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Appearance of catecholamine-synthesizing enzymes during development of rat sympathetic nervous system: Possible role of tissue environment

[immunocytochemistry/tyrosine 3-monooxygenase (tyrosine hydroxylase)/dopamine-β-hydroxylase/phenylethanolamine *N*-methyltransferase]

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ABSTRACT **We sought to determine, in rat embryo, when and at what site in their migration cells derived from the neural crest differentiate into sympathetic neuroblasts. This has been accomplished by immunocytochemical detection, within the cells, of the enzymes catalyzing catecholamine biosynthesis—tyrosine hydroxylase [TH; tyrosine 3-monooxygenase, L-tyrosine,tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] dopamine-β-hydroxylase [DBH; 3,4-dihydroxyphenylethylamine,ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1)]—and, as a marker of prospective adrenal medullary cells, the enzyme phenylethanolamine *N*-methyltransferase (PNMT; *S*-adenosyl-L-methionine:phenylethanolamine *N*-methyltransferase, EC 2.1.1.28). TH and DBH, not detected in the neural crest, appear almost simultaneously in cells of the thoracic sympathetic ganglia in 11-day-old embryos, and in abdominal and lumbar ganglia 1–2 days later, thereby exhibiting a characteristic rostral–caudal gradient of differentiation. Cells stained for TH and DBH are seen in the gut wall from day 11 to day 14, but not thereafter. Cells stained for TH and DBH appear in the adrenal anlage at day 15. However, PNMT is not detected in the adrenal until day 17 of development, and is present only in the sympathoblasts in contact with the adrenal cortex. Treatment of pregnant rats with dexamethasone failed to accelerate the appearance of PNMT in the embryo or to initiate its expression in cells of other sympathetic organs. We conclude that neural crest cells express a noradrenergic phenotype only after leaving the neural crest and that these cells are labile with respect to their neurotransmitter and are capable of transformation in response to environmental stimuli.**

The neural crest is a transient embryonic structure formed early in development along the dorsal surface of the neural tube. Soon after it forms, cells from the crest migrate widely and subsequently differentiate into a variety of cell types. Those cells that move dorsolaterally spread under the ectoderm to differentiate into melanocytes. Those cells that progress dorsoventrally give rise to chromaffin cells of the adrenal medulla and neurons of sensory and autonomic ganglia (1–3).

A critical issue in developmental neurobiology revolves around the question of when crest cells differentiate into their adult phenotypes. It has been variously proposed that they become committed either prior to (4) or during migration (5, 6), or after the cells arrive at their destination, possibly as a consequence of interaction with the end organ (7, 8).

A population of cells of neural crest origin that has been singled out for intensive study is the sympathoblasts,† cells that ultimately form the neurons of sympathetic ganglia and chromaffin cells of the adrenal medulla. Because, when differentiated, these cells synthesize and store catecholamines (norepinephrine in ganglia, and norepinephrine and epinephrine in the adrenal), it has been possible to track their biochemical differentiation by detection of the amines by histofluorescence. Thus, the development of sympathetic neuroblasts has been studied by fluorescence histochemistry in developing chicken (9), rabbit (10, 11), rat (12), and human (13, 14). In this manner it has been proposed that sympathoblasts differentiate after migration at a time when they aggregate to form primary sympathetic ganglia.

However, the histofluorescence technique as applied to these problems has, by its limitations, left several questions unresolved. First, the presence of catecholamines in sympathetic cells is not *a priori* proof that they are synthesized *in situ*. Conceivably they could be formed elsewhere and only stored in these cells.

