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Transformation of catecholaminergic precursors into glucagon (A) cells in mouse embryonic pancreas

[tyrosine 3-monooxygenase (tyrosine hydroxylase)/peptides/development/immunocytochemistry]

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ABSTRACT **In embryonic mice, the catecholamine biosynthetic enzyme tyrosine hydroxylase [L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] can be visualized immunocytochemically in a population of cells in epithelial cords of the developing pancreas. These embryonic catecholamine cells, first seen by day 11, are large and vacuolated and have a folded nuclear membrane. One day later, at day 12, glucagon is first detected immunocytochemically in pancreatic cells similar in location and morphology to the embryonic catecholamine cells. By use of a method for detecting both antigens in the same cell, both the hydroxylase and glucagon can be visualized between day 12 and day 14 in 10–40% of stained cells. From day 14, the number of cells stained for hydroxylase decreases; they cannot be detected after day 18. In contrast, the cells containing glucagon increase during development and persist throughout life. Endocrine cells of the embryonic pancreas also contain dopa decarboxylase but not dopamine-β-hydroxylase or phenylethanolamine-*N*-methyl transferase. In adult mice, small cells containing tyrosine hydroxylase but differing in location and morphology from the embryonic catecholaminergic cells are seen in pancreatic islets. The adult catecholaminergic cells never store glucagon. We suggest that adult glucagon (A)-containing cells arise from transformation *in situ* of cells that transiently express a catecholaminergic (probably dopaminergic) phenotype. These results suggest that one class of peptidergic cells may arise from transformation of an aminergic precursor.**

We have recently discovered that, during prenatal development of rats and mice, tyrosine hydroxylase [TyrOHase; tyrosine 3-monooxygenase, L-tyrosine,tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2], which catalyzes the first step in the biosynthesis of catecholamines, appears in cells in the primordial gut (1, 2).* The expression of the catecholaminergic phenotype is transient; in 2 or 3 days, the enzyme can no longer be detected in enteric cells (1–3).* As catecholaminergic cells are not present in adult gut, the observation raises the question: Do the TyrOHase-containing cells transform into gut cells, possibly neurons, that contain other neurotransmitters or neuropeptides (or both)? However, as the peptides are first visualized in gut cells after the disappearance of TyrOHase (4), it is technically difficult *in vivo* to establish such a transition.

Also, we have detected cells containing TyrOHase in the pancreatic anlage in embryonic mice at day 11 of development. As the pancreatic polypeptide, glucagon, appears in embryonal cells at about the same time (5–7), we sought to determine, by immunocytochemical localization of two antigens in the same histological section, whether the transient catecholaminergic cells of mouse pancreas also contain glucagon.

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MATERIALS AND METHODS

Preparation of Tissues. Pregnant CD-1 mice (white outbred) were received during the first week of gestation and housed individually. The day of appearance of the vaginal plug was considered the first day of pregnancy. In all, 45 embryos were studied. Mice were killed at the appropriate day of gestation by cervical dislocation. The embryos were removed and fixed in formalin [4% (wt/vol) paraformaldehyde]/0.1 M Na phosphate, pH 7.4 for 3 hr at 5°C. Tissues were embedded in 30% sucrose overnight and the following day were sectioned at 15 μm in a cryostat microtome at −20°C. The sections were melted onto glass slides precoated with gelatin. Other embryos were fixed in a similar way, dehydrated in alcohol, embedded in paraffin at 56°C for 30 min at reduced pressure, and sectioned at 5 μm. Adult mice were anesthesized with pentobarbitol (40 mg/kg) and perfused through the heart with formalin/Na phosphate for 5 min. The pancreas was removed, postfixed in cold formalin for 1 hr, washed twice with 0.1 M Na phosphate, embedded in 30% sucrose, and sectioned in a cryostat microtome.

Source of Antibodies. TyrOHase and aromatic-L-amino-acid decarboxylase [dopaDCase (dopa, 3,4-dihydroxyphenylalanine), aromatic-L-amino-acid carboxy-lyase, EC 4.1.1.28] were purified from adrenal medulla of rat, and dopamine-β-hydroxylase [3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1] and phenylethanolamine-*N*-methyltransferase (S-adenosyl-L-methionine: phenylethanolamine *N*-methyltransferase, EC 2.1.1.28) were purified from bovine adrenal. The techniques for purification of enzymes, production of antibodies, and criteria for establishment of specificity have been described (8–10). Antibodies against glucagon were purchased from Calbiochem.

