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Ten Text-figures

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It has been thought that the hypothalamic neurosecretory cells are of a cholinergic nature, since the neurosecretory cells of several species of vertebrates show a high activity of acetylcholinesterase (Abrahams *et al.*, 1957; Arvy, 1962; Kobayashi and Farner, 1964; Uemura, 1964 and 1965). On the other hand, strong monoamine oxidase (MAO) activity bas been shown in the neurosecretory cells of the guinea pig (Léonardelli, 1966) and squirrel monkey (Iijima *et al.*, 1967), and a weak MAO activity was observed in the neurosecretory cells of the tree sparrow and rat (Matsui and Kobayashi, 1965). These findings suggest that the neurosecretory cells may also be of a monoaminergic character.

In order to clarify whether the neurosecretory cells have cholinergic or monoaminergic character, the author has examined the hypothalamic neurosecretory system more precisely and in detail by using Glenner's method with some modifications for monoamine oxidase.

Materials and Methods

Six male and five female adult Japanese quails (*Coturnix coturnix japonica*), weighing from 84 to 107 g, were purchased from a commercial source. They were maintained on 12-hour daily photoperiods for more than two weeks before sacrifice for experimental use. Three estrous and three diestrous adult female mice and four adult male mice, weighing 20 to 23 g, were also used. They were maintained on natural daily photoperiods in autumn.

Demonstration of Monoamine Oxidase Activity. Tryptamine-tetrazolium method (Glenner *et al.*, 1957) was used with some modifications. The tissues of the hypothalamo-hypophysial region were cut out as a small block upon

decapitation and frozen in isopentane maintained at −160°C with liquid nitrogen. Fresh frozen sections, with a thickness of 10 μ (quail) or 20 μ (mouse), were prepared on a cryostat and mounted on cover slips. In the quail, alternate sections were used for demonstration of MAO activity and paraldehyde fuchsin (AF) staining (Gomori, 1950), respectively. Comparing two successive sections stained with two methods, neurosecretory cells in the MAO preparation were identified. Immediately after removal of sections from the cryostat, they were dried with a hair-drier at room temperature for MAO activity. The quail tissue was incubated for 30 minutes, and the mouse tissue for 1 hour in the following freshly prepared reaction mixture at 37°C. The reaction mixture consisted of tetra-nitro-blue tetrazolium (TNBT, 5 mg in the quail and 7.5 mg in the mouse), tryptamine-HCl (25 mg), sodium sulfate anhydrous (1.67 g), 0.1 M Sörensen-Gomori phosphate buffer (5.0 ml, pH 7.6), and deionized water (15 ml). TNBT was used as a tetrazole component in place of nitro-blue tetrazolium (NBT) which was used by Glenner *et al.* TNBT was added to the reaction mixture through the use of solvent, N, N-dimethyl-formamide (DMF). To dissolve 5 mg TNBT, 0.1 ml of purified DMF was used. When precipitates were formed in the reaction mixture after addition of TNBT, the mixture was filtered through a filter paper (Toyo Roshi, No. 4) before use. For the purification of DMF, granular activated charcoal (30 g), 5 A molecular sieve (60 g) and DMF (100 ml) were mixed and shaken vigorously until an aromatic aroma was obtained, and then filtered through a hard filter paper (Toyo Roshi, No. 4).

Staining reaction was first compared between a reaction mixture containing TNBT and that containing NBT on the tissues of the quail hypothalamo-hypophysial region. Although two reaction mixtures did not give any marked difference in the distribution of formazan deposits, granular precipitates of dark brown TNBT formezan were finer and less diffused than those of purple NBT formazan. Furthermore, dark coarse granular precipitates, which were seen in NBT-stained specimens, rarely occured in TNBT-stained preparations. Thus, TNBT gave a sharper staining picture than NBT. Moreover, MAO inhibitors blocked the staining reaction of TNBT more specifically than those of NBT.

The specificity of MAO reaction was confirmed on the tissues of the quail and mouse by (1) incubation of sections exposed to 100°C water vapor for 1 minute, (2) incubation of sections in reaction mixture without substrate, (3) incubation of sections pretreated with MAO inhibitor (p-phenyl-isopropylhydrazine 10^{-3}M or tranylcypromine 10^{-4}M) in a saline solution for 30 to 50 minutes, and (4) incubation of sections in the reaction mixture containing MAO inhibitor (p-phenyl-isopropylhydrazine 10^{-3}M or tranylcypromine 10^{-4}M). In all cases, granular precipitates of tetrazolium formazan were prevented.