Second, the cellular biosynthesis of catecholamines is sequential (15), involving the conversion of the amino acid precursor L-tyrosine to L-dopa by the enzyme tyrosine hydroxylase [TH; tyrosine 3-monooxygenase, L-tyrosine,tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2], the subsequent conversion of L-dopa to dopamine by aromatic-L-amino-acid decarboxylase (aromatic-L-amino-acid carboxy-lyase, EC 4.1.1.28), and hydroxylation of dopamine by the enzyme dopamine-β-hydroxylase [DBH; (dopamine β-monooxygenase, 3,4-dihydroxyphenylethylamine,ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1] to norepinephrine, the principal transmitter in ganglia. In the adrenal medulla norepinephrine can be further converted to epinephrine by the enzyme phenylethanolamine *N*-methyltransferase (PNMT; *S*-adenosyl-L-methionine:phenylethanolamine *N*-methyltransferase, EC 2.1.1.28). Thus, ganglia contain at least two, and chromaffin cells three, catecholamines, all of which fluoresce. It is not possible to distinguish between the various catecholamines (16–18) without more specialized histochemical techniques than have heretofore been applied in developmental studies. Hence, it is not known which catecholamines are present in various end organs populated by sympathoblasts, and whether the amine is the same as in adult cells.

A third and related question concerns the time of appearance of the chain of catecholamine-synthesizing enzymes in developing sympathoblasts. It has been proposed on the basis of biochemical evidence (19) that the enzymes appear sequentially

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Abbreviations: TH, tyrosine hydroxylase; DBH, dopamine-β-hydroxylase; PNMT, phenylethanolamine *N*-methyltransferase; PAP, peroxidase-antiperoxidase.

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† Sympathoblasts are defined as those progenitor cells originating in the neural crest destined to form the catecholamine-containing cells of the sympathetic ganglia, paraganglia, and adrenal medulla.

during development, suggesting that the adult phenotype is expressed progressively. However, the appearance of an enzyme protein may precede its detection by biochemical techniques. Therefore the possibility remains that some or all of the catecholamine enzymes appear in synchrony. Finally, PNMT is presumably expressed primarily in those sympathoblasts that populate the adrenal gland (20). The question is therefore raised as to whether this enzyme is expressed in cells prior to their contact with the adrenal anlage or only after they interact with the end organ.

It is evident that much more information with respect to the time and place of differentiation of sympathoblasts can be obtained by identification of the intracellular expression of the enzymes required for catecholamine biosynthesis. This approach has now been made feasible by the availability of highly specific antibodies against TH, DBH, and PNMT and techniques for localizing them immunochemically (21–25). In the present study we have therefore applied this method to monitor the differentiation of cells of neural crest origin into sympathetic ganglia and chromaffin cells. We shall demonstrate that during prenatal development: (*i*) TH and DBH appear simultaneously in crest cells at the time of aggregation into ganglia; (*ii*) PNMT is expressed only in those sympathoblasts that are in contact with the adrenal anlage; and (*iii*) a population of sympathoblasts is found to populate the gut transiently. These findings suggest that crest cells after migration first express a labile noradrenergic phenotype, which may be modified by influences exerted in part by the environment.

MATERIALS AND METHODS

Pregnant Sprague–Dawley rats were received after the first week of gestation and were housed individually. The day of appearance of the vaginal plug was considered the first day of pregnancy. The crown–rump length of an embryo from each pregnant female was measured to account for differences in the rate of development between different litters.

At the appropriate day of gestation, the females were anesthetized with pentobarbitol (40 mg/kg) and a small incision was made in the abdomen. The embryos were removed individually from the uterus and fixed in formalin [4%(wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4] for 2 hr. Embryos younger than 15 days were fixed whole; in older embryos the upper abdominal region was isolated under formalin. Tissues were embedded in 30% sucrose overnight at 5°C, and on the following day were mounted on tissue holders in 30% sucrose, frozen with dry ice, and serially sectioned at 15 μm in a cryostat microtome at −20°C. At least 10 embryos were examined at each gestational age from 11 days to birth. In all, 128 embryos were studied. The sections were melted onto glass slides that had been precoated with a solution of gelatin. They were then kept at room temperature for up to 2 weeks until processed for staining with antibody, a procedure which did not alter the specificity or intensity of the reaction.