Immunohistochemical Staining. *Single label.* Immunohistochemical staining was performed by using the soluble peroxidase–antiperoxidase method (11) as modified for use in embryos (2). The antibodies were diluted (vol/vol) in Tris/saline/1% goat serum—TyrOHase, 1:1000; dopaDCase, 1:500; dopamine-β-hydroxylase, 1:1000; the methyltransferase, 1:1000. The glucagon antibody was purchased at a 1:50 dilution and further diluted 1:20 so that the total dilution was 1:1000. The bound antibody was visualized by incubation with 0.043% 3,3-diaminobenzidine (Sigma)/0.01% H_2O_2 (30% solution) in 0.2 M Tris·HCl, pH 7.6, to form a brown reaction product. The sections then were dehydrated and mounted in Permount. In sections chosen for sequential staining, 4-chloro-1-naphthol, (Sigma) was used as reducing agent rather than diaminobenzidine, which is insoluble in organic solvents.

Double label. To perform sequential staining for TyrOHase

Abbreviations: TyrOHase, tyrosine hydroxylase; dopa, 3,4-dihydroxyphenylalanine; dopaDCase, aromatic-L-amino-acid carboxy-lyase.

* Gershon, M., Teitelman, G., Rothman, T., Joh, T. H. & Reis, D. J. (1979) *Society for Neuroscience*, Atlanta, GA, p. 334 (abstr.).

and glucagon by the indirect immunoperoxidase technique, first, the sections were incubated with antibodies against glucagon, the bound antibody was visualized by reaction with 0.043% 4-chloro-1-naphthol/0.01% H_2O_2 (12), and the sections were mounted in buffered glycerine and photographed. Then, the sections were immersed in phosphate-buffered saline, the coverslips were removed, and the blue reaction product of the chloronaphthol was removed by dehydration to xylene. The sections were then rehydrated and the tissue-bound antibodies were removed as described by Tramu *et al.* (13) and modified by Nilaver *et al.* (14). Essentially, the slides were immersed first in 2.5% $KMnO_4$/5% H_2SO_4, distilled H_2O, 1:1:30 (vol/vol) for 1 min and then in Na metabisulfite in distilled water for 2 or 3 min. The tissues were washed for 2 hr in running tap water, washed twice with phosphate-buffered saline and incubated overnight with antibody against TyrOHase. The following day, the bound antibody was localized by the brown precipitate of the diaminobenzidine reaction product. The sections were rephotographed for comparison with the photomicrographs from the first immunocytochemical sequence.

Controls of the Elution Technique. Control experiments were performed to test the efficiency of the elution technique. Sections were processed for localization of a primary antibody (TyrOHase or glucagon). After elution, the sections were incubated with phosphate-buffered saline or rabbit preimmune serum and again processed for localization of bound antibodies. This procedure will stain the sections if the antibodies used prior to the elution step are not completely removed. However, in all cases, the sections were blank after the staining sequences, indicating complete elution of the first antibody. In another control experiment, cross-sections of mouse embryos were first incubated with a TyrOHase antibody that stained sympathetic ganglia and pancreatic cells. After elution and subsequent incubation with antibody to glucagon, only the pancreatic cells were stained.

RESULTS

Appearance of TyrOHase in Pancreas. In agreement with previous results,* immunoreactive TyrOHase first appeared in the mouse embryo on day 10 of gestation. The enzyme was localized in cells in the mesenchyme of the gut. On day 11, a few cells containing TyrOHase were observed in the sympathetic ganglia and in the dorsal pancreatic anlage.

By day 12, 12–50 stained cells were present in each section of the pancreatic primordia (Fig. 1). These embryonic catecholamine cells were localized in clusters in the cord-like epithelial evaginations that form the pancreatic primordia and were contiguous with unstained cells. The intensity of the immunohistochemical staining of TyrOHase in the embryonic pancreatic cells was less than that in sympathetic cells in the same section.

The pancreatic TyrOHase-containing cells had a characteristic morphology: they were large (18–22 μm), they contained numerous cytoplasmic vacuoles that appeared empty when the tissue was processed through organic solvents, and they had a folded nuclear membrane (Fig. 1). We have termed these cells embryonic catecholaminergic cells.

