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Pancreatic Polypeptide (PP)- and Glucagon Cells in the Pancreatic Islet of *Xiphophorus helleri* H. (Teleostei)

Correlative Immunohistochemistry and Electron Microscopy*

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Summary. Correlative immunohistochemical and electron microscopical studies on the pancreatic islet of the teleost fish *Xiphophorus helleri* using antibodies to pancreatic polypeptide (PP) and glucagon show that separate cell types are responsible for the production of these peptides. The PP-cells correspond to the previously described "A2-cells with round granules", while the "A2-cells with crystalline granules" are the true glucagon cells. An earlier suggestion that there are two types of glucagon cells in teleost islets is therefore withdrawn.

Key words: Pancreatic polypeptide (PP) – Glucagon – Pancreatic islet – *Xiphophorus helleri* – Immunohistochemistry – Electron microscopy.

Résumé. L'étude immunohistochimique de l'îlot pancréatique du poisson téléostéen *Xiphophorus helleri,* à l'aide de sérums anti-polypeptide pancréatique (PP) et anti-glucagon a permis de montrer que deux populations cellulaires distinctes sont responsables de la sécrétion de ces hormones. L'observation comparée de coupes sériées, ultrafines et semifines, traitées par la technique immunohistochimique, a démontré que les cellules à PP correspondent aux cellules qui avaient été précédemment définies, dans cette espèce, comme "cellules A2 à grains ronds" et que les cellules sécrétrices de glucagon sont les "cellules A2 à grains cristallins". L'hypothèse de l'existence de deux catégories de cellules à glucagon chez les téléostéens est abandonnée.

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In addition to insulin, glucagon and somatostatin, a fourth hormone, pancreatic polypeptide (PP), is secreted by the endocrine pancreas. Discovered first in the chicken (Kimmel et al., 1968, 1971) and later in mammals (Lin and Chance, 1972), the production of PP has been attributed to a fourth endocrine cell type, the PP-cell, in birds (Larsson et al., 1974) and in mammals (Larsson et al., 1975, 1976; Polak et al., 1976). PP-cells have been demonstrated, with histochemical methods, in various vertebrates (for review, see Van Noorden and Falkmer, 1979), including cartilaginous and bony fish (Stefan et al., 1978; Van Noorden and Patent, 1978).

In *Xiphophorus helleri,* Van Noorden and Patent (1978) have demonstrated, by immunostaining, the occurrence of a PP-like substance in islet cells located at the periphery of the islet. These results raised the problem of interpreting this "new cell type" in relation to the islet cell classification previously proposed for *Xiphophorus helleri* (Klein, 1975; Klein and Lange, 1977). The PP-cells showed a striking analogy in location and shape with the cells described, in this classification, as "A-cells (or A2-cells) with round granules". It was thus necessary to carry out a more detailed investigation. Correlative light and electron microscopy, with immunostaining on semithin sections, appeared to be the most accurate method of approaching this problem and, moreover, should provide details of the still undescribed ultrastructure of the PP-cells. Indeed, most of the investigations undertaken so far have been on paraffin-embedded material and no correlative light and electron microscopical observations have been published on PP-cells in teleosts.

Materials and Methods

Fish were reared in laboratory aquaria. Adult males and females were killed by decapitation.

I. Preparation of the Sections

Islets were fixed in a variety of solutions including glutaraldehyde, glutaraldehyde with formaldehyde, and formaldehyde. Of the fixatives tested, the most satisfactory with regard to both the immunoreaction and ultrastructural preservation were the following:

1. 1% methanol-free formaldehyde (MFF) + 1.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, at 4° C, for 45 min;

2. 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, at 4° C, for 35 min.

The material was embedded in Araldite. Serial semithin (500 nm to 1,000 nm) and ultrathin sections were cut. The ultrathin sections were collected on central slot grids, which provided an uninterrupted viewing range of 2 mm × 1 mm. These conditions were extremely favourable for finding the cells that corresponded to those previously stained on the adjacent semithin sections.

