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Catecholamine biosynthetic enzymes are expressed in replicating cells of the peripheral but not the central nervous system

[neural development/[^{3}H]thymidine/immunocytochemistry/tyrosine 3-monooxygenase (tyrosine hydroxylase)/dopamine β-hydroxylase]

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ABSTRACT **We sought to determine whether the precursors of catecholamine-containing neurons in the developing peripheral and central nervous systems of chickens and rats express the biosynthetic enzymes tyrosine hydroxylase [THase; tyrosine 3-monooxygenase; L-tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] or dopamine β-hydroxylase [DBHase; 3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1], prior to the time they withdraw from the cell cycle. Chicken embryos (stages 26–27) were injected with [^{3}H]thymidine and 4 hr later were prepared for the simultaneous demonstration of radioautographically labeled nuclei in immunoreactive THase cells. The brains and sympathetic chains of rat fetuses (days E12–E14), exposed for 2 hr to [^{3}H]thymidine, were treated similarly except that peripheral tissues were stained with a specific antibody to DBHase as well as anti-THase. In the peripheral nervous system of both chicken and rat, nuclei of THase-containing cells were radioautographically labeled. DBHase-containing cells in the peripheral nervous system of rats were also labeled and thus are noradrenergic. THase was localized in cells of the brain of the same rat fetuses beginning on day E12 (no THase was detected on day E11 or E11.5) in the mantle layer of the ventral mesencephalic and rostrolateral rhombocephalic cellular groups; however, THase-containing cells in the central nervous system did not incorporate [^{3}H]thymidine. We conclude that, during development, the adrenergic neuronal precursors of the peripheral nervous system but not of the central, have the capacity to synthesize catecholamines before they withdraw from the cell cycle. Differences in the maturation of peripheral and central neurons may be related to differences in their embryological origin.**

Until recently it was believed that none of the properties that characterize mature neurons are expressed by developing precursor cells until after they cease to divide (1–4); however, studies of the developing peripheral nervous system of chickens have indicated that some properties of mature adrenergic neurons may be expressed by the precursor cells while these cells are still replicating (5, 6). Thus, precursor cells in primary and secondary sympathetic ganglia (and the adrenal medulla) continue to incorporate [^{3}H]thymidine after they acquire several of the phenotypic characteristics expressed by adult sympathetic cells. These cells contain characteristic subcellular catecholamine (CA) storage vesicles (6), a specific uptake mechanism for norepinephrine (6, 7), and a store of CA (5, 6, 8) while still remaining in the cell cycle.

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Several questions still persist with respect to the relationship between the phenotypic expression of adrenergic characteristics and the ability of precursor neurons to replicate. First, the presence of uptake and storage mechanisms in peripheral CA-containing cells does not necessarily prove that these cells have the capacity to synthesize the neurotransmitter while they are dividing. Conceivably, CA could be synthesized elsewhere and only stored in developing adrenergic cells. The demonstration that replicating CA-containing cells can express the specific proteins responsible for transmitter biosynthesis—i.e., the CA biosynthetic enzymes tyrosine hydroxylase [THase; tyrosine 3-monoxygenase; L-tyrosine tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] and dopamine β-hydroxylase [DBHase; dopamine β-monooxygenase; 3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1]—would provide more direct evidence that the CA found in developing sympathicoblasts is synthesized *in situ*. Second, it is not known whether developing CA-containing neurons in the central nervous system also continue to divide after acquiring adrenergic neurotransmitter related characteristics. The fact that CA can be detected in those regions of the mantle layer that give rise to adult CA cells (9, 10) at a time when some cells in the neural tube are still dividing (11) raises the possibility that CA neurons of the central nervous system, like those of the peripheral, may acquire phenotypic characteristics before withdrawing from the cell cycle.