Appearance of Pancreatic Glucagon. At day 12, cells containing glucagon were detected in the pancreatic anlage (Fig. 2A). Cytologically, these cells were identical to those containing TyrOHase in that they were of comparable size (18–22 μm) and vacuolated, they contained a folded nuclear membrane, and they were localized in the epithelial-like cords of the pancreatic rudiment. The presence of numerous cytoplasmic vacuoles and the restriction of the staining to the basal pole of the cells resulted, in many instances, in a characteristic lack of definition of the cytoplasmic boundaries.

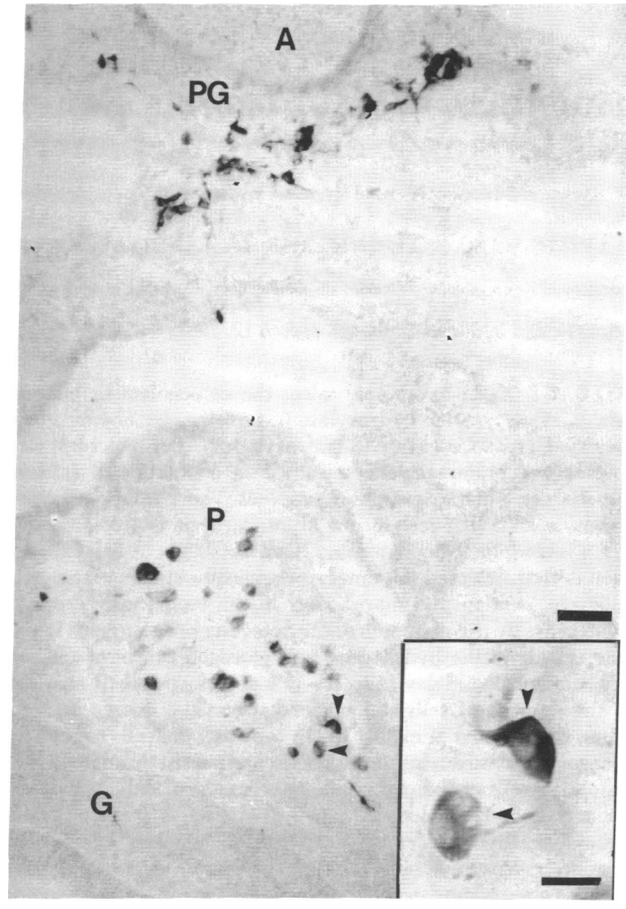

FIG. 1. Immunocytochemical localization of TyrOHase-containing cells in a cross-section of day 12 mouse embryo. Stained cells are found in the perivisceral ganglia (PG) and in the pancreas (P). A, dorsal aorta; G, gut. (Bar = 120 μm.) (*Inset*) High-magnification photomicrograph of TyrOHase-containing-cells in the pancreas. Arrows indicate cells having a folded nuclear membrane. (Bar = 14 μm.)

Coexistence of TyrOHase and Glucagon in the Same Pancreatic Cells. To determine whether TyrOHase and glucagon coexisted in the same cell, sections of pancreas from day 12 embryos were first processed for localization of glucagon and photographed. Then, the antibodies were eluted, and the tissues were processed for localization of TyrOHase. As seen in Fig. 2, in which localization of glucagon is compared with localization of TyrOHase on the same section, a population of cells in the pancreas contains both antigens. At day 12, the proportion of cells that contained both antigens (phenotypic hybrids) varied, in different sections, from 0% to 40% of all cells that stained for either antigen. When the results from 10 different double-labeled experiments were averaged, ≈10% of all stained cells were double labeled. The remainder of the stained cells were usually divided equally between those having only TyrOHase and those having only glucagon. In all cases, however, the cells containing TyrOHase, those containing glucagon, and those having both were always clustered in the same region of the developing pancreas, while other areas of the organ lacked stained cells of any kind.