For routine electron microscopy, standard double fixation was employed: 3% glutaraldehyde in 0.1 M cacodylate buffer, 35 min, followed by 1% OsO_4 in the same buffer, 45 min.

II. Immunostaining

Semithin sections were mounted on glass slides. The resin was removed with a saturated solution of sodium hydroxide in absolute ethanol (Lane and Europa, 1965). The indirect immunofluorescence technique (Coons et al., 1955) or the indirect peroxidase-labelled antibody method (Nakane and Pierce, 1967) were then applied.

Anti-PP and anti-glucagon sera were used for comparative immunostaining.

A. Staining for PP-like Substance. 1) For specific incubation, the following sera were used as the first layer: anti-avian pancreatic polypeptide (APP) (dilution 1 : 700), or anti-bovine pancreatic polypeptide (BPP) (dilution 1 : 1000 or 1 : 1500 for the peroxidase method), for 18 to 24 h at 4° C.
2) Controls: a) prior absorption of the diluted antisera with APP or BPP (10 nmol/ml), b) prior absorption of the diluted antisera with glucagon and/or insulin (10 nmol/ml), c) non-immune serum as first layer.

B. Staining for Glucagon-like Substance. 1) For specific incubation, anti-glucagon serum (dilution 1 : 50 or 1 : 100) was used as the first layer, for 18 to 24 h at 4° C.
2) Controls: a) prior absorption of the diluted antiserum with glucagon (10 nmol/ml), b) prior absorption of the diluted antiserum with BPP (10 nmol/ml), c) non-immune serum as first layer

III. Correlative Study

1) Consecutive semithin sections were stained with the various antisera.
2) Comparison of photographs of the semithin sections with their adjacent ultrathin sections allowed the immunostained cells to be identified in the electron microscope.

Results[1]

I. Immunostaining – Comparative Observations

The use of serial semithin sections was particularly instructive, since this allowed the comparison of results obtained with the various antisera. Indeed, the cells were sufficiently large to be observed on three or four serial semithin sections.

A. Use of Anti-PP Serum. 1. Anti-BPP. After immunostaining with anti-BPP serum, positive cells appeared to be located at the extreme periphery of the islet (Fig. 1). Absorption with glucagon or insulin did not affect the staining. The other controls were negative (absorption of the antiserum prior to staining with BPP, use of non-immune serum).

2. Anti-APP. In sections treated with anti-APP serum, the stained area was peripheral but extended much farther into the islet. However, when the antiserum was absorbed with glucagon prior to staining, the positive cells were restricted to a narrow peripheral rim and corresponded to those stained with anti-BPP serum. Pre-absorption with insulin did not affect the staining, which was, however, completely removed by pre-absorption with APP.

B. Use of Anti-Glucagon Serum. After staining with anti-glucagon serum, the positive cells were distributed in a large peripheral area, which was very similar to the positive area observed when the reaction was carried out with anti-APP serum used without any pre-absorption. However, when the serum was employed after pre-absorption with PP, the cells located at the extreme periphery were no longer positive and the positive cells were restricted to a more interior area (Fig. 2).