The procedure for preparation of antibodies to TH, DBH, and PNMT, as well as the criteria used to judge their specificity, has been previously described (21–23). Immunohistochemical staining by the soluble peroxidase-antiperoxidase (PAP) method of Sternberger *et al.* (24) was carried out essentially as described by Pickel *et al.* (25). The sections were first incubated for 15 min in a 0.25% solution of Triton X-100 in 0.5 M Tris·HCl/saline buffer, pH 7.6, and then for 30 min in a 30% solution of goat serum in Tris/saline buffer to reduce nonspecific binding. The sections were then incubated overnight in a humid chamber at 5°C with a rabbit anti-enzyme. The antibodies were diluted (vol/vol) in the Tris/saline buffer plus 1% goat serum as follows: TH, 1:1200; DBH, 1:1000; rabbit anti-rat PNMT, 1:2000; rabbit anti-bovine PNMT, 1:1000.

Groups of three consecutive slides obtained from different regions of the embryo were incubated, individually, with each type of antibody. The next day the sections were sequentially incubated with goat anti-rabbit IgG (1:10 dilution with Tris/saline plus 1% goat serum) and PAP (1:30 dilution with Tris/saline plus 1% goat serum). Each step was followed by two 5-min washes in Tris/saline plus 1% goat serum. The bound peroxidase was allowed to react for 6 min with 0.013% 3,3′-diaminobenzidine (Sigma) and 0.01% hydrogen peroxide (30% solution) in 0.5 M Tris·HCl buffer, pH 7.6, to form a brown precipitate. After a 10-min wash in distilled water the sections were dehydrated and mounted in Permount.

To examine whether the expression of PNMT immunoreactivity could be influenced by glucocorticoids, six pregnant rats (approximate weight 250 g) were injected on days 12–16 of gestation with 2–4 mg/kg per day of dexamethasone (Decadron; Merck, Sharp and Dohme) and sacrificed one day later. The embryos were removed and processed for immunocytochemical localization of the enzymes TH, DBH, and PNMT.

The timing of differentiation was determined by examination of rostro–caudal gradients in the developing embryo. This method, introduced by Detwiler (26) and extensively used in experimental neuroembryology, takes advantage of the fact that in young embryos there is a continuous gradient of differentiation along the rostro–caudal axis. Thus the neural crest, first appearing in the head, gradually proceeds to form, over time, towards the tail. Differentiation of neural crest cells also shows the gradient. Thus it is possible, in the same embryo, to sample cellular differentiation along the evolving crest at stages of small spatial intervals, reflecting in turn equally small temporal intervals. The technique has the advantage for timing maturation over a method in which different embryos from different litters are sampled at different days of gestation in that it has higher temporal and spatial resolution, is not susceptible to variations in maturation between littermates, and permits use of fewer animals and hence conserves antibodies.

RESULTS

Cells containing TH and DBH were first detected in the anterior region of 11-day-old embryos (approximately 6-mm crown–rump length). These cells were localized in a dorsolateral position with respect to the paired descending aorta and represent the primordia of the thoracic ganglia. At the same time, no labeled cells were detected in the abdominal region, following the well known rostral–caudal gradient of differentiation (26).

While both TH and DBH were present in consecutive sections of the rostral ganglia (Fig. 1*A*), neither of these enzymes was detected in progressively more caudal areas. At no time did primary ganglia stain for one enzyme but not for the other. Thus, these two enzymes appear simultaneously or nearly so in the developing sympathetic chain.

In immature ganglia (Fig. 1), cells containing TH and DBH had an irregular shape and loosely contacted each other by long processes (Fig. 1*B*). After the 13th day of development, small numbers of cells containing the enzymes were found in the abdominal region along the ventrolateral aspects of the aorta (Fig. 1*C*). Presumably these cells migrated ventrally from the primary ganglia and formed, at later stages, the paraganglionic system and the prevertebral ganglia (11, 27).