In the present study we have sought to determine whether replicating cells in the peripheral and central nervous systems express the enzymes THase and DBHase before withdrawing from the cell cycle. This was examined by combined use of immunocytochemical staining of the developing nervous systems of chickens and rats, with specific antisera to THase and DBHase and [^{3}H]thymidine radioautography. We shall demonstrate that cells of the peripheral nervous system, but not the central, continue to divide after the enzymes are expressed.

METHODS AND MATERIALS

Preparation of Animals. *Chickens.* Fertilized eggs of White Leghorn chickens (purchased from Shamrock Poultry and Breeding Farms, North Brunswick, NJ) were maintained in a humidified, forced-draft, egg incubator at 38 ± 1°C for 4.5–5.5 days. For administration of [^{3}H]thymidine (6), a small hole was

Abbreviations: CA, catecholamine; THase, tyrosine hydroxylase; DBHase, dopamine β-hydroxylase.

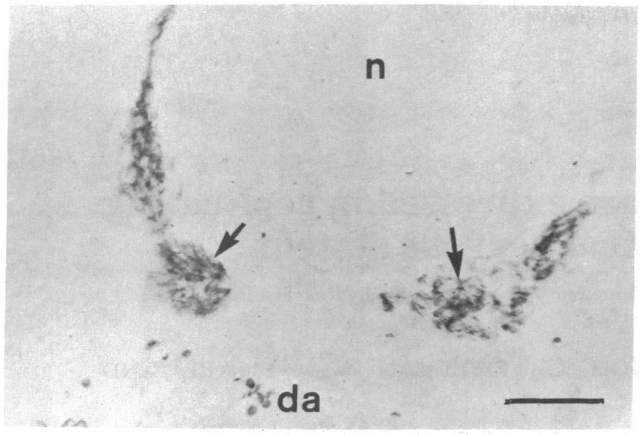

FIG. 1. Immunocytochemical localization of THase in cells of primary sympathetic ganglia (arrows) from chicken embryo (4.5–5 days of incubation; stage 26). Surrounding tissues are not reactive. At the left an extension of immunoreactive cells lies between the primary ganglion and the site where secondary ganglia will form. n, Notochord; da, dorsal aorta. Bar = 50 μm.

made in the narrow end of an egg with a sterile needle. After withdrawal of 0.8 ml of albumen, 0.2 ml of sterile saline containing [^{3}H]thymidine (20 or 30 μCi per egg; specific activity, 50–65 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels; New England Nuclear) was injected directly into the yolk sac. Embryos were killed 4 hr later. It has been shown (6) that the replicating neuronal precursors do not complete G_2, mitosis, and cell division in this time period. Four embryos were tested with [^{3}H]thymidine and, as controls, four embryos received only vehicle. The embryos were staged (12), decapitated, and fixed for 2 hr at room temperature with 4.0% formaldehyde (generated from paraformaldehyde) in 0.1 M phosphate buffer (pH 7.2). They were then infiltrated overnight at 4°C with 30% sucrose and frozen. Transverse or sagittal sections 10–16 μm thick were cut in a cryostat (13, 14), cooled to −25°C, and mounted on glass slides that had previously been coated with an adhesive chrome alum/gelatin solution.