Fate of the Labeled Embryonal Cells. At day 14, the last developmental stage in which the double-staining technique was performed, the embryonic pancreatic cells containing TyrOHase, glucagon, or both were still found in the pancreatic primordia in approximately the same numbers and proportions as on day 12. By day 16, however, the number of embryonic cells containing TyrOHase had diminished greatly; by day 18,

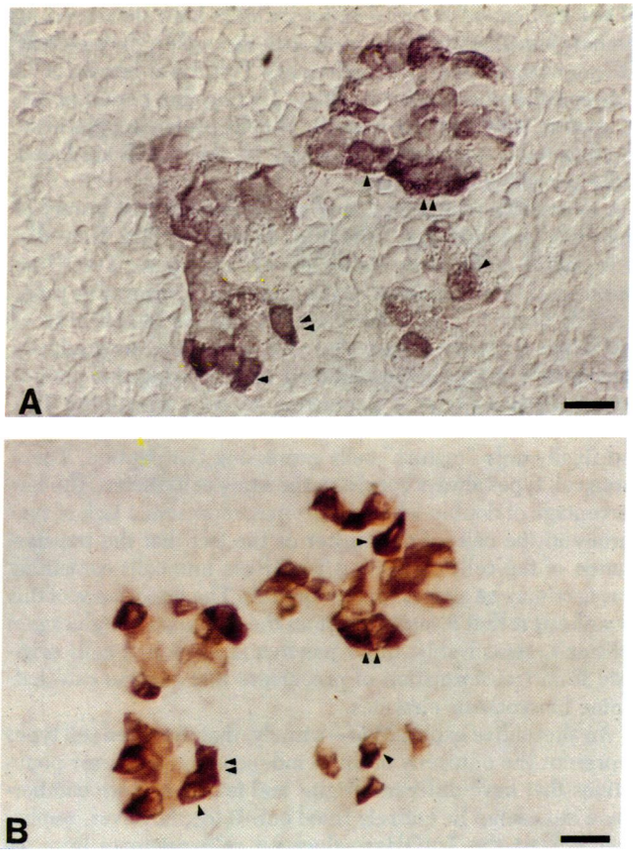

FIG. 2. Immunocytochemical localization of glucagon (*A*) and TyrOHase (*B*) in the same section of pancreas of day 12 embryo. Note the presence of cells containing only glucagon (single arrows in *A*), only TyrOHase (single arrows in *B*), and TyrOHase-glucagon (double arrow). Cytoplasmic vacuoles were visualized in *A*, but removed in *B* by organic solvents. (Bars = 22 μm.)

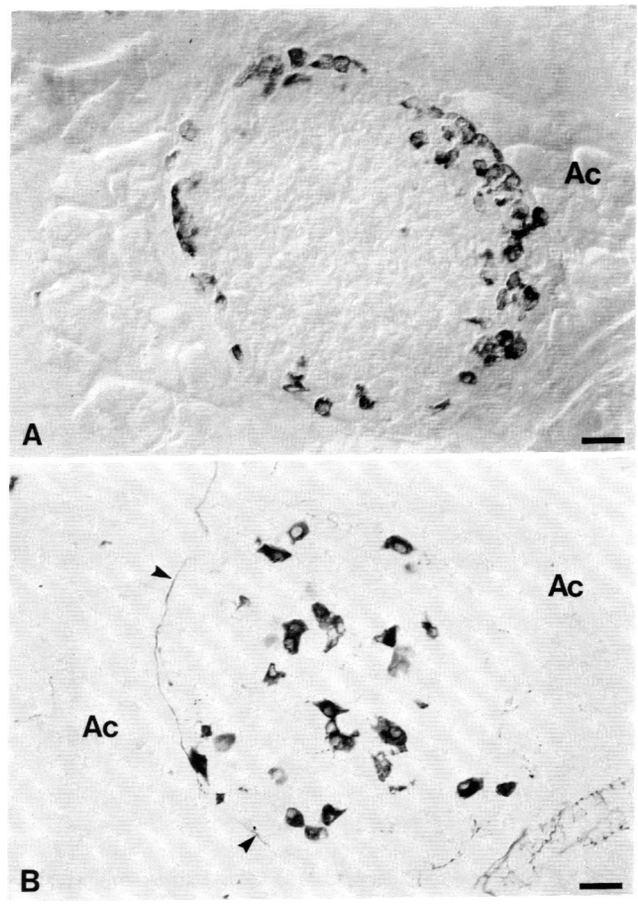

FIG. 3. Immunocytochemical localization of glucagon (*A*) and TyrOHase (*B*) in the same section of pancreas of adult mouse. Note noradrenergic nerves (arrows) surrounding islet. Ac, acinii. (Bars = 15 μm.)

these were seen only rarely; and in the newborn and adult, they were absent.

In contrast, the number of cells containing glucagon progressively increased. By day 18, most of the glucagon-storing cells were small (13–15 μm) and unvacuolated and had assumed a position characteristic of the adult glucagon cells in the periphery of the islets (15).

