibers. Norberg (156) observed that the adrenergic postganglionic fibers of the intestine form terminal networks around the cholinergic cell bodies of the intramural ganglia only; the smooth muscle is innervated exclusively by cholinergic terminals originating from these cells.

Hamberger & Norberg (109) found intramural ganglia also in the bladder, in which some of the ganglion cell bodies were catecholamine-containing and the others nonfluorescent but surrounded by an adrenergic nerve net. Only in the trigone area was the smooth muscle supplied with adrenergic nerve fibers; elsewhere in the bladder muscle adrenergic nerves were found in the blood vessels only.

The innervation of the vas deferens, the seminal vesicle, and the prostate likewise originates from a multitude of small peripheral ganglia scattered around the internal genitals, as was observed by Owman & Sjöstrand (162) and by Sjöstrand (183). These ganglia supply the genitals with a dense adrenergic nerve net and their catecholamine content is therefore unaffected by hypogastric denervation. In the vas deferens, the seminal vesicles, and the prostate, the same authors (162, 183) found clusters of small, intensely fluorescent cells, apparently chromaffin cells which contained epinephrine. Chromaffin cells were earlier described in the hypogastric nerves by Vanov & Vogt (191), and their presence explains many apparently anomalous results obtained in depletion experiments.

Central aminergic neurons.—The pioneer work by Vogt (192) and Amin, Crawford & Gaddum (1) showed that monoamines are present in the central nervous system; it was then of obvious interest to investigate their microscopic distribution in the brain. Fine varicose fibers showing an intense fluorescence were indeed found already in the first histochemical studies of the central nervous system by Carlsson et al. (25, 26), while fluorescent nerve cell bodies were not observed. Subsequent extensive studies, notably by Andén, Dahlström, and Fuxe, who have published detailed data, have indicated that the cell bodies of the monoamine-containing neurons are almost exclusively localized in the brain stem, from which originate the ascending aminergic pathways to the higher brain nuclei and the descending pathways to the spinal cord (2, 3, 40, 42, 90, 91). For details, the original papers should be consulted.

Several large neuron systems have been described using the fluorescence method. Thus, norepinephrine- and 5-HT-containing terminals have been found in the spinal cord and traced to definite cell groups in the medulla oblongata (42). The 5-HT-containing terminals surrounding the preganglionic sympathetic cells in the lateral horn of the spinal cord, which innervate the adrenal medulla, have been proposed to have an inhibitory function (2). Further, two large ascending neuron systems have been reported, one of which is composed of DOPAmine-containing neurons originating in the substantia nigra and supplying the neostriatum with a vast number of terminals (3). The other, which is mainly noradrenergic, forms extensive ipsilateral mesencephalo-hypothalamic, mesencephalo-limbic, and mesencephalocortical pathways (40).

Median eminence is the only area above the mesencephalon whose fluorescent terminals are not affected by ipsilateral transection of the crus cerebri and the medial forebrain bundle; its fibers have been traced to tuberoinfundibular cell bodies (90).

In the area postrema, long known for its particularly high content of norepinephrine and 5-HT (1, 192), both green and yellow fluorescent nerve cell bodies and green fibers were found, thus providing the explanation for the presence of both amines (94).

Neurons of nonvertebrates.—Monoamine-containing neurons have been described not only in vertebrates but also in other animals, including quite primitive ones. Thus, numerous catecholamine-containing neurons were found in the cockroach brain by Frontali & Norberg (89). Adrenergic sensory cells in mollusks, turbellaria, earthworms, and leeches were observed by Dahl et al. (37), and adrenergic neurons with a combined sensory and motor function were reported in sea anemones by the same authors (38).

Nerve division and axon reaction.—Several studies have shown that division or compression of a peripheral or central adrenergic axon results in rapid accumulation of fluorogenic amines in the proximal side of the lesion and disappearance of the fluorescence from the peripheral part of the neuron, including the nerve terminals (16, 39, 41, 42, 67, 115, 128). The accumulation of amines in the proximal part of the neuron has indeed made it possible to make intensely fluorescent such central aminergic fibers both in the central (42) and the peripheral (41) nervous system, which are not normally demonstrable because of the low amine concentration in the nonterminal axon. It is of interest that some fluorescent material may accumulate also at the peripheral side of the lesion, which suggests the presence of ascending adrenergic fibers in peripheral nerves (39).