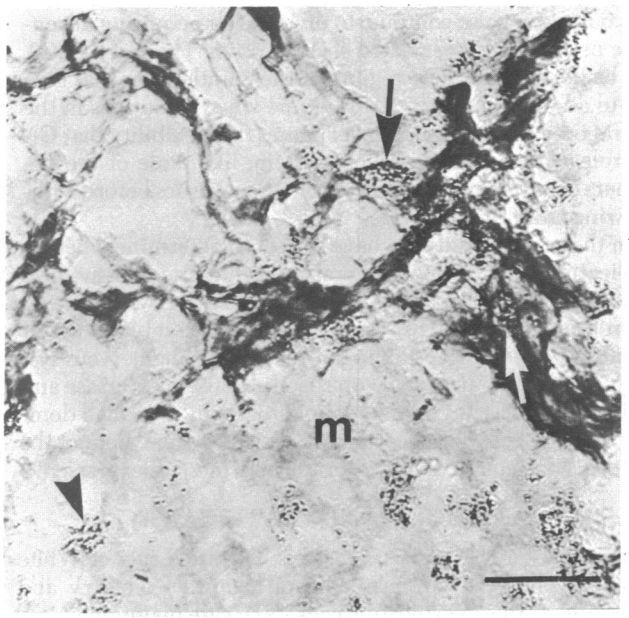

FIG. 2. Immunoreactive THase in chicken embryo (stage 27) cells fixed while migrating ventrally to the aorta. The nuclei of some immunoreactive cells are labeled by [^{3}H]thymidine (arrows). Cells in the mesenchyme (m) are also labeled by [^{3}H]thymidine (arrowhead) but these do not contain THase immunoreactivity. Bar = 30 μm.

FIG. 3. (*A*) Sympathetic ganglion cells of a developing chicken (5–5.5 days; stage 27), demonstrating THase immunoreactivity and labeling by [^{3}H]thymidine (arrows). Bar = 20 μm. (*B*) Higher magnification of cells in the primary sympathetic ganglia. Arrow indicates cell showing both THase immunoreactivity and nuclear labeling by [^{3}H]thymidine. Bar = 20 μm.

Rat. Pregnant Sprague–Dawley rats were obtained from Hilltop Lab Animals (Scotsdale, PA). The date of conception was established by the presence of a vaginal plug and was designated as day 1 of gestation (E1). Pregnant rats were housed in individual cages and allowed food and water ad lib. Rats pregnant for 12.0, 13.5, and 14.0 days were injected intraperitoneally with [^{3}H]thymidine (7 μCi/g; specific activity, 50.3 Ci/mmol, New England Nuclear). Two hours later they were anesthetized (Equithesin; 3 ml/kg intraperitoneally; Jensen Salsbury Laboratories, Kansas City, MO) and the fetuses were exposed. The fetuses of these animals and of rats pregnant for 11 and 11.5 days but not exposed to [^{3}H]thymidine were measured for crown–rump length and then perfused for 3 min (15) through the heart with 4.0% formaldehyde (generated from paraformaldehyde) in 0.1 M phosphate buffer (pH 7.2–7.4). Fetuses were subsequently immersed in the same fixative for an additional 20 min, infiltrated overnight at 4°C with 30% sucrose, and frozen. Five fetuses at day E12, three at day E13.5, and two at day E14 were prepared for the simultaneous demonstration of incorporation of [^{3}H]thymidine and THase as described below.

Immunocytochemical Localization of THase and DBHase. Procedures for the purification of THase and DBHase