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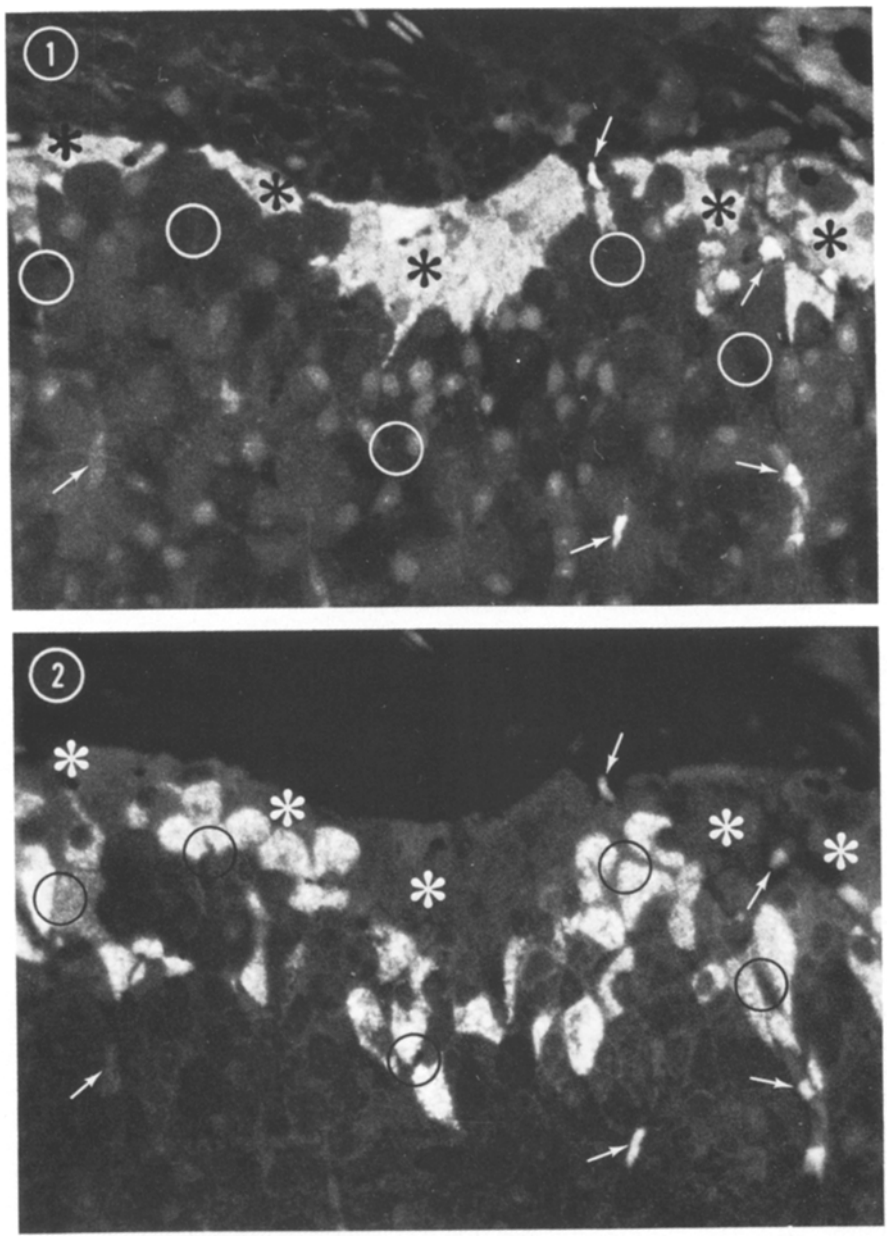
[1] In *Xiphophorus helleri*, a single islet contains all the endocrine pancreatic tissue; there is usually very little or no exocrine pancreas surrounding this islet (Klein, 1975; Klein and Lange, 1977)



Figs. 1 and 2. PP-cells and A-cells: comparison between two serial semithin sections, treated with anti-BPP serum (**Fig. 1**) and anti-glucagon serum pre-absorbed with BPP (**Fig. 2**) (1% MFF + 1.25% glutaraldehyde in 0.1 M phosphate buffer, 45 min, PP-cells (*asterisks*) and A-cells (*circles*) are clearly recognizable as separate cells due to their specific immunoreactivity. *Arrows* indicate capillaries. × 900

The cells demonstrated in *Xiphophorus* by using antibodies to APP, BPP and glucagon were located in the peripheral part of the islet; no positive cell was observed in the central area of the islet. Comparison of the results obtained with the various antisera applied to serial semithin sections showed the existence of two distinct cell categories (Figs. 1, 2):

1. the cells with PP-like activity, located at the extreme periphery of the islet;
2. the cells with glucagon-like activity, distributed more centrally.

II. Correlative Light and Electron Microscopy

Correlation of the light and electron microscopical observations (Figs. 3–5) emphasized the distinctness between the two categories of cells, which, in fact, displayed completely different ultrastructural features.