Adult Pancreas. In the adult, the pancreatic islets were once again populated with cells that stained for TyrOHase. However, these differed in several respects from the embryonic catecholaminergic cells. The adult TyrOHase-containing cells (*i*) were smaller (13–15 μm); (*ii*) were distributed between rather than within the cords; (*iii*) lacked cytoplasmic vacuoles; and (*iv*) had an unfolded nuclear membrane (Fig. 3*B*).

Moreover, when the adult pancreas was processed for localization of both TyrOHase and glucagon, the two antigens were never observed in the same cells. Rather, the two cell types were topographically distinct: the glucagon-containing cells surrounded the islets (Fig. 3*A*), while the TyrOHase-containing cells were scattered at random within them (Fig. 3*B*).

Other Catecholamine Biosynthetic Enzymes. Sections of the pancreas from day 12, 14, 16, and 18 embryos and from adult mice were processed for localization of other enzymes required for catecholamine biosynthesis—i.e., dopaDCase, dopamine-β-hydroxylase, and the methyltransferase. At day 12, clusters of cells lightly stained for dopaDCase were visualized in the pancreatic rudiment. At this stage, the cells were indistinguishable topographically and cytologically from cells that contained TyrOHase, glucagon, or both antigens: they were localized in the epithelial cords, were of comparable sizes and vacuolated, and had indented nuclear membranes (Fig. 4*A*).

The number of cell clusters containing dopaDCase and the intensity of staining in each cell increased during development. In the adult mouse, almost all islet cells contained dopaDCase (Fig. 4*B*). On the other hand, at any time, cells of the endocrine pancreas contained dopamine-β-hydroxylase or the methyltransferase.

DISCUSSION

We have shown, by use of an immunocytochemical technique that a population of cells containing the catecholamine biosynthetic enzyme TyrOHase appears in the anlage of the mouse pancreas on day 11 of development. This is 1 day after the enzyme can first be detected anywhere in the mouse embryo (in gut) and about the same time at which it appears in sympathetic ganglia.* At this stage, dopaDCase also is present in pancreatic cells but dopamine-β-hydroxylase and the methyltransferase are not.

On the basis of the enzymatic profile, the only catecholamine that could be synthesized by embryonic catecholaminergic cells is dopamine. As the mouse pancreas at this stage lacks endogenous histofluorescence (16), the concentration of dopamine is probably too low to be visualized by histochemical techniques. However, dopamine histofluorescence has been detected in pancreatic cells as early as day 11 after administration of L-dopa. (6, 16–19).

The origin of the embryonic catecholaminergic cells is un-

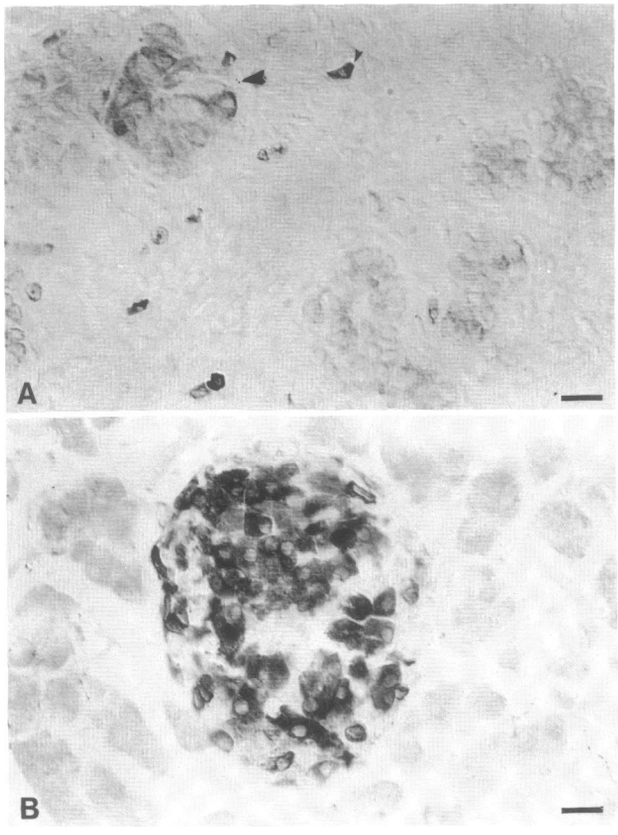

FIG. 4. Localization of dopaDCase by the peroxidase–antiperoxidase method in sections through the pancreas. (*A*) Day 12 embryo. (Bar = 22 μm.) (*B*) Adult. Note that most cells of the islet contain dopaDCase. (Bar = 15 μm.)