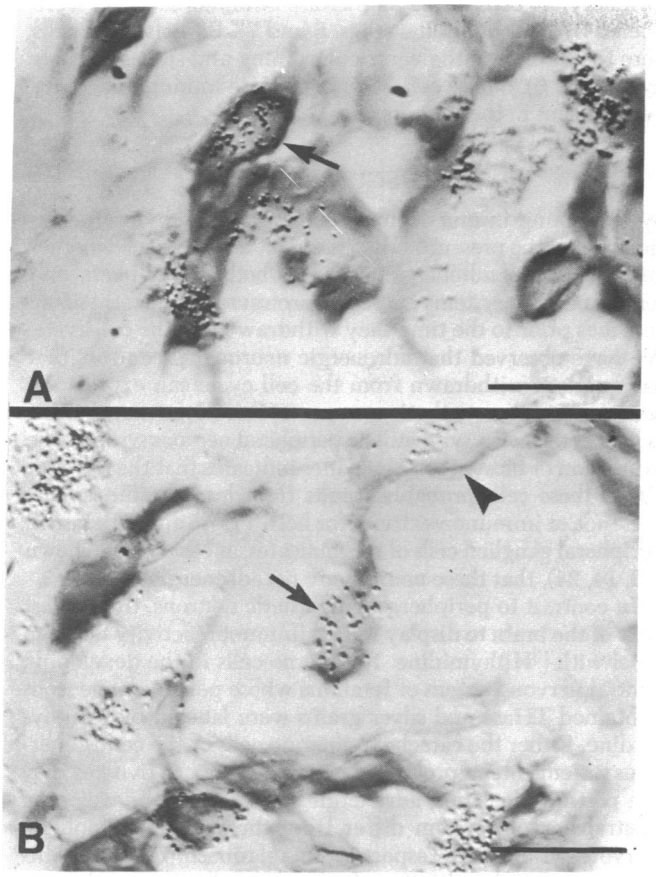

FIG. 4. Immunocytochemical localization of THase in the developing sympathetic chain of rat fetus at day E14. Pregnant mothers were injected with [^{3}H]thymidine (7 μCi/g) and killed 2 hr later. Silver grains are seen over the nuclei of doubly labeled cells (arrows). Some of these doubly labeled cells exhibit THase containing processes (n). Bar = 20 μm.

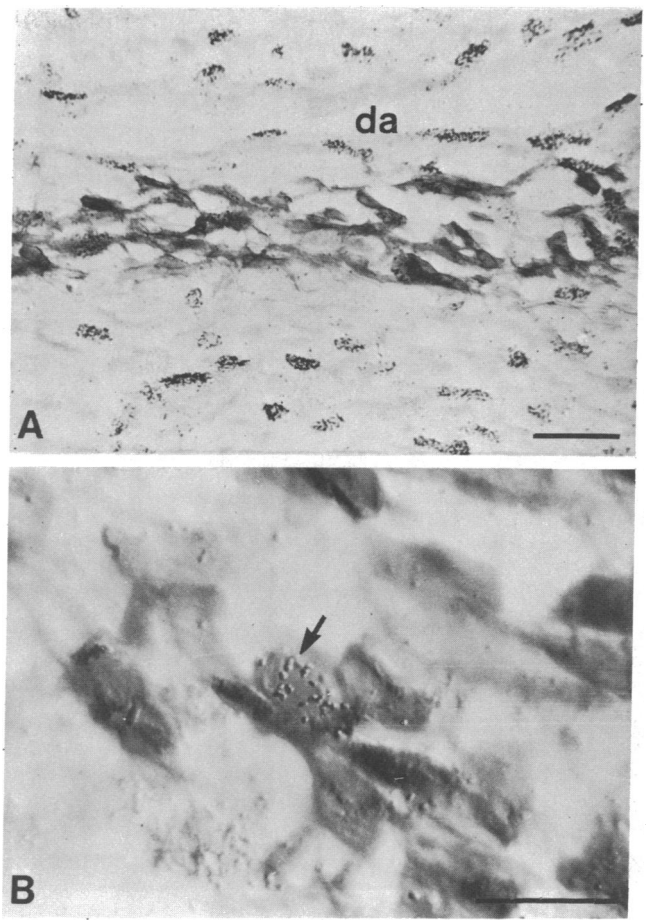

FIG. 5. Immunocytochemical localization of DBHase in cells of the sympathetic chain of the same rat fetuses as in Fig. 4. Doubly labeled cells are hard to discern at low magnification (*A*) but are apparent at higher magnification (*B*). da, Dorsal aorta. Bar = 50 μm. (*B*) Higher magnification, showing DBHase localized in a cell labeled by [^{3}H]thymidine (arrow) within the sympathetic chain of a rat fetus. Bar = 20 μm.

and the production of specific antibodies to these enzymes have been described (16, 17). THase and DBHase immunoreactivities were localized in sympathetic ganglia and THase immunoreactivity was localized in the brain by using the peroxidase-antiperoxidase method of Sternberger (18) as modified by Pickel *et al.* (19–21). Sections were washed in 0.1 M Tris/saline buffer (pH 7.6) and incubated for 15 min with 0.25% Triton X-100 detergent to facilitate penetration of the antiserum into the tissues. The sections were then incubated sequentially with rabbit antiserum to THase (or DBHase), goat anti-rabbit IgG (30 min), and rabbit peroxidase-antiperoxidase complex (30 min). All dilutions were made in Tris·HCl/saline (0.1 M) buffer containing 1.0% goat serum. THase and DBHase antisera were diluted 1:500 and applied at 4°C for 24 hr. Bound peroxidase was demonstrated by incubation with 0.01% hydrogen peroxide and 0.013% 3,3-diaminobenzidine in 0.1 M Tris·HCl buffer (pH 7.6) for 6 min. Control sections were incubated with preimmune rabbit serum.