Identification of Neurosecretory Cells. Fresh frozen sections of the quail tissue were fixed with Bouin's fluid immediately after being taken out from the cryostat and then stained with paraldehyde fuchsin (AF) method (Gomori, 1950). In some cases paraffin sections (10 μ) of brain tissue of the quail and mouse were stained with the same method.

Observations

Hypothalamus General.

The hypothalamus of both quail and mouse generally showed MAO activity. There was no sexual difference in MAO activity. Although the general pattern of MAO distribution in the hypothalamus was almost the same in both species, the quail showed stronger activity than the mouse. In the mouse hypothalamus, longer incubation and thicker sections were necessary to obtain the same amount of formazan precipitates as in the quail hypothalamus.

In the anterior hypothalamus of both species, MAO activity was generally weak and mainly localized in the neuropil around the cell bodies of many neurons. However, in the posterior hypothalamus, cell bodies of many neurons showed moderate MAO activity and the neuropils around them demonstrated strong MAO activity. The neuropils in the quail tuberal nucleus (Fig. 4) and

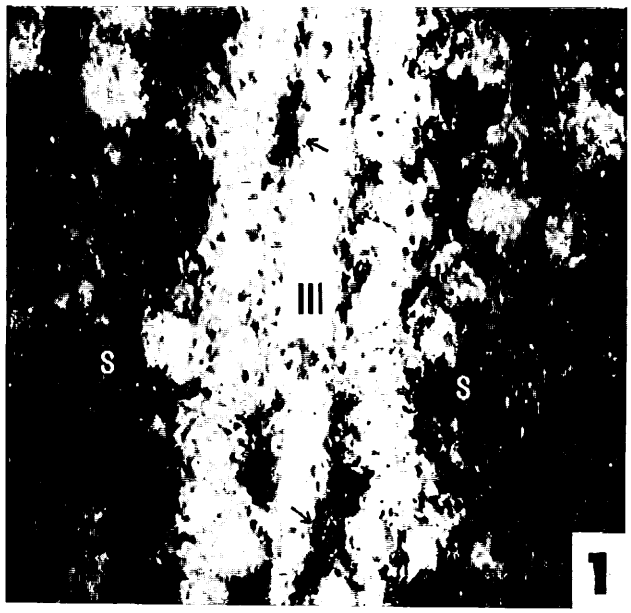

Fig. 1. Monoamine oxidase in the ependymal cells and the subependymal layer (S). Note moderate MAO activity in the apical portion (arrows) of the ependymal cells lining the third ventricle (III), and strong activity in the neuropil of the subependymal layer. ×960.

in the mouse arcuate nucleus showed strong MAO activity (Figs. 6, 7 and 10). Although most of the cell bodies of these nuclei showed weak activity, some of them had moderate MAO activity (Figs. 4 and 6). Ependymal cells lining the third ventricle generally showed weak or moderate MAO activity in their apical portions (Figs. 1 and 7). The neuropil of the subependymal layer showed rather strong enzymatic activity (Fig. 1).

Neurosecretory Cells.

Neurosecretory cells of both species exhibited very weak MAO activity in the cytoplasm of their parikarya and axon hillocks. The cell nuclei showed slight or no enzymatic activity. The neruopil around the neurosecretory cells gave strong MAO activity. In this neuropil, there were numerous fibers beaded with heavy formazan deposits. These fibers forming a fine network were sometimes in close contact with the cell membrane of the neurosecretory cells (Figs. 2 and 3).

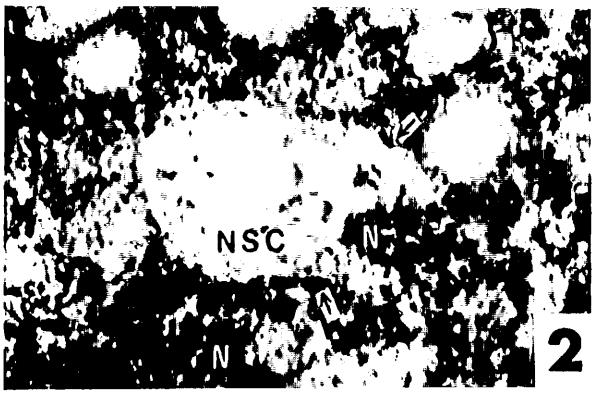

Fig. 2. Monoamine oxidase in a AF-positive neurosecretory cell (NSC) of the quail paraventricular nucleus. Note very weak activity in the perikarya and the axon hillock, and almost no activity in the nucleus. Neuropils (N) around the neuroscretory cell show rather strong activity. Arrows show beaded fibers with intensely colored formazan deposits which are in close contact with the cell membrane of the neurosecretory cell. ×960.

Median Eminence and Infundibular Stem.