In 11-day-old embryos, cells containing TH and DBH were also observed in the mesoderm of the gut wall. These cells were not detected after the 14th day of development (Fig. 2).

In embryos of 14 days of gestation, cells stained for TH and DBH were found in a plexus that had formed near the anlage of the adrenal gland and at the level of the kidneys. In 15-

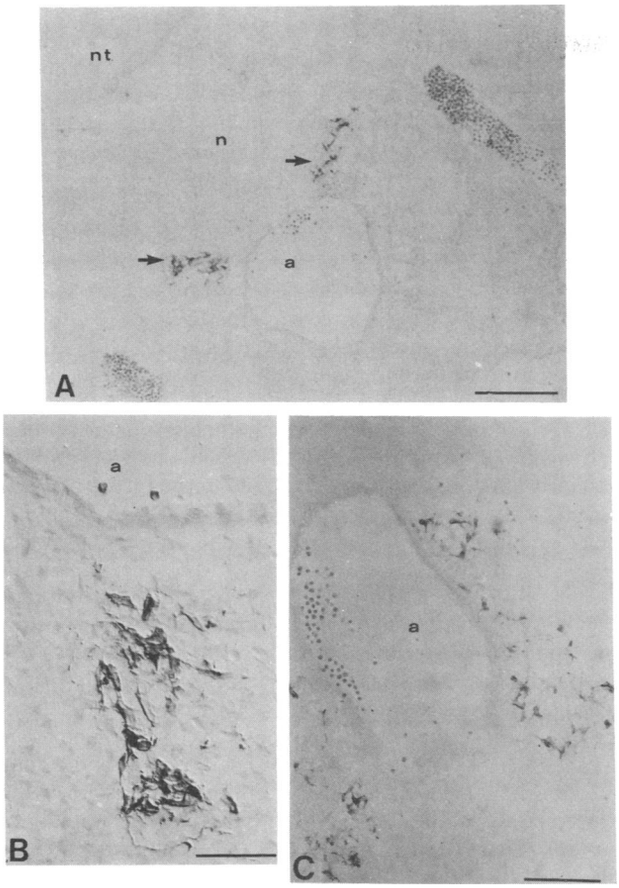

FIG. 1. Immunocytochemical localization of TH and DBH in sympathoblasts of young rat embryos. a, Aorta; nt, neural tube; n, notochord. (*A*) TH-containing cells (arrows) present in the region of the future sympathetic chain of 13-day-old embryos. Bar = 100 μm. (*B*) Photomicrograph of lower ganglionic group of *A* obtained with Nomarski interference optics. Bar = 40 μm. (*C*) DBH-containing cells are shown alongside the dorsal aorta in 14-day-old rat embryos. Bar = 100 μm.

day-old embryos, cells of the adrenal anlage formed an oval mass that in older embryos protruded into the dorsal coelomic cavity. From this stage on, strands of cells containing TH and DBH were found between the suprarenal ganglion and the closely associated adrenal anlage. On the 16th day of development the stained fusiform cells were poorly organized within the cortical tissue (Fig. 3). In the newborn the sympathoblasts had sorted out from the mesodermal cells, forming rosettes (Fig. 4); the cells had assumed the shape of the mature chromaffin cells, with a large nucleus and scanty cytoplasm.

PNMT was first detected in a few adrenal medullary cells in 17-day-old embryos. In older embryos the number of cells containing the enzyme had greatly increased. Cells containing PNMT were observed in contact with the outer surface of the adrenal cortex but were never found in either sympathetic ganglia or aortic plexii.

In newborn pups the number and distribution of stained cells were very similar in sections incubated, respectively, with antibodies against TH, DBH, or PNMT (Fig. 4). Unfortunately, it was not possible by the present technique to determine whether these enzymes were all present in the same cells.