Radioautography. Immediately after incubation with diaminobenzidine, stained sections were transferred to 0.1 M phosphate buffer (pH 7.2–7.4). They then were washed twice with distilled H_2O, dipped in Kodak NTB_2 emulsion (diluted 1:1 with water), and maintained at 4°C in a light-proof box containing a drying agent (Drierite) for 8–22 days. They were then developed with Kodak D19 developer and, for the simultaneous demonstration of silver grains and immunoreactivity, were viewed with Normarski interference or phase contrast optics. Sections of ganglia from embryos that did not receive [^{3}H]thymidine were sequentially stained and processed for radioautography to rule out chemography.

RESULTS

Chicken. Developing ganglion cells in the primary sympathetic chain of chicken embryos (4.5–5 days incubation; stage 26) contained THase (Fig. 1). Specific reaction product was seen in cells in the primary ganglia as well as in individual cells fixed while migrating dorsally toward their definitive positions in the secondary paravertebral ganglia. In some embryos, immunoreactive cells were also seen ventral to the primary ganglia. These presumably were fixed while migrating toward the adrenal medulla and prevertebral aortic plexus (Fig. 2). The cells containing THase were similar in appearance to the CA-containing cells seen with formaldehyde-induced fluorescence in embryos of the same developmental age (6, 22, 23). No cells were labeled by antibody to DBHase, probably because of limited species crossreactivity (unpublished data).

In embryos injected with 20 or 30 μCi of [^{3}H]thymidine 4 hr prior to fixation, the nuclei of many immunoreactive THase-containing cells were labeled. Cells containing THase and nuclear labeling by [^{3}H]thymidine were observed within

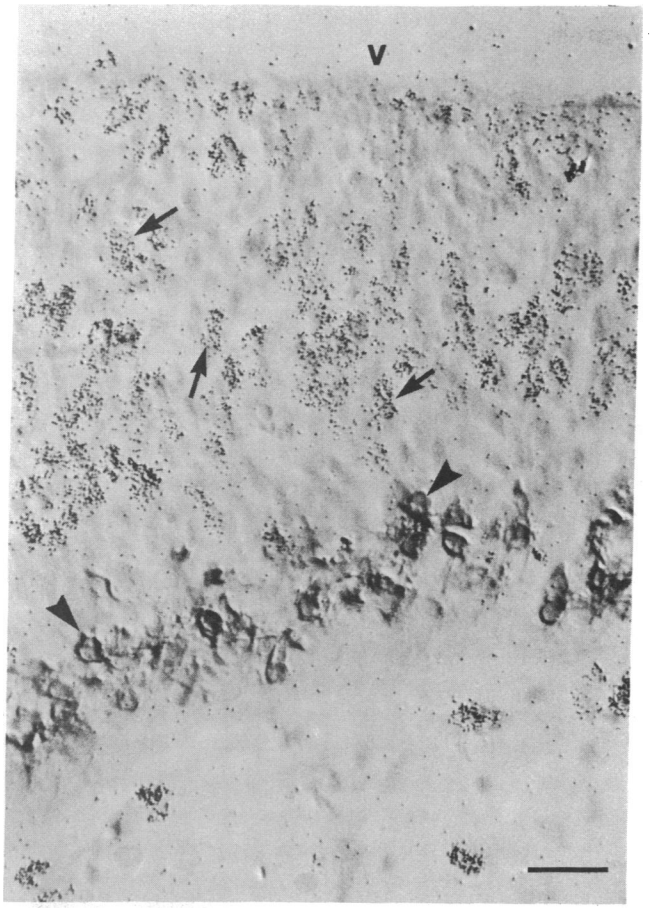

FIG. 6. Sagittal section through the fourth ventricle of the brain of the same rat fetus (day E14) as in Figs. 4 and 5. THase immunoreactivity can be seen in cells in the mantle layer in the ventral mesencephalic cellular group (arrowheads). Radiolabeling of nuclei in the ventricular and subventricular zones is frequent (arrows). v, Ventricular cavity. Bar = 30 μm.