While accumulation of amines in the proximal stump has always been observed after transection of the axon, such operation has different effects on the fluorescence of the cell body from which it originates, depending on the site of the division. Härkönen (115) reported that division of the postganglionic nerves near the ganglion causes a complete loss of amine fluorescence from all the ganglion cells of the superior cervical ganglion. On the

other hand, Owman (160) found after pinealectomy an increase in the fluorescence intensity of some cells in the same ganglion. Dahlström & Fuxe (42) reported that the transection of aminergic pathways in the spinal cord likewise resulted in a clear retrograde increase of the fluorescence in the corresponding nerve cell bodies; this phenomenon can be used to study the spinal connections of the different brain stem nuclei. The differences in the axon reaction probably depend on the distance of the lesion from the cell body.

Electrical stimulation.—Malmfors observed (149) but a slight decrease in the fluorescence of the varicosities of the iris nerve net after stimulation of the cervical sympathetic trunk of normal rats. After administration of amine synthesis inhibitors such as H 22/54 and stimulation as above, the fluorescence of most terminals disappeared. On the other hand, Dahlström et al. (46) reported a marked decrease in the fluorescence of the norepinephrine and 5-HT stores of the varicose terminals in the spinal cord after stimulation of the medulla oblongata in normal animals. Obviously, the length and the mode of stimulation affect the degree of depletion.

Effects of drugs.—Authoritative reviews have been published recently on this subject (23, 25, 40). After total depletion by reserpine, the catecholamine fluorescence rapidly reappears in the cell bodies of both central and peripheral adrenergic neurons; a distinct fluorescence is demonstrable around the cell nucleus a few hours after the injection, and the fluorescence is very intense in the whole perikaryon a day later (40, 44, 158). However, the amines in the terminals become demonstrable only after several days. This effect of reserpine has been explained by long-lasting inhibition of the granular uptake of amines; this prevents recovery in the terminals, while the rapid reappearance of amines in the cell body is supposed to be due to formation of new granules in it (44). This is also the main evidence presented for the view that all neuronal catecholamines are bound in granules (23). Electron microscopic studies on the perikaryon are needed before this view can be finally accepted. After depletion with tetrabenazine, the recovery of the amine fluorescence is rapid both in the cell body and in the terminal, presumably because this drug blocks the granular uptake mechanism only for a short time.

All parts of all catecholamine-containing neurons recover their catecholamine content quickly after displacement of the endogenous amines by *m*-tyrosine (40, 44). After administration of α-methyl-*m*-tyrosine, the recovery in the terminals of some neurons is rapid, in those of others slow. The slowly recovering cells have been concluded to be norepinephrine neurons, in which α-methyl-*m*-tyrosine is converted into metaraminol, which for a long time blocks the norepinephrine uptake. DOPAmine neurons recover rapidly because such oxidation does not occur. This differential response to α-methyl-*m*-tyrosine has been used to discriminate histochemically between noradrenergic and DOPAminergic neurons in the central nervous system (25, 40, 91). After administration of α-methyl-dihydroxyphenylalanine, reserpine fails to

produce disappearance of amine fluorescence, presumably because α-methylnorepinephrine, which gives the fluorescence reaction, replaces endogenous norepinephrine and is resistant to reserpine (24).

Amine uptake in vivo.—Hillarp & Malmfors (118, 149) demonstrated that norepinephrine injected into the lingual vein or directly in the eye is rapidly taken up by the preterminal and terminal nerve fibers of the iris. This uptake through the axon membrane resulted in an increased fluorescence along the whole axon. It was inhibited by cocaine but not by reserpine and it was very transient unless monoaminoxidase had been inhibited by nialamide. The slower uptake in the synaptic varicosities is apparently due to active uptake by granular vesicles, which is inhibited by reserpine.

Nonterminal axons, normally difficult to demonstrate by fluorescence microscopy, can be made fluorescent by increasing experimentally their amine content (23, 25, 40, 91, 149, 158). Interesting differences have been found in the amine uptake between catecholamine- and 5-HT-containing neurons. The latter have been found to become intensely fluorescent throughout after treatment with a monoaminoxidase inhibitor alone, both in normal and in reserpinized animals. Because of the blood-brain barrier, administration of both dihydroxyphenylalanine and monoaminoxidase inhibitor fails to cause such fluorescence in the central DOPAmine or norepinephrine neurons of reserpinized animals except for the area postrema, where such a barrier is lacking (92). It is therefore possible to demonstrate the 5-HT neurons selectively by first depleting all amines with reserpine and subsequently administering nialamide, which restitutes the 5-HT neurons (40).