As is well known, the median eminence, as well as infundibular stem, of higher vartebrates consists of the ependymal layer, internal layer (hypendymal and fiber layers) and external layer. In the quail the external layer of the median eminence is further divisible into the reticular and palisade layers. Along the antero-posterior axis, the quail median eminence is divisible into the anterior region containing AF-positive material and the posterior region

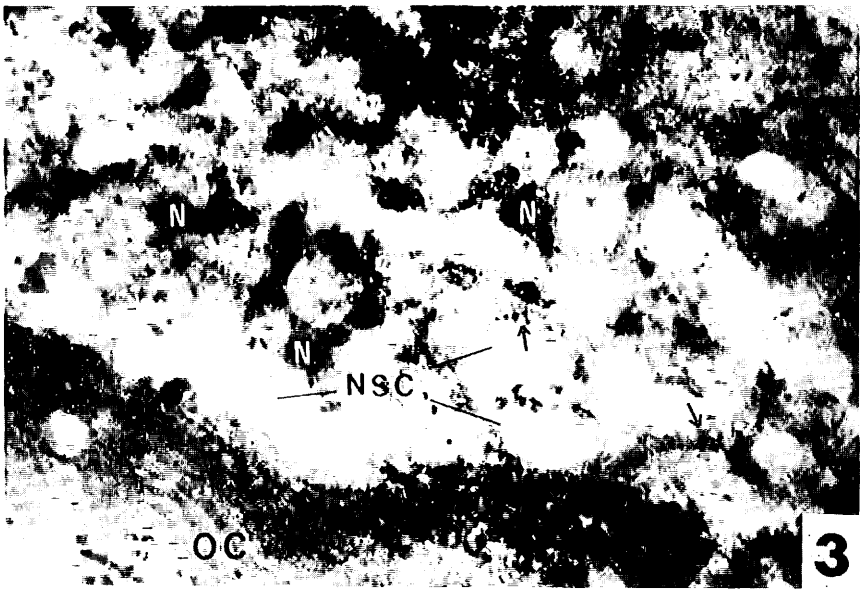

Fig. 3. Monoamine oxidase in the mouse supraoptic nucleus. Note weak MAO activity in the perikarya of the neurosecretory cells (NSC), slight activity in the nuclei and rather strong activity in the neuropils (N) around the cells. Arrows show fibers with heavy formazan deposits. OC, optic chiasma. ×960.

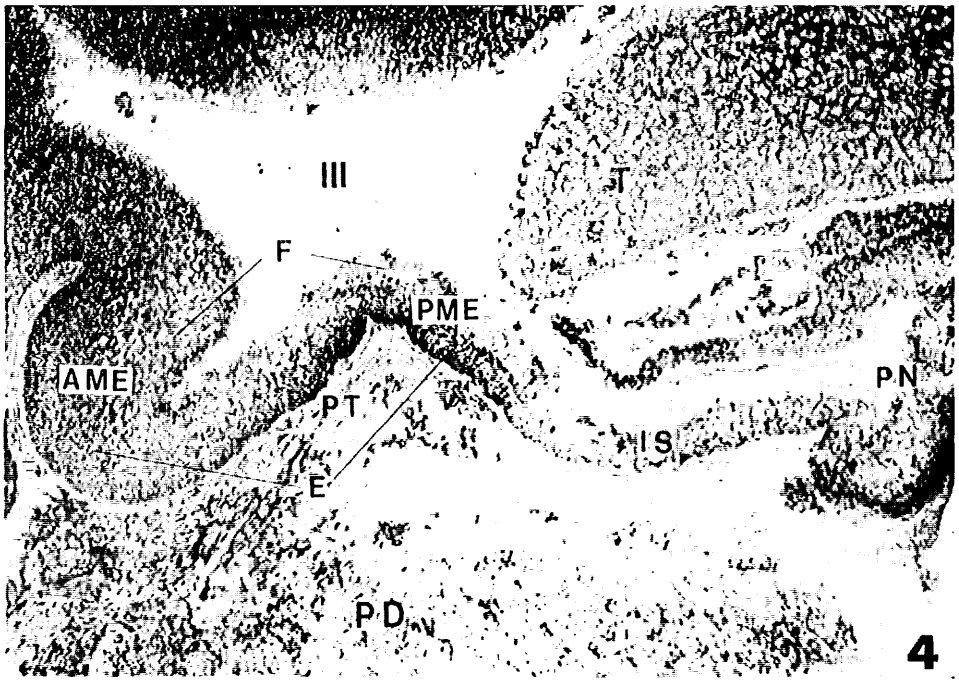

Fig. 4. Monoamine oxidase in the median eminence-pituitary region of the quail (sagittal section). Note the difference between the fiber layer (F) and the external layer (E) in MAO activity. The external layer of the median eminence and the pars nervosa (PN) show strong activity. The cell bodies in the tuberal nucleus (T) show moderate or weak MAO activity. AME, anterior median eminence; IS, infundibular stem; PD, pars distalis; PME, posterior median eminence; PT, pars tuberalis; III, third ventricle. ×120.

without AF-positive material (see Oksche *et al.*, 1964).