The number of cells containing TH, DBH, and PNMT in the adrenal medulla increases significantly after the 18th day of development. This augmentation can be accounted for by the incorporation of newly arrived sympathoblasts into the adrenal gland and, perhaps, their proliferation *in situ* (28, 29).

While most of the cells of the immature sympathetic ganglia contain TH and DBH, it was observed that the number of stained cells decreased by the newborn stage, in particular in the suprarenal ganglia. This finding may reflect the beginning of the degenerative changes that most extra-adrenal chromaffin tissue is known to undergo after birth (30, 31).

Because in adults the synthesis and accumulation of PNMT

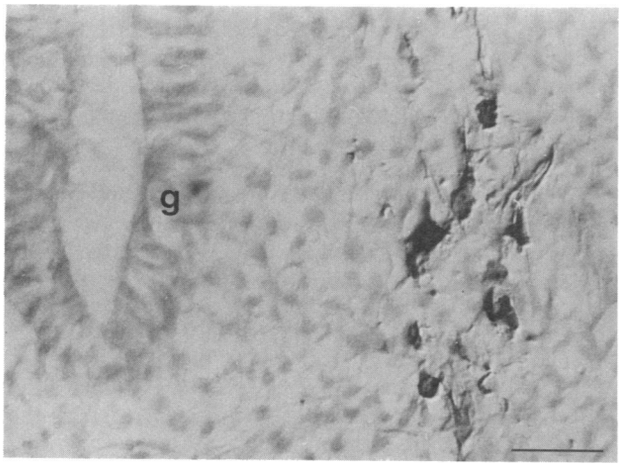

FIG. 2. Immunocytochemical localization of TH in cells present in the gut wall of 14-day-old rat embryo. Photomicrograph obtained with Nomarski interference optics. g, Gut. Bar = 20 μm.

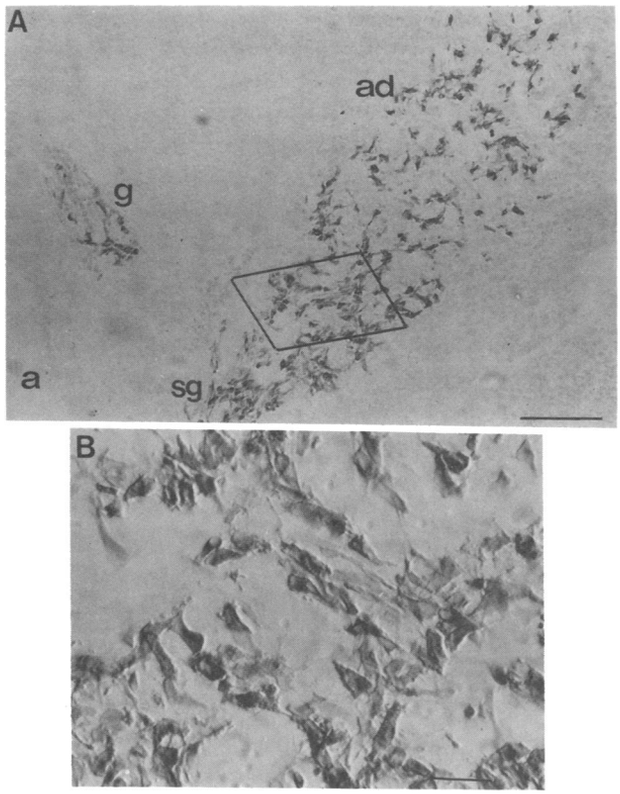

FIG. 3. DBH localized by the PAP method in frozen sections through the adrenal anlage of 16-day-old rat embryos. (*A*) Low magnification photomicrograph shows stained cells scattered within the adrenal primordium (ad). g, Primary sympathetic ganglion; sg, suprarenal ganglion; a, aorta. Bar = 100 μm. (*B*) Photomicrograph obtained with Nomarski interference optics of the area within the rhombus in *A*. Note the specific cytoplasmic localization of the antigenic precipitate as indicated by presence of negatively stained nuclei. Bar = 20 μm.