Amine uptake in vitro.—Angelakos & King (5, 9) were the first to study histochemically the amine uptake of the adrenergic tissues *in vitro*. They incubated isolated preparations of rat iris in Tyrode's solution with DOPAmine, norepinephrine, or epinephrine. In each case, a distinct increase was observed in the catecholamine fluorescence of the nerve fibers. The presence of adenosine mono-, di-, or triphosphate was necessary but not the presence of glucose.

Experiments by Eränkö & Räisänen (73) furnished somewhat different results: incubation with norepinephrine in Ringer's solution caused but a slight increase in the intensity of the formaldehyde-induced fluorescence of the iris, whether adenosine triphosphate was present or not. On the other hand, incubation with tyramine, DOPAmine, epinephrine, or 5-HT caused a marked decrease in the amine fluorescence, which was readily restituted to normal by subsequent incubation in 1 μg/ml of norepinephrine. This restitution occurred in the absence of adenosine triphosphate, but the presence of glucose was essential. In accordance with our incubation experiments, Angelakos et al. (6) observed a drastic depletion of norepinephrine in an isolated atrium of the guinea pig heart after perfusion with epinephrine.

Since injected catecholamines do not penetrate the blood-brain barrier, except for certain brain regions such as the area postrema (92, 94), it is usually not possible to study the amine uptake of the central adrenergic

neurons of living animals. Hamberger & Masuoka (113) have incubated brain slices with monoamines and observed such an uptake *in vitro*. After an experimental lesion of the blood-brain barrier, amine uptake by the brain cells has been histochemically demonstrated in living animals (106).

Monoamines and electron microscopic granules.—The pioneer studies by Von Euler and associates (78, 79) clearly showed more than ten years ago that catecholamines are highly concentrated in granules prepared from homogenates of nervous tissue. Subsequently, numerous electron microscopic studies on sympathetic synapses revealed, in addition to the small "empty" vesicles which are normally present in the synapses, other vesicles containing an electron-dense core (49, 54, 103, 143, 167, 186, 187). Several observations indicate that such granular vesicles are carriers of synaptic monoamines: (*a*) fixatives and stains employed in electron microscopy form electron-dense compounds with catecholamines (194); (*b*) the granular vesicles are found in regions rich in aminergic nerve fibers (49, 102, 143, 169, 170, 177, 194); (*c*) sympathetic denervation is associated with a loss of both amines and granular vesicles (48, 102, 165); (*d*) amine depletion after administration of drugs is accompanied by a loss of the granular vesicles (36, 48, 152, 165, 176); (*e*) monoaminoxidase inhibitors which prevent such depletion also prevent the loss of granular vesicles (36, 48); (*f*) after constriction of an adrenergic nerve, both catecholamines and granular vesicles accumulate above the lesion (16, 127); (*g*) administration of amines increases the number of granular vesicles (165); and (*h*) catecholamines are taken up by the granular vesicles in the sympathetic nerves, as was elegantly shown by Wolfe et al. (195), using autoradiographic localization of tritiated norepinephrine at the electron microscopic level.

Other pools of amines.—Several other observations suggest that the granular vesicles may not be the only ultrastructural storage element of transmitter amines: (*a*) granular vesicles have not been found in all such synapses which are aminergic (120, 155); (*b*) there are always agranular vesicles in synapses which contain granular ones [e.g. (48, 49, 54, 102, 143, 167, 169, 170, 186)]; (*c*) efforts to isolate pure fractions of granular vesicles have been unsuccessful, and vesicle fractions with a high norepinephrine content have but a low percentage of granular vesicles (168, 194); (*d*) increase in norepinephrine concentration due to administration of monominoxidase inhibitors is not always accompanied by an increase in the number of granular vesicles (36); (*e*) the proportion of granular vesicles does not increase when such fractions are induced to take up norepinephrine *in vitro* (194); (*f*) vesicles obtained from catecholamine-poor regions of the brain may be shown to possess dense cores when suitably prepared (193, 194); and (*g*) the number of granular vesicles is too small in the intersynaptic parts of terminal nerve fibers and in the perikaryons of the adrenergic neurons to account for their intense formaldehyde-induced fluorescence (142).