In both species, the ependymal cells of the median eminence gave two types of staining in their cytoplasm, one was very slight and the other was weak in MAO activity (Figs. 5 and 7). Some of the hypendymal cells were in close contact with fibers beaded with heavy formazan deposits. The commissura tuberis ventralis in the hypendymal layer of the quail and the mouse

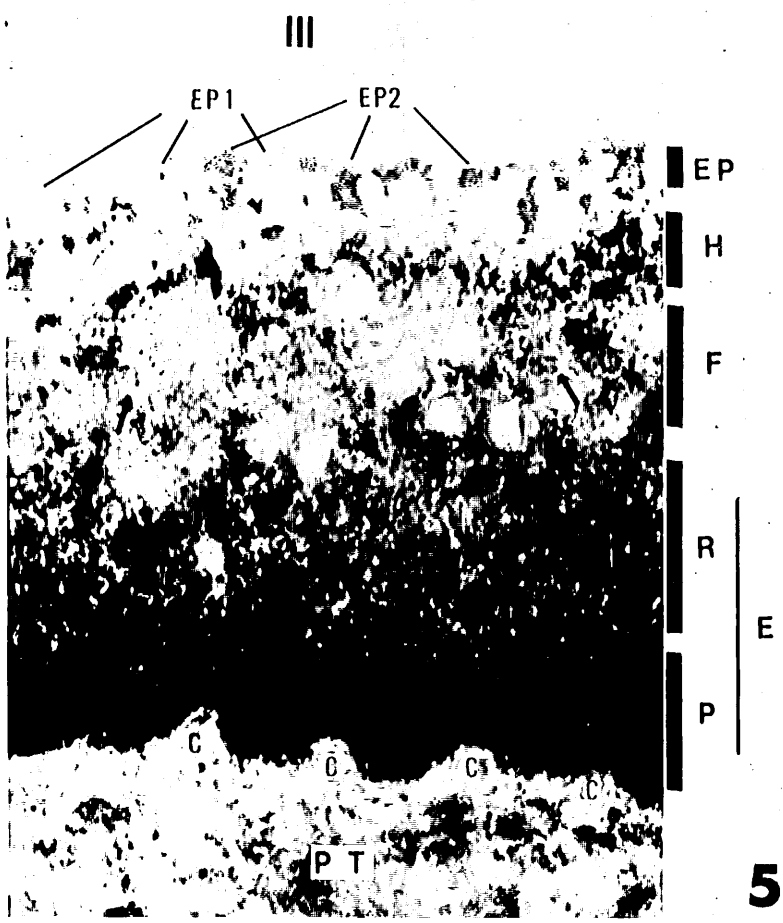

Fig. 5. Higher magnification of the quail median eminence showing MAO activity (sagittal section). Note the fibers (arrows) beaded with formazan deposits running cephalo-caudally in the fiber layer (F), strong activity in the external layer (E) and intence formazan deposits around the blood capillaries (C). EP, ependymal layer; EP1, ependymal cells showing very slight reaction; EP2, ependymal cells giving weak activity; H, hypendymal layer; P, palisade layer; PT, pars tuberalis; R, reticular layer; III, third ventricle. ×960.

contained a number of fibers beaded with intensely colored-formazan precipitates (Figs. 5 and 6). MAO activity of the fiber layer in the quail median eminence and infundibular stem was rather moderate compared with other layers in the median eminence (Figs. 4 and 5). In the mouse median eminence, the fiber layer showed moderate MAO activity (Fig. 6). In this fiber layer of both species, a considerable number of fibers beaded with formazan deposits, running cephalo-caudally, were observed.