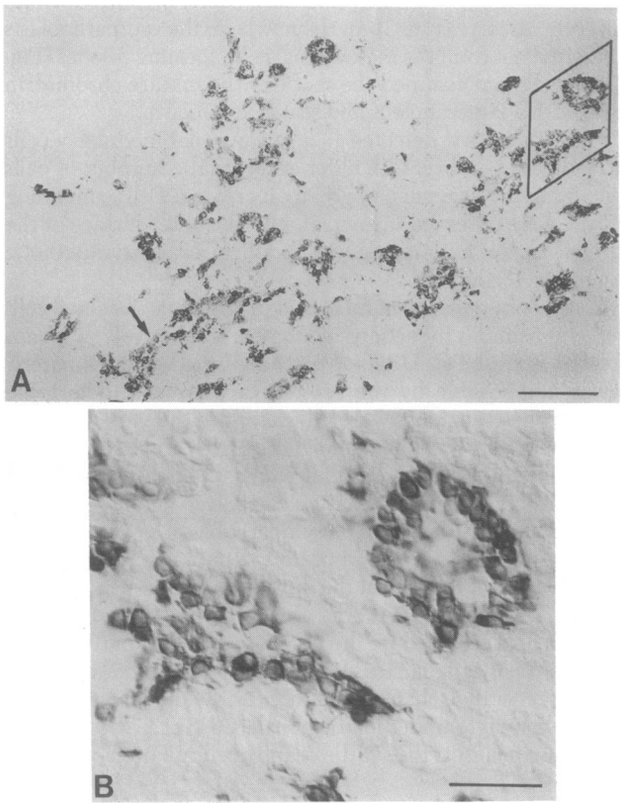

FIG. 4. (*A*) PNMT-containing cells visualized by the PAP method in the adrenal gland of newborn rats. Note that the number and organization of the cells are similar to those shown in Fig. 3. Bar = 100 μm. (*B*) Photomicrograph obtained with Nomarski interference optics of the area within the rhombus in *A*, showing precise cytoplasmic localization of the staining. Lower cell group is still poorly organized. Bar = 30 μm.

is controlled by glucocorticoids (20), we investigated whether the administration of dexamethasone to pregnant rats could accelerate the appearance of PNMT in embryonic adrenal cells and even promote its expression in extra-adrenal sympathoblasts (32). Dexamethasone (2–4 mg/kg per day) was administered to pregnant rats from day 12 to day 16 of gestation, and the animals were sacrificed one day later. In this dose range dexamethasone will restore PNMT activity to normal levels in adult hypophysectomized rats (20), and enhance the activity of PNMT and content of epinephrine in the superior cervical ganglia of newborn rats (32, 33). Dexamethasone did not elicit the appearance of PNMT prior to the 17th day of development in the sympathoblasts in adrenal or in cells of the abdominal sympathetic ganglia or paraganglia.

DISCUSSION

The present study has sought to determine when and where cells of the neural crest differentiate biochemically into sympathetic neuroblasts. This has been achieved by the immunocytochemical localization of the enzymes that specifically subserve the biosynthesis of catecholamines—i.e., TH, DBH, and PNMT. The underlying assumption has been that expression of these enzymes is evidence of differentiation. By testing for the presence of all three cell-specific macromolecules, it has been possible to determine when sympathoblasts express a differentiated phenotype, and where in the pathway of migration differentiation occurs. Moreover, because in adults PNMT is primarily restricted to the chromaffin cells of the adrenal medulla (31, 34), it has been possible to establish if expression of this enzyme depends upon an interaction of sympathoblasts with their end organ.