These objections certainly deserve further examination. However, many failures to demonstrate granular vesicles may be due to inadequate tech-

niques. This has been emphasized by two successful investigators, De Robertis (48, 101) who considers fixation by perfusion necessary in studying central synapses, and Richardson (171) who has recently found that capriciousness in demonstrating the granular vesicles can be avoided by using 3 per cent potassium permanganate as a fixative. Even these authors have always found the smaller "empty" vesicles with the granular ones.

New histochemical amine methods.—Several combination methods have recently been proposed suitable for the histochemical demonstration of catecholamines at the ultrastructural level: (*a*) formaldehyde and ammoniacal silver (60); (*b*) formaldehyde and osmium tetroxide (13, 48, 101); (*c*) glutaraldehyde and potassium dichromate (15, 196); (*d*) glutaraldehyde and osmium tetroxide (35); (*e*) glutaraldehyde and ammoniacal silver (190). The methods have provided satisfactory results with the adrenal medulla, but most of them still await application to nervous structures. However, Bloom & Barrnett (15) and Wood (196) have recently reported promising results with glutaraldehyde and potassium dichromate in demonstrating the dense-core vesicles in adrenergic nerve endings. Preliminary experiments with formaldehyde vapor fixation for electron microscopy suggest that diffusible pools of catecholamines are easily lost by fixation in aqueous solutions (75).

To conclude, the evidence now available indicates that granular vesicles are an important but probably not the only structure for monoamine storage. It is of interest that Kopin (137) has presented a hypothetical model of a nerve ending which incorporates, in addition to granules, a membranous structure, which is necessary to explain some biochemical and pharmacological observations. Finally, Von Euler (78) has emphasized the potential importance of reversible binding of amines by membrane phospholipids.

Cholinesterases

Development of methods.—In 1949, Koelle & Friedenwald (134) described a method for histochemical demonstration of cholinesterase activity in tissue sections. The method was subsequently improved by Koelle (130) to eliminate diffusion artifacts, and the new method was used to study the distribution of cholinesterases in peripheral (132) and central (131) nervous structures. The method has since then been extensively used, as such or in a slightly modified form. Acetyl- and butyrylthiocholine serve as substrates in the original method and its many modifications. Since copper and sulfate ions are present in the incubation medium, enzymatically liberated thiocholine is precipitated as copper thiocholine sulfate, a colorless, poorly soluble compound, whose distribution can be examined as such by phase contrast microscopy, as proposed by Holmstedt (121), or after treatment with sulfide solution to render the precipitate brown (130).

Discrimination between esterases.—Specific inhibitors play an important role in histochemical esterase techniques. Acetylthiocholine is readily split not only by acetylcholinesterase but also by nonspecific cholinesterase, and the latter must be inhibited by a selective inhibitor. The specificity problem

and the use of the inhibitors have recently been discussed by several authors (50, 100, 122, 133, 135, 164). Substrates other than thiocholine esters have also been used (20, 69, 115, 123, 136, 164).

Acetylcholinesterase as a marker of cholinergic neurons.—Acetylcholinesterase has been found to be a very consistent enzyme in being present selectively in nervous structures and specifically in the cell bodies, axons, and terminals of all cholinergic neurons, while nonspecific cholinesterase is present predominantly in glial cells, and in some neurons and in non-nervous structures as well (115, 133, 189). There is a fairly good correlation between acetylcholinesterase activity, cholinacetylase activity, and acetylcholine (ACh) content in the brain, as was shown already by Feldberg & Vogt (88) and recently by Lewis, Shute & Silver (148) [see also (117, 133, 154)]. However, Shute & Lewis (180, 181) have emphasized that the cell bodies of many neurons exhibit acetylcholinesterase activity, although their axons are devoid of cholinesterase and cholinacetylase activity, which proves them noncholinergic; the presence of acetylcholinesterase on the axon membrane is the essential histochemical criterion of a cholinergic neuron (146). The best criterion of a cholinergic neuron is of course the presence of high concentrations of ACh in its terminal. In view of Whittaker's (194) recent electron microscopic observations on ACh-containing synaptic vesicles, histochemical demonstration of ACh does not seem impossible. For the time being, an intense acetylcholinesterase activity can nevertheless be considered a tentative suggestion of the cholinergic nature of a neuron (125).