certain. To date, all cells in the periphery that express one or more specific catecholamine biosynthetic enzymes (TyrOHase, dopamine-β-hydroxylase, or the methyltransferase) are of neuroectodermal origin (20–25). On this basis, it seems possible that the embryonic pancreatic catecholaminergic cells also arise from the ectodermal germ layer. It is unlikely, however, that they originate from the neural crest. The only neural crest derivatives so far detected in the pancreas differentiate into parasympathetic ganglion cells and do not display catecholamine histofluorescence (20, 23). However, the possibility that the cells are endodermal must also be considered as present evidence suggests that all pancreatic cells arise from that germ layer (25). If indeed the embryonic catecholaminergic cells are endodermal, this would show that cells expressing catecholamine biosynthetic enzyme(s) can arise from layers other than the ectoderm.

By using a technique for identifying two antigens in the same cell, we have discovered that, by day 12, some of the embryonic dopamine cells also store glucagon. These double-labeled cells are contiguous in pancreatic cords with other cells, some of which contain only TyrOHase, others only glucagon, and still others neither antigen. Each section of the embryonic pancreas typically contains only one or two clusters of stained cells. Morphologically, the three positively stained cell types (i.e., TyrOHase-glucagon, TyrOHase alone, and glucagon alone) are similar in size, have folded nuclear membranes, and are vacuolated. Interestingly, the characteristics of these cells are similar to those of pancreatic A (glucagon) cells during their early embryonic growth (25).

For the first few days after they appear, there are few double-labeled cells, an average of 10% of all stained cells but, by day 16, the embryonal catecholaminergic cells have decreased in number, presumably to disappear soon thereafter; the pancreas of the newborn mouse does not contain TyrOHase-labeled cells of comparable location or cytology. During this period of development, however, the number of cells containing glucagon simultaneously increases and, by day 18, these cells are found in the periphery of the islets.

A reasonable interpretation of the developmental history of pancreatic dopaminergic cells (Fig. 5) assumes that, from day 11 to day 14 or 15, a population of cells expressing TyrOHase continuously differentiates in the pancreas. During this time, the cells begin to synthesize glucagon and the biosynthesis of TyrOHase gradually ceases. The cells, therefore, are gradually transformed to glucagon-containing cells. During this period, the pancreas contains a population of cells containing TyrOHase, others in transition containing TyrOHase and glucagon, and finally only "mature" cells expressing the peptide. These three cell types always coexist in the same cell cluster. The low percentage of double-labeled cells may represent a lack of synchrony in the cells of the cluster or the fact that the transformation of the cells containing TyrOHase into cells containing glucagon may be a rapid process or both. The importance of this hypothesis is that it implies that all the pancreatic A (glucagon) cells and, conceivably, other pancreatic endocrine cells originate from transformation of precursors that express catecholamine biosynthetic enzymes.

An alternative explanation—namely, that the three cell types represent the ontogeny of three independent precursor populations that have different origins and fates—is more cumbersome but cannot be entirely ruled out. It too, however, implies that at least the TyrOHase-glucagon cells originate from catecholaminergic precursors.

In the adult, a population of cells containing TyrOHase is again seen in the pancreas. As all the islet cells of the adult pancreas contain dopaDCase, the cells containing TyrOHase are probably dopaminergic. These adult dopaminergic cells differ cytologically and topographically from the embryonic catecholaminergic cells: they are smaller and unvacuolated, have an unfolded nuclear membrane, and are localized between rather than within epithelial cords. However, we found no clear transitional forms between embryonic and adult TyrOHase-containing cells, and the origin of the adult dopamine cell remains uncertain. These cells could (*i*) arise from embryonic cells that moved out of the cords and had transiently reduced the expression of TyrOHase, (*ii*) represent a migration of different populations of precursor catecholaminergic cells into the pancreas, or (*iii*) arise from differentiation of intrinsic pancreatic precursor cells late in development.