In the quail median eminence the external layer showed strong MAO activity (Figs. 4 and 5). In the reticular layer, heavy formazan deposits had no regularity in arrangement. In the palisade layer, heavy formazan deposits were arranged in beads, running dorso-ventrally toward the capillaries of the primary plexus. Accordingly, MAO activity was especially strong around the blood capillaries (Fig. 5). Between the anterior and posterior divisions of the median eminence, no marked difference was observed except that the external layer of the posterior median eminence had stronger MAO activity than that of the anterior median eminence. This difference may be due to the larger number of capillaries accompanying the fibers showing MAO activity in the

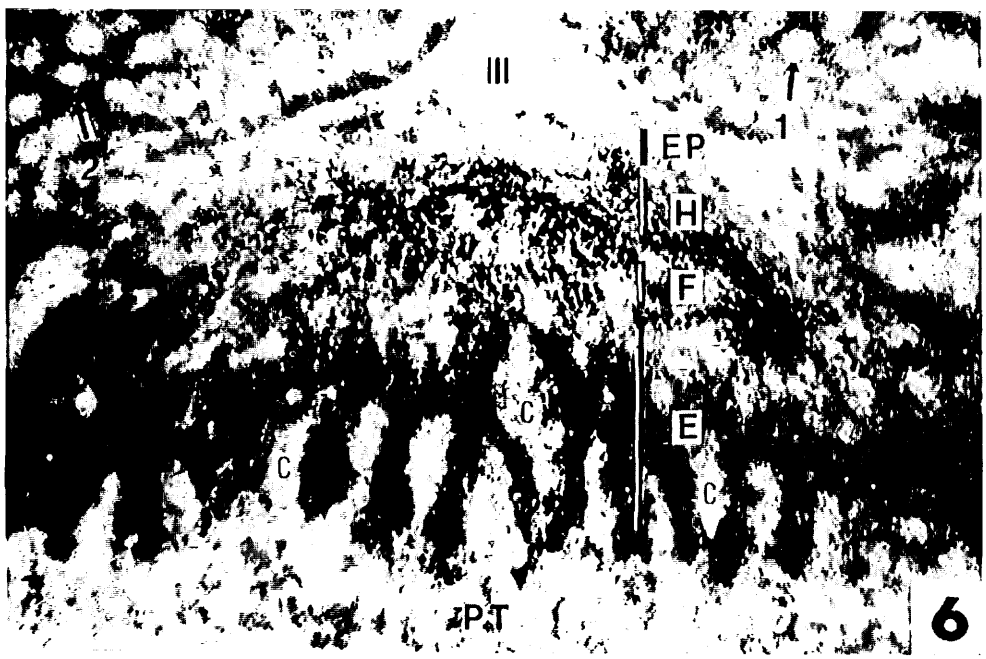

Fig. 6. Monoamine oxidase in the mouse median eminence. Note fibers beaded with intensely colored formazan deposits in the hypendymal layer (H), and strong activity around the capillaries (C). Cells in the arcuate nucleus show weak activity (arrow 1) or moderate activity (arrow 2). EP, ependymal layer; E, external layer; F, fiber layer; H, hypendymal layer; PT, pars tuberalis; III, third ventricle. ×500.

Fig. 7. Higher magnification of the ependymal cells in the mouse median eminence. In the median eminence, there are two types of ependymal cells, one showing very slight MAO activity (EP1) and those giving weak activity (EP2). Arrows show ependymal cells of the other region giving moderate MAO activity. A, arcuate nucleus; H, hypendymal layer; III, third ventricle. ×960.

posterior median eminence than in the anterior median eminence. In general, a similar pattern of distribution of MAO activity was also observed in the infundibular stem (Fig. 4). The only difference from the median eminence is that the heavy formazan deposits were rare around the capillaries covering the stem.

In the median eminence and infundibular stem of the mouse, there were moderate MAO activity in the external layer and strong activity in the neural tissue around the capillaries of the primary plexus (Fig. 6). There were many fibers beaded with formazan deposits, terminating at the capillaries of the primary plexus, as well as the median eminence of the quail.

According to the difference in stainability, two types of glial cells were distinguishable in the median eminence of both species. The glial cells of one type, which were commonly present in all layers, had weak MAO activity in their cytoplasm, and there was no heavy formazan deposits around the cells. The glial cells of the other type, which were mainly present in the hypendymal layer, showed rather moderate activity in the cytoplasm, and the heavy bead-like formazan deposits were found in close contact with the

cells (see Figs. 4 and 6).

Pars Nervosa.