Axon reaction.—After division of the axon, acetylcholinesterase activity of the associated cell body decreases at the same time that the activity in the proximal stump of the divided nerve increases (115, 145, 185). Härkönen (115) examined the superior cervical ganglion of the rat after division of the postganglionic nerves near the ganglion. This resulted in an almost complete disappearance of the acetylcholinesterase activity, not only from the ganglion cells but also from the preganglionic fibers and synapses. Less marked changes in the activities of other enzymes and a complete restitution indicated that the changes were not due to interference with the blood supply of the ganglion. A distinct decrease in the acetylcholinesterase activity was seen in the ventral horn cells of the spinal cord after division of the sciatic nerve by Söderholm (185) and a similar decrease in the hypoglossal nucleus was observed after division of the hypoglossal nerve by Lewis & Shute (145). Use has been made of the piling up of acetylcholinesterase activity in the severed axons to trace their origin in the central nervous system (180).

Cholinergic pathways in the brain.—Previous work on the brain with the cholinesterase method has been reviewed in several articles (131, 133, 138, 180, 181, 185). Therefore, only some newer ones are mentioned here. Krnjevic & Silver (138, 139) carried out a thorough study on the distribution of acetylcholinesterase in the cerebral cortex of the cat. A previously unknown tangential system of fine fibers was observed, the terminal network of which was closely related to deep pyramidal cells. Since many of these cells are readily

excitable with ACh, it was concluded that the acetylcholinesterase-containing tangential system provided cholinergic innervation for these cells. Many fibers of this system probably belong to medial and lateral projections from the basal corpus striatum and the septal region. This again has been suggested by Shute & Lewis (179) to be the forebrain extension of the midbrain reticular formation. Thus, the tangential cortical system of acetylcholinesterase-positive fibers may be a final link in the reticular ascending, activating pathway, which is fully consistent with the pharmacology of cortical arousal.

Distribution of acetylcholinesterase in the hippocampal region of the rat was studied by Mathisen & Blackstad (151) and by Shute & Lewis (181) who, in another paper (180), studied the cholinesterase-containing pathways of the hindbrain. In all these studies, use was made of experimental lesions to control the validity of the histochemical observations.

Blood-brain barrier and cholinesterases.—In many species, the brain capillaries exhibit an intense nonspecific cholinesterase activity. Joo & Csillik (126) have recently observed that cholinesterase activity is lacking from such areas of the rat brain, e.g., the area postrema, from which the blood-brain barrier is known to be absent. It was concluded that the barrier function is correlated to the cholinesterase activity, which was electron microscopically localized in pinocytotic vesicles of the vascular endothelium in the barrier-protected brain areas.

Peripheral acetylcholinesterase-positive structures.—Earlier studies have been admirably described in a recent review (133). Other reviews include a symposium publication (175) and three monographs, one by Gerebtzoff (97) on cholinesterases and two others by Zacks (197) and Csillik (33) on the muscle end plate. Cholinesterases have also been thoroughly examined in recent histochemical papers on the retina (76), the superior cervical ganglion (115), and the spinal cord (185). Some other recent observations are mentioned here.

Biscoe & Silver (11) studied the cat carotid body and found both acetylcholinesterase and nonspecific cholinesterase in nerve fibers around the glomus cells, which themselves were nonreactive. Division of the preganglionic trunk to the superior cervical ganglion or of the sinus nerve had no effect on these fibers, but cutting of the postganglionic nerve resulted in their disappearance. The observations strongly suggest cholinergic sympathetic innervation of the glomus cells, and electron microscopic observations have indeed demonstrated typical synapses on them (12). Fibers exhibiting both types of cholinesterase activity were reported by Palkama (163) also in the carotid body of the rabbit.

Large variations in the acetylcholinesterase activity of individual cell bodies in the spinal ganglion of the rat were observed by Kokko (136). She quantitated by microscopic cytophotometry the acetylcholinesterase activity and compared it with the activity of some other enzymes in the same cells by examination of the neighboring sections. A significant positive correlation was thus found between the acetylcholinesterase and acid phosphatase activities in individual cells. Both of these enzymes were negatively

correlated to the cell size. Giacobini (98) determined quantitatively the cholinesterase activity of individual spinal ganglion cells which were separated by microdissection. He reported two populations of cells, one exhibiting an intense, the other a weak, cholinesterase activity. The former was considered truly cholinergic.