It has been established that many pancreatic islet cells have the ability to take up, store, and decarboxylate amine precursors—notably, L-dopa to dopamine and 5-hydroxytryptophan to serotonin (19). The capacity to transform L-dopa has been detected by histofluorescence in the mouse as early as day 11 of development (6, 7) and persists in the adult (26). Pearse (27–29) has proposed that these pancreatic endocrine cells, as well as other widely distributed endocrine-like cells, were part of the diffuse neuroendocrine system. Such cells, Pearse postulates, are of neural origin and share a capacity for synthesizing amines and peptides. He has grouped these cells together into what he terms an APUD series (*A*mine *P*recursors *U*ptake and *D*ecarboxylation).

This generalization of Pearse's APUD concept has been criticized, particularly with respect to the pancreas. On embryonic grounds, the most cogent criticism has derived from studies indicating that endocrine cells of the enteropancreatic system do not originate from the neural crest (20–25, 30). Biochemi-

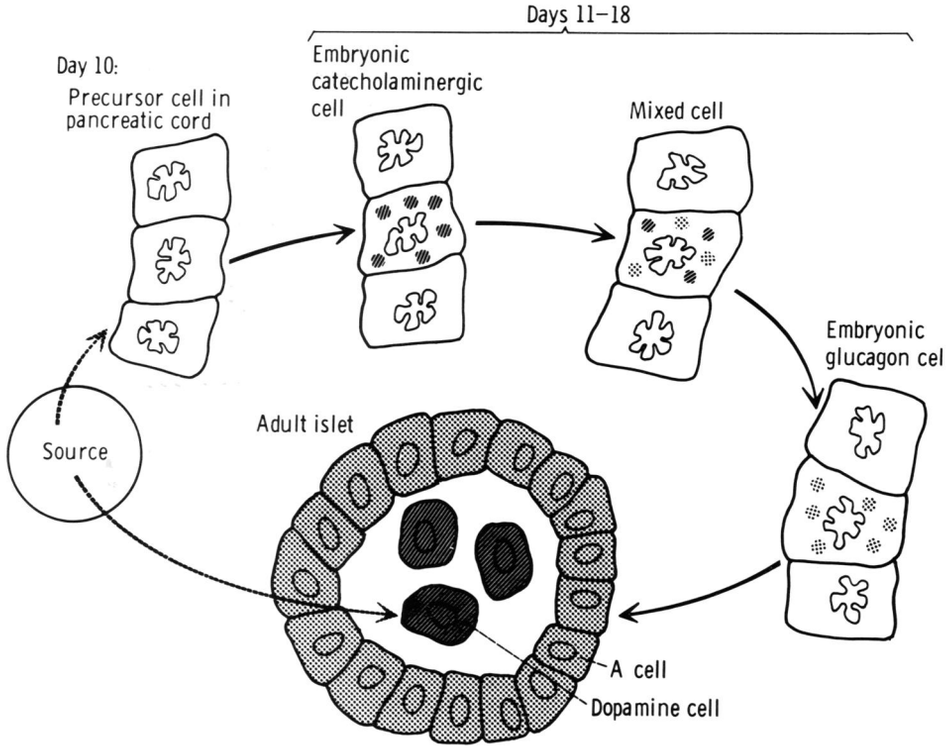

FIG. 5. Representation of the possible history of differentiation of pancreatic A (glucagon) cells from catecholaminergic precursors. ■, TyrOHase; ⊠, glucagon.

cally, there has not been direct evidence that the enzyme responsible for the decarboxylation of the amine precursors L-dopa or 5-hydroxytryptophan—i.e., dopaDCase—is present in pancreatic endocrine cells; thus, the accumulated amines could be taken up and stored after their biosynthesis elsewhere.

Our investigation, however, demonstrates that some, and probably all, of the pancreatic A (glucagon) cells originate from precursors that transiently express a catecholaminergic phenotype. Moreover, it provides direct immunological evidence of dopaDCase in pancreatic cells, indicating thereby that L-dopa or 5-hydroxytryptophan can be converted to the corresponding amine *in situ*. Finally, by using double labeling, we have proved that catecholamine cells also contain a peptide hormone. Although these findings would tend to support Pearse's hypothesis, full validation of the developmental aspect of the APUD concept would require the identification of the germ layer from which the TyrOHase cells of the pancreas are derived.

Although it is now evident that some catecholamine cells in brain, sympathetic ganglia, and adrenal medulla also store neuropeptides (31), the present study establishes the fact that catecholaminergic cells can interconvert into cells containing peptides. This finding raises the question of whether the transitory coexistence of catecholamine biosynthetic enzymes and peptides is also a characteristic of other peptide-containing cells in enteropancreatic systems and, possibly, brain.

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