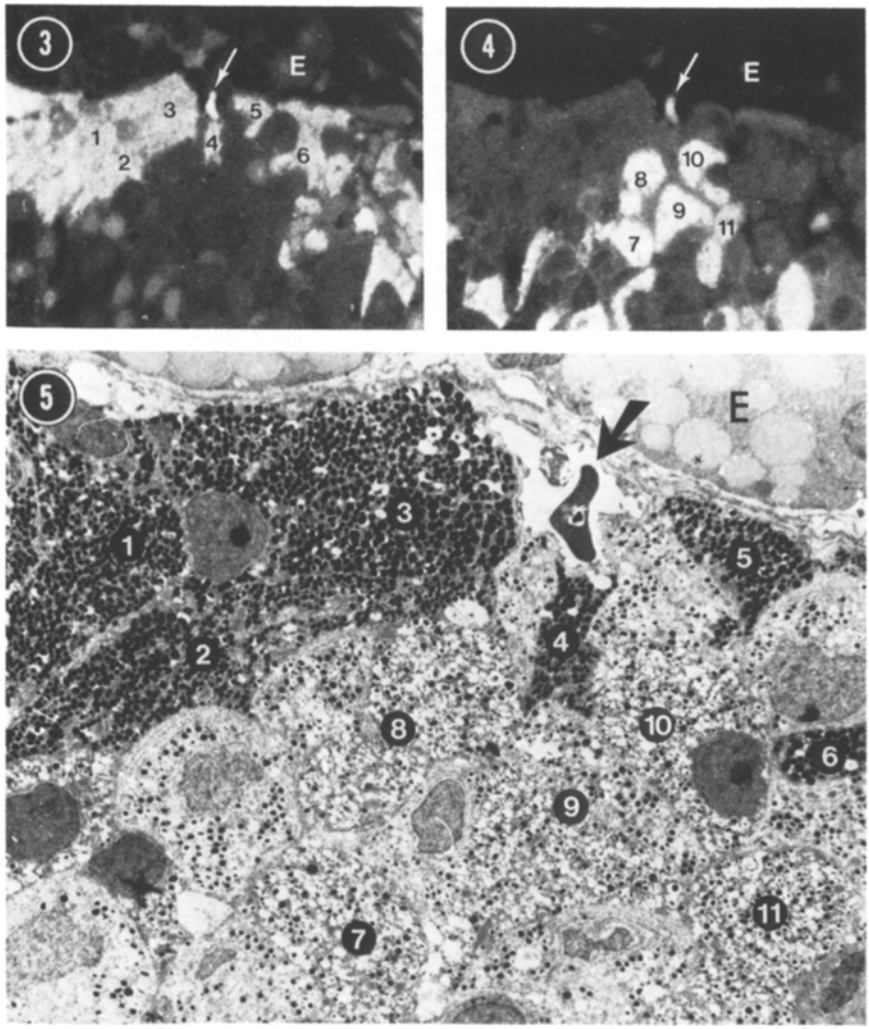
A. Cells with PP-like Activity. In these cells, numerous secretory granules were observed, closely packed in the cytoplasm (Figs. 5, 6). Due to the lack of osmium in the fixative, which was the condition for immunostaining, the granule membrane was not visible. The secretory granules displayed highly electron-dense circular cores (Fig. 6).

B. Cells with Glucagon-like Activity. These cells appeared quite different. Secretory granules were more scattered in the cellular cytoplasm (Figs. 5, 7). As explained above, the granule membrane was not visible but the dense cores were surrounded by a wide clear halo. Many secretory granules displayed hexagonally-shaped dense cores. As described elsewhere (Klein, 1975, 1977; Klein and Lange, 1977), these granules are characteristic of this cell type, regardless of the type of fixation.

Discussion

I. Identity of the Two Cell Types with Those Previously Described in Xiphophorus

The first results demonstrating the existence of PP-like activity in peripheral cells of the *Xiphophorus* islet raised the problem of the correlation of these cells with some of the cell types previously described in this species (Klein, 1975; Klein and Lange, 1977). It had been previously shown that an intense glucagon-like immunoreactivity was located in, and restricted to this peripheral area (Klein and Lange, 1972). The specificity of the reaction had been tested by various controls: absorption with glucagon prior to staining, use of non-immune serum as first layer, use of FITC-labelled globulins alone. However, it was quickly realized that the population of cells positive after specific incubation with anti-glucagon serum was heterogeneous, consisting of two categories of cells, each of which could be defined by precise and constant features (Klein and Lange, 1972, 1974; Klein, 1975; Klein and Lange, 1977). Some cells were positive with Adams' tryptophan staining (DMAB-sodium nitrite) and negative with Grimelius' silver staining. They were always located at the extreme periphery of the islet and displayed, in the electron



Figs. 3–5. PP-cells and A-cells, correlative light and electron microscopy (1% MFF + 1.25% glutaraldehyde in 0.1 M phosphate buffer, 45 min). **Fig. 3:** semithin section, treated with anti-BPP serum. ×900. **Fig. 4:** adjacent semithin section, treated with anti-glucagon serum pre-absorbed with BPP. ×900. **Fig. 5:** adjacent ultrathin section. ×3,600. In the electron microscope (Fig. 5), PP-cells (n° 1–6) and A-cells (n° 7–11) appear completely different. *E* exocrine pancreas; *arrow* indicates erythrocyte in a capillary

microscope, amorphous granules, with a circular dense core. The other cells, located in a more interior area, were Adams-negative and Grimelius-positive. Their secretory granules very often exhibited crystalline dense cores. At that time, these two categories were respectively called "A-cells (or A2-cells) with round granules" and "A-cells (or A2-cells) with crystalline granules".

When PP-like activity was demonstrated in the *Xiphophorus* islet, the possibility that it was located in the so-called "A-cells with round granules" had to be explored

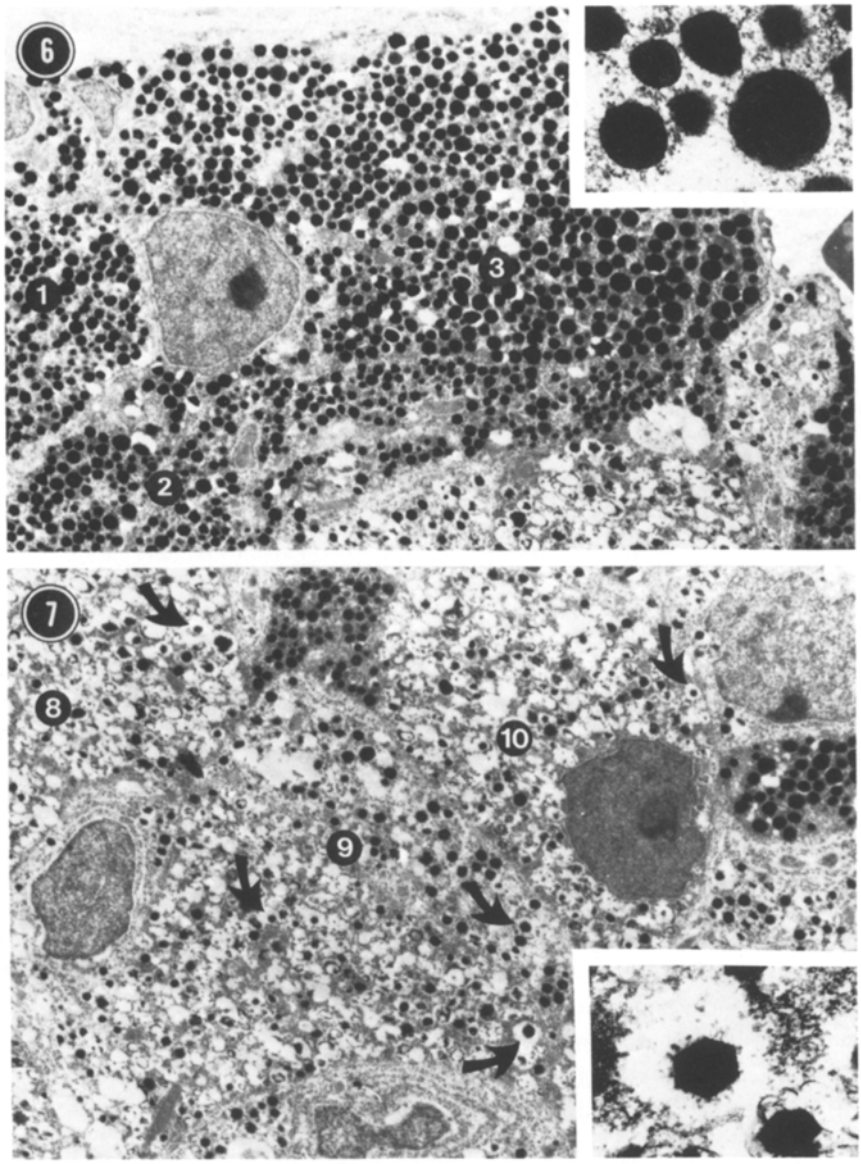


Fig. 6. PP-cells, detail of Fig. 5 (numbers refer to cells identified in Fig. 5). Secretory granules, closely packed in the cytoplasm, exhibit dense cores circular in section (*inset*); the membrane is not visible due to the lack of osmium in the fixative. ×7,200; *inset:* ×39,000

Fig. 7. A-cells, detail of Fig. 5 (numbers refer to cells identified in Fig. 5). Secretory granules are more scattered within the cell; many of them display a hexagonally-shaped dense core (*arrows* and *inset*); as in Fig. 6, the granule membrane is not visible. ×7,200; *inset:* ×39,000

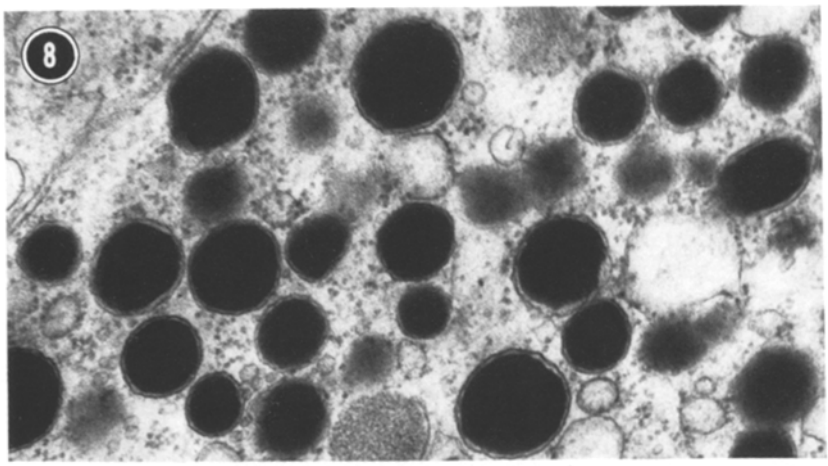


Fig. 8. PP-cell, aspect of the secretory granules after the standard double fixation (3% glutaraldehyde in 0.1 M cacodylate buffer, 35 min, 1% OsO_4 in the same buffer, 45 min). Secretory granules appear very similar to those observed in Fig. 6, but here the granule membrane is visible. × 39,000

carefully. The use of material prepared for electron microscopy insured an accurate approach to this problem. First, the same cells could be compared on three or four semithin sections. With this method and using various sera with appropriate pre-absorption (this point will be discussed further), it was clear that the cells with PP-like activity were distinct from the cells with glucagon-like activity (Figs. 1, 2). Secondly, the results obtained with correlative light and electron microscopy underlined a striking similarity, regarding the secretory granules, between the cells with PP-like activity and the "A-cells with round granules" on the one hand, the cells