Electron microscopic methods.—While Koelle's (130) thiocholine method is admirable for light microscopic demonstration of cholinesterases, its localizing power is insufficient for electron microscopic studies. Barrnett (10) proposed thiolacetic acid for this purpose, with satisfactory results as far as accurate localization was concerned, but lack of specificity was admitted.

Recently, several successful modifications have been reported in which the more specific substrate acetylthiocholine has been employed for electron microscopy. Lewis & Shute (144, 147) described an incubation medium containing, like the Koelle (130) method, copper sulfate for capturing the liberated thiocholine. Karnovsky & Roots (129) incorporated ferricyanide into the substrate mixture; it is reduced by liberated thiocholine, and the enzymatic activity is revealed by a fine precipitate of brown copper ferrocyanide. Koelle & Gromadzki (135) have recently proposed aurous gold as a capturing ion, instead of copper, to obtain a fine precipitate with either thiocholine or thiolacetate, and the preliminary results have been promising.

Electron microscopic observations.—Relatively little work has as yet been done with the electron microscopic cholinesterase methods. However, there is a fair agreement between the results obtained with the different methods. Thus, in the muscle end plate, the acetylcholinesterase activity has been localized in the axon and muscle plasma membranes within the synaptic region by Barrnett (10) with thiolacetic acid and by Lewis & Shute (144) with acetylthiocholine. Pre- and postsynaptic membranes, axon membranes, and the endoplasmic reticulum of the perikaryons of the cholinergic neurons are likewise positive with both substrates, as was observed in the brain of the rat (153, 189), the cockroach (184), and the wood ant (141) with the thiolacetic acid method, and in the rat brain with the thiocholine method (144) and its copper ferrocyanide modification (178). This modification has also been observed to stain the axon membranes of all nonmyelinated fibers in the sciatic nerve (174) and in the salivary gland (96).

The synaptic vesicles have been reported to give a positive reaction toward thiolacetic acid in the muscle end plate (10) and the nervous synapses (141, 153, 181, 184), but not toward acetylthiocholine either in the muscle end plate (144) or in the nervous synapses (178, 181). However, vesicles exhibiting a positive acetylcholinesterase reaction with the copper ferrocyanide modification of the thiocholine method have been observed inside the sciatic axons (174).

Correlation of Catecholamines and Cholinesterases

Sympathetic ganglia.—Early studies on the acetylcholinesterase activity in the sympathetic ganglia revealed marked differences in the intensity of individual ganglion cells, which ranged from strong to weak [see (133)]. The

strongly positive ones were proposed by Koelle (132) to be cholinergic sympathetic cells, and confirmatory evidence of this assumption in the cat was presented by Sjöqvist (182). However, in the ganglia of many other species the majority of cells, necessarily including many adrenergic cones, exhibit a moderate or intense acetylcholinesterase activity (132). The problem became especially interesting when Burn & Rand (21, 22) presented the hypothesis that the release of norepinephrine from adrenergic fibers is mediated by ACh. When subsequent work showed that also the catecholamine fluorescence of individual cells of sympathetic ganglia is very variable (65, 110), it was of obvious interest to correlate the acetylcholinesterase activity and the amine content in individual ganglion cells.

Modification of an earlier method by Eränkö (59) proved successful for this purpose: a short low-temperature exposure to formaldehyde vapor of frozen-dried ganglia made norepinephrine sufficiently fluorescent for photomicrography, while the acetylcholinesterase activity was preserved and subsequently demonstrated in the same section (61, 62). Jacobowitz & Koelle (124, 125) independently presented a similar method, applied on cryostat sections of fresh tissue. Hamberger & Norberg (107) proposed the use of two neighboring cryostat sections for the same purpose.