MAO activity was not detected in the ependymal cells of the quail pars nervosa. The hypendymal layer showed rather strong activity around some hypendymal cells (Fig. 8). The internal layer of the diverticular wall of the pars nervosa showed rather weak MAO activity. The external layer showed generally moderate activity, but strong MAO activity was observed in the

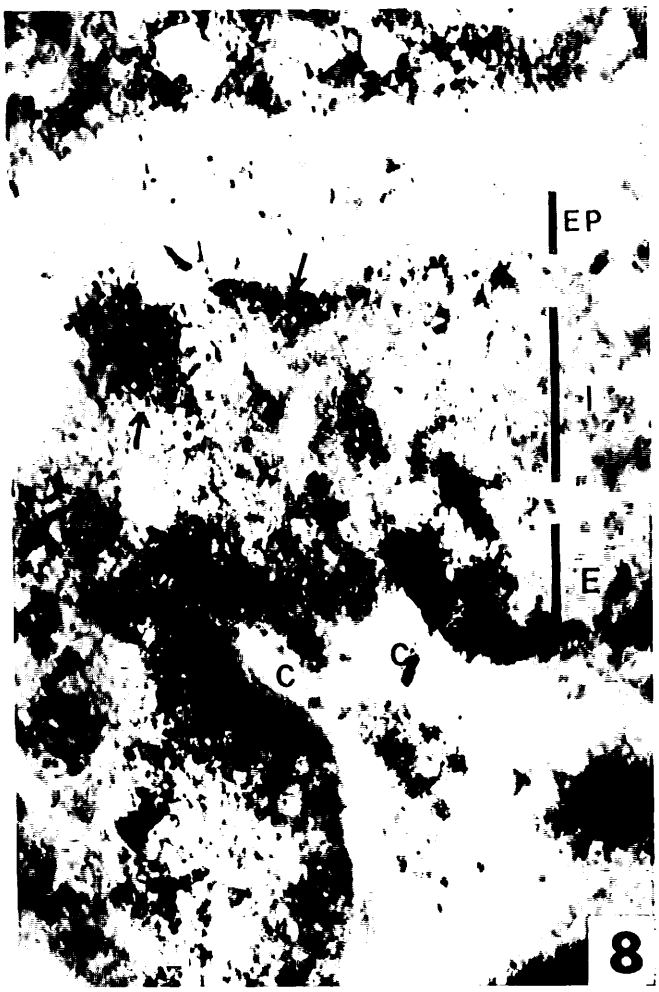

Fig. 8. Monoamine oxidase in the quail pars nervosa. Note strong activity in the neural tissue around the capillaries (C), and rather strong activity around the hypendymal cells (arrows). E, external layer; EP, ependymal layer; I, internal layer. ×960.

neural tissues around the capillaries in the pars nervosa (Fig. 8).

The diverticular wall of the mouse pars nervosa showed generally moderate MAO activity. Around the blood capillaries in the pars nervosa, fibers beaded with formazan deposits were often obsered (Fig. 9). The MAO activity in the ependymal cells of the mouse pars nervosa could not be discerned.

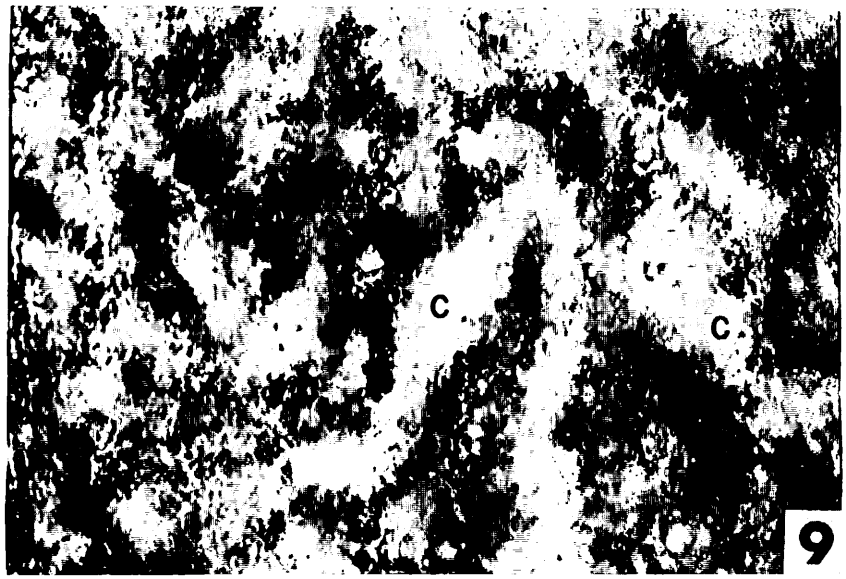

Fig. 9. Monoamine oxidase in the mouse pars nervosa. Formazan deposits are rather dense around the capillaries (C). ×840.

Pars Intermedia, Pars Distalis and Pars Tuberalis.

In the mouse, cells of the pars intermedia showed moderate MAO activity in their cytoplasm. Bead-like formazan deposits were infrequently observed around the cells. The cells of pars distalis and pars tuberalis of both species showed slight or weak MAO activity. In the mouse, fibers beaded with heavy formazan deposits, running to the capillaries of the primary plexus through the arcuate nucleus, were observed in the regions lateral to the median eminence (Fig. 10). It was not known whether these fibers originated in the arcuate nucleus or not.