It is not until the 11th day of development that TH and DBH can be detected, almost simultaneously, in cells within the primordia of the sympathetic ganglia. In contrast, catecholamine-synthesizing enzymes could not be detected in cells still within the neural crest. The time of appearance of the enzymes is approximately coincident with the time when catecholamines can first be detected in the same cell population by histofluorescence (9–14). Taken together, the immunocytochemical and histofluorescence findings suggest that: (*i*) overt differentiation of crest cells into sympathoblasts does not occur prior to their arrival at the site of the future sympathetic chain; (*ii*) the catalytic activity and immunoreactivity of TH and DBH are expressed at the same time; (*iii*) sympathoblasts can synthesize and store catecholamines at this early stage of development; (*iv*) the catecholamine contained in sympathoblasts in the sympathetic chain (9–14) is most probably norepinephrine; and (*v*) as soon as sympathoblasts reach the site of the future ganglia, they express a noradrenergic phenotype.

The simultaneous appearance of TH and DBH in developing sympathetic neurons is also of interest. In a biochemical study of whole chicken embryos, Ignarro and Shideman (19) found that the catecholamine enzymes appear sequentially during development, with a time interval of even a day or more between the appearance of each consecutive enzyme in the chain. However, differences in sensitivity of the enzymatic reactions or regional variations could account for the delayed detection of some of these enzymes. In the present study, the coincident appearance of TH and DBH revealed by immunocytochemistry suggests that their biosynthesis is probably activated in a coordinate fashion, perhaps by the same environmental signals.

While cells containing TH and DBH do not appear within the adrenal anlage until the 15th day of development (four days after their appearance in the sympathetic ganglia), PNMT is not detected until the 17th day. The delay in the appearance of PNMT, and its restriction to sympathoblasts that contact the fetal adrenal cortex, suggests that the expression of this enzyme may depend upon an interaction with the end organ. The nature of the initiating signal is unknown. Possibly it may be mediated by adrenal corticoids, because it has been proposed (20, 35, 36) that glucocorticoids normally induce PNMT. However, the failure of large doses of dexamethasone administered to pregnant rats to elicit the appearance of PNMT before the 17th day of development suggests that factors other than glucocorticoids may be required for the expression of the enzyme.

Another population of cells containing TH and DBH was found in the gut wall of rat embryos. These noradrenergic cells were detected only from day 11 to day 14 of development. Because these cells disappear at later stages of development and are not detected in the adult rat (37), questions are raised as to their fate. Conceivably, their presence in anomalous locations results in their death. Alternatively, the transient population of noradrenergic cells may be transformed, either prior to or after birth, into enteric ganglioblasts. This assumption is supported by reports that crest cells from the vagal level of the neural axis populate the gut and differentiate into enteric (probably cholinergic) neurons (38).

In summary, our study indicates that the cells from the neural crest destined to populate the autonomic nervous system express an overt noradrenergic phenotype only upon reaching the primordium of the sympathetic chain. However, the initial metabolic commitment is apparently mutable, because it can be modified in response to environmental cues, thereby affecting the character of the adult cell. For example, those cells that populate the sympathetic ganglia remain noradrenergic,

those that invade the adrenal gland synthesize PNMT and hence become adrenergic, while those that colonize the gut lose their catecholaminergic character and perhaps develop into neurons containing other transmitters.

The contention that developing autonomic neurons are initially pluripotent is supported by evidence from studies of isolated neural crest cells (39–42) and by recent *in vitro* (43–46) and transplantation (7, 8) studies which have shown that the neurotransmitter produced by sympathetic ganglion cells can be influenced by the cellular environment. Our experiments provide *in vivo* support for the contention that autonomic neurons are initially pluripotent and that their differentiation may be influenced by their environment.

Note Added in Proof. After submission of this paper for review, Cochard *et al.* (47) demonstrated that TH-containing sympathoblasts appear in the primordia of sympathetic ganglia and transiently in the gut at the same times in development as those reported here.

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