primary sympathetic ganglia (Fig. 3) and also in single cells outside of the ganglionic mass (Fig. 2). Cells containing THase immunoreactivity were seen in sections from embryos that did not receive [^{3}H]thymidine (although no silver grains were observed over the nuclei of these cells). Conversely, labeled nuclei were seen in all sections from embryos that received [^{3}H]thymidine and were incubated with preimmune rabbit serum. Therefore, the combined immunocytochemical and radioautographic techniques did not interfere with one another and the presence of doubly labeled cells in animals given [^{3}H]thymidine was not due to chemography or other artifacts.

Rat. THase and DBHase were seen in neurons in the sympathetic chain of rat fetuses at days E11, -11.5, -12, -13.5, and -14. The enzyme was localized to the cytoplasm of cell bodies and neuritic processes. In animals injected with [^{3}H]thymidine [days E12, E13.5, and E14 (crown–rump length 9, 10.5, and 13 mm, respectively)], many of the immunoreactive neurons also had labeled nuclei (Figs. 4 and 5).

At days E11 and E11.5, no THase-containing cells could be detected in the developing brain; they appeared for the first time on day E12. These cells were located in the mantle layer in the ventral mesencephalic and rostrolateral rhombocephalic cellular groups that give rise to CA neurons of the substantia nigra and locus ceruleus, respectively (15). In contrast to the periphery, no cells displaying THase immunoreactivity were labeled by [^{3}H]thymidine (Figs. 6 and 7). Radiolabeled cells were found only in the ventricular lining and subventricular layers (Fig. 6), but no cells showing THase immunoreactivity were present in these layers.

DISCUSSION

By combining immunocytochemical and [^{3}H]thymidine radiolabeling, the present study has sought to determine whether the precursors of adrenergic neurons of both the peripheral and central nervous systems express neurotransmitter-synthesizing enzymes prior to the time they withdraw from the cell cycle. We have observed that adrenergic neuronal precursors that have not yet withdrawn from the cell cycle can express the neurotransmitter synthetic enzyme THase in the mammalian as well as the avian sympathetic peripheral nervous system. The expression of biosynthetic enzymes indicates that the stores of CA in these cells probably results from local synthesis. The presence of immunoreactivity for both THase and DBHase in peripheral ganglion cells of rats indicates, as others have shown (13, 14, 24), that these neurons are noradrenergic.

In contrast to peripheral sympathetic neurons, the earliest cells of the brain to display THase immunoreactivity failed to label with [^{3}H]thymidine. In fact, no cells in the developing central nervous system of fetal rats whose peripheral neurons contained THase and silver grains were labeled by [^{3}H]thymidine. Either the catecholaminergic cells of the central nervous system enter a prolonged pause in mitotic activity or they are postmitotic. Thus, adrenergic neurons of the developing central nervous system differ from those of the peripheral nervous system with respect to the relationship between differentiation and replication of precursor cells. The mechanism responsible for the ability of peripheral CA neuroblasts to divide after expressing biosynthetic enzymes, although those of the central nervous system do not, is unknown. Conceivably, it could relate to the different embryological origin of the two types of cell. The peripheral catecholaminergic cells are derived from neural crest (25–27) whereas those of the central nervous system arise from the neural tube (28). Thus, there appear to be significant differences, during development, between neurons derived from the neural crest and those derived from the neural tube, even if these neurons share a common neurotransmitter. The nature of this difference remains to be determined. There may be an intrinsic, preprogrammed difference between the two cell types. Alternatively, the microenvironment within which differentiation occurs may be responsible for the persistent replication of peripheral catecholaminergic cells.