In several sympathetic ganglia of the cat, Hamberger, Norberg & Sjöqvist (110, 111) observed that the majority of cells exhibited a weak acetylcholinesterase reaction but an intense catecholamine fluorescence, while the minority showed a weak fluorescence but an intense acetylcholinesterase activity. The results were concluded to indicate the presence of distinct adrenergic and cholinergic neurons in the ganglia. Entirely different results were obtained by Eränkö & Härkönen (68) in the superior cervical ganglion of the rat: weak, moderate, or strong norepinephrine fluorescence of individual ganglion cells was indiscriminately associated with weak, moderate, or strong acetylcholinesterase activity. The high amine content and the intense acetylcholinesterase activity in some of the cells were taken to suggest that these cells are at the same time both adrenergic and cholinergic, which fits in well with the Burn & Rand (21, 22) hypothesis. However, the cytoplasmic cholinesterase of these cells may be taken to reflect their cholinoceptive rather than cholinergic nature (181). Furthermore, it is of interest, although somewhat confusing, that nonspecific cholinesterase has been shown by Härkönen (115) to be inversely correlated with the intensity of the catecholamine fluorescence in the superior cervical ganglion of the rat, in the same way as acetylcholinesterase is correlated in the cat (110).

Peripheral sympathetic fibers.—The same problem has been approached by studying catecholamines and acetylcholinesterase in peripheral sympathetic nerve fibers. Eränkö, Härkönen & Räisänen (66, 72) observed that there were many fine fibers in the nerve net of the dilator muscle in the rat iris which exhibited both catecholamine fluorescence and acetylcholinesterase activity, while other fibers were found to contain either catecholamine or acetylcholinesterase but not both. It was tentatively concluded that the

fibers containing both catecholamine and acetylcholinesterase originated from similar cells of the superior cervical ganglion. However, the reservation was expressly made that catecholamine and acetylcholinesterase may be in closely concomitant fibers which cannot be resolved by light microscopy. Our observations were confirmed by Ehinger & Falck (53).

In an independent study, Jacobowitz & Koelle (125) observed, likewise, fibers containing both catecholamine and acetylcholinesterase in the vas deferens of the guinea pig, as well as in the uterus and the tube of the cat. Such fibers could not be found in the vas deferens or in the nictitating membrane of the cat, and their presence remained uncertain in the same organ of the rabbit. The findings were considered compatible with a restricted version of the Burn & Rand hypothesis, according to which liberation of ACh may cause subsequent release of norepinephrine from the same or adjacent fiber.

Denervation experiments.—Csillik & Koelle (34) observed that removal of the superior cervical ganglion of the rat caused a rapid disappearance of all the fluorescent fibers from the iris and also a slower degeneration and disappearance of some 15 to 20 per cent of the acetylcholinesterase positive fibers. These fibers were assumed to originate from those cell bodies in the superior cervical ganglion which possess a moderate or high acetylcholinesterase activity. In a similar study, Ehinger & Falck (53) failed to observe any appreciable reduction in the number of acetylcholinesterase positive fibers after excision of the cervical sympathetic chain. On the other hand, a considerable reduction in the number of acetylcholinesterase-containing fibers was observed after removal of ciliary ganglion, although no overt reduction of adrenergic nerves was detected.

All the above cited authors agree concerning several important points: (*a*) some sympathetic fibers contain catecholamine but not acetylcholinesterase; (*b*) other fibers contain acetylcholinesterase but not catecholamine; (*c*) fibers which appear single in the light microscope, but may in fact enclose several axons, contain both acetylcholinesterase and catecholamine; (*d*) liberation of catecholamines through a cholinergic link is possible through either of the structural mechanisms given in (*c*).

Electron microscopic clues.—Terminal sympathetic axons are known to run close together enclosed in the same Schwann's sheath (170). Therefore, electron microscopic studies are imperative before it can be confirmed or rejected that an axon may be at the same time adrenergic and cholinergic.

Since "empty" nongranular vesicles can always be demonstrated in adrenergic synapses, in addition to granular vesicles (48, 102, 169, 170), and since relatively pure fractions of such "empty" vesicles contain high concentrations of ACh (48, 194), it is possible throughout that adrenergic synapses also contain ACh. However, the empty vesicles of the adrenergic synapses may serve some other function. More reliable criteria are therefore necessary.

Intense acetylcholinesterase activity of the axon membrane has been proposed as a reliable criterion of a cholinergic neuron (180, 181). Looking for the presence or absence of such an activity in axons known to contain

catecholamine can therefore be expected to provide valuable information. Preliminary electron histochemical studies in our laboratory (unpublished) suggest indeed the presence of high acetylcholinesterase activity in the terminal adrenergic axons of the rat iris.

However, final solution of the problem can be expected only when ACh and catecholamine can be demonstrated at the ultrastructural level in a single synapse. In view of the rapid development of electron histochemical techniques, the solution may be found in the not too distant future.

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