The cells in the pars tuberalis, closely facing the regions mentioned above, showed stronger MAO activity than those in other regions of the pars tuberalis (Fig. 10). It seems likely that there is a direct innervation of monoaminergic fibers in certain cells of the pars tuberalis.

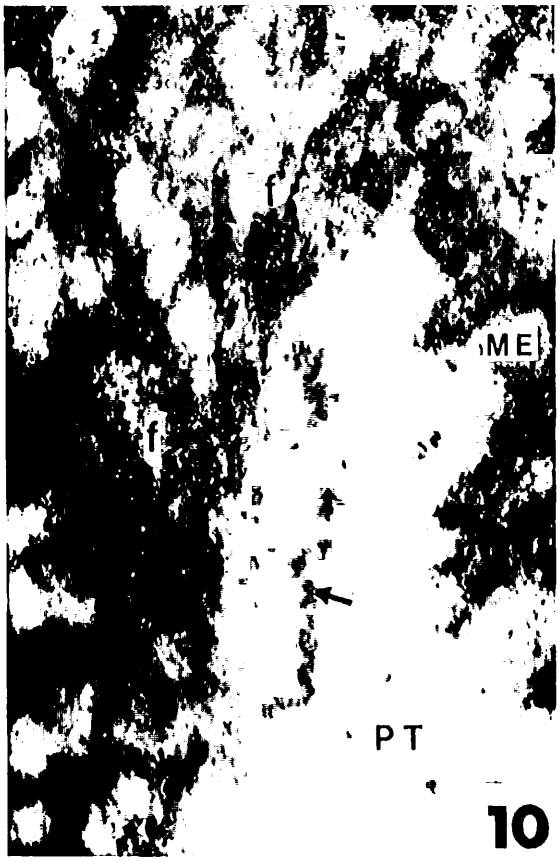

Fig. 10. Monoamine oxidase in the area lateral to the median eminence (ME) of the mouse. Note the fibers (f) beaded with formazan deposits, and the cells (arrow) showing weak MAO activity in the pars tuberalis (PT). ×840.

Discussion

The neurosecretory cells of both quail and mouse brains showed weak MAO activity. However, neuropils around them showed rather strong activity. The fibers showing strong MAO activity often contacted the cell membrane of the neurosecretory cells. Since it has been said that regions rich in free monoamines in the mammalian brain show high activity of MAO (see Zolovick *et al.*, 1966) and that sympathetic nerve endings have high MAO activity (Snyder *et al.*, 1964), the fibers showing MAO activity around the neurosecretory cell membrane may be monoaminergic fibers which innervate at the neurosecretory cells. This assumption is also supported by the follow-

ing observations: (1) monoaminergic nerve fibers revealed by fluorescence microscopy are numerous in both supraoptic and paraventricular nuclei of the pigeon (Fuxe and Ljunggren, 1965), and some species of mammals (Carlsson *et al.*, 1962), (2) a considerable number of synapses exist between neurosecretory cell bodies and axons containing possible monoamine granules (Murakami, 1962; Peterson, 1965; Polenov and Senchik, 1966; Oota *et al.*, 1966). From the observations mentioned above, it is highly probable that the neurosecretory cells are not of a monoaminergic nature but they receive a number of monoaminergic innervation.

As was mentioned above, the neurosecretory cells of several species of vertebrates including the quail and mouse show strong reaction of acetylcholinesterase (AChE) in their cytoplasm (Abrahams *et al.*, 1957; Arvy, 1962; Kobayashi and Farner, 1964; Uemura, 1964 and 1965; Yokoyama, unpublished data). This suggests that the neurosecretory cells are regulated by cholinergic fibers. In favor of this idea, Pickford (1947) reported that acetylcholine injected in the supraoptic nuclei induced release of neurohypophysial hormones from the dog pars nervosa. However, there is another possible explanation for the presence of high AChE activity in the neurosecretory cells. The monoaminergic nerve endings terminating at the neurosecretory cells might first release acetylcholine and change the permeability of the cell membrane of the endings, thus facilitating the release of monoamines through the membrane (see Burn and Rand, 1965). If so, AChE in the neurosecretory cells may destroy acetylcholine liberated from the endings and prevent its accumulation. This idea may be more plausible than the first one, since the presence of monoaminergic fibers around the neurosecretory cells is suggested in the present studies. Then, a question is raised as to the role of monoamines in the monoaminergic nerve endings forming synapses with neurosecretory cells. They may stimulate neurosecretory cells to synthesize neurohypophysial hormones. It has been reported that catecholamines stimulate the formation or accumulation of cyclic 3′, 5′-AMP, which may be involved in the hormone synthesis through activation of adenyl cyclase (see Sutherland and Robison, 1966). Another possibility is that monoamines act as transmitter substances of nerve impulses to the neurosecretory cells to release neurohypophysial hormones.

The distribution of MAO activity in the median eminence of the mouse and Japanese quail generally coincides with those of the rat, tree sparrow (*Passer montanus saturatus*) (Matsui and Kobayashi, 1965), and white-crowned sparrow (*Zonotrichia leucophrys gambelii*) (Follett *et al.*, 1966). In the present studies, however, more precise and exact localization of MAO was demonstrated by the use of TNBT instead of NBT. Two different types were distinguished by staining reaction of MAO among ependymal cells of the median

eminence; one showed slight and the other weak activity. We cannot attach any biological meaning to the difference in stainability between two types of cells. The close contacts between the hypendymal or glial cells with fibers beaded with heavy formazan deposits suggest innervations of monoaminergic axons at these cells. Supporting this suggestion, Matsui (1966a, b) and Kobayashi and Matsui (1967) observed the synapses between the axons containing possible monoamine granules and the hypendymal or glial cell bodies in the hypendymal layer. The fibers beaded with formazan deposits found in the external layer of the median eminence may be monoaminergic fibers, since the presence of small granules possibly carrying monoamines (Kobayashi, 1964) and the existence of catecholamines (Fuxe, 1964) have been reported in the neurohemal region of the rat median eminence. The monoamines in the median eminence may be involved in the secretory mechanisms of releasing factors from the median eminence (Kobayashi *et al.*, 1967), or may have roles in the pars distalis in mechanisms releasing adenohypophysial hormones (Fuxe and Hökfelt 1967).

Strong MAO activity found around the capillaries in the pars nervosa of both species suggest the presence of monoaminergic fibers in the pars nervosa. Govyrin *et al.* (1966) and Konstantinova (1967) have demonstrated intensely fluorescent adrenergic fibers around the capillaries in the rat pars nervosa. The fibers beaded with formazan deposits, which proceed cephalo-caudally in the fiber layer of the median eminence, may be monoaminergic nerve fibers terminating at the capillaries of the pars nervosa. The monoaminergic fibers in the pars nervosa may be involved in the release of neurohypophysial hormones, since Konstantinova (1967) has shown that the intraventricular injection of acetylcholine caused the rat to release antidiuretic hormones from the pars nervosa with concomitant increase of fluorescence in the monoaminergic nerves.

Beaded fibers with formazan deposits found in the pars intermedia may suggest monoaminergic innervation in this gland. This observation agrees with the results obtained by fluorescence microscopy in the frog and rat (Enemer and Falck, 1965), and by electron microscopy in the cat (Bargmann *et al.*, 1967).

Distribution of MAO activity was different from that of AF-positive materials in the hypothalamic neurosecretory system of both mouse and quail in the following points: 1) neurosecretory cells showed very weak MAO activity in their cytoplasm, 2) distribution pattern of the AF-positive materials in the median eminence of the quail and mouse did not coincide with that of MAO activity. Therefore, AF-positive neurosecretory neurons may not be of a monoaminergic nature, but of a cholinergic one. However, as mentioned above, the neurosecretory cells seem to be regulated by monoaminergic fibers.

Summary

Distribution of monoamine oxidase (MAO) was histochemically examined in the hypothalamo-hypophysial region of the Japanese quail and mouse. As a substrate, tetra-nitro-blue tetrazorium gave more pricise localization of MAO activity than nitro-blue tetrazorium. Distribution pattern of MAO was similar in both species, although the quail showed generally stronger MAO activity than the mouse. The hypothalamic neurosecretory cells showed weak MAO activity in their perikarya. However, rather strong activity was observed in the neuropils around them, and furthermore, fibers beaded with formazan deposits were often in contact with the cell membrane of the neurosecretory cells. This suggests the innervation of monoaminergic fibers at the neurosecretory cell. Possible roles of the monoaminergic fibers are discussed in relation to the regulation of neurosecretory cells.

In the median eminence, some ependymal or glial cells showed weak MAO activity, but others did not. Strong MAO activity was found in the subependymal layer of the median eminence, which may be due to monoaminergic fibers in the commissura tuberis ventralis. Fibers containing heavy formazan deposits, which were considered monoaminergic, were observed around the capillaries of the primary plexus of the median eminence. In the pars nervosa of both species, MAO activity was rather strong around the capillaries. Monoaminergic innervations in the pars intermedia and the pars tuberalis are suggested.

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