A remaining unanswered question is whether cell division persists in CA cells of adult sympathetic ganglia or adrenal medulla or whether the ability to proliferate disappears as the animal matures. Although replication of mature adrenal medullary pheochromocytes (8) has been found, the proportion of replicating CA-containing cells decreases as ontogeny progresses (6, 8). Hendry has reported that, in rats, sympathetic neurons ultimately do withdraw from the cell cycle (29). Thus, replicating cells might represent an intermediate stage that peripheral neuronal precursors pass through. If so, the characteristic that marks the final passage from intermediate to mature neuron has not yet been identified. Moreover, the factor or factors responsible for the ultimate withdrawal of sympathetic neurons from the cell cycle are as yet unknown.

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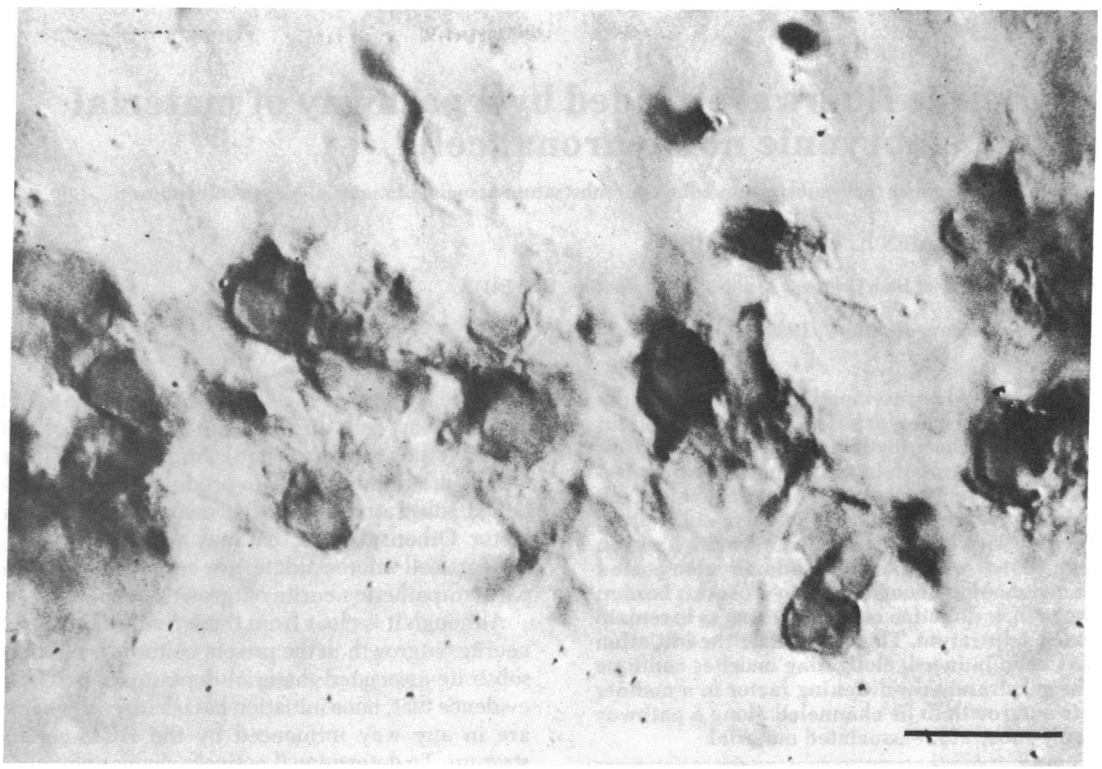

FIG. 7. Higher magnification of the cells in the mantle layer of the fetal rat brain, showing THase immunoreactivity. The nuclei of these neurons are not labeled by [^{3}H]thymidine. Bar = 20 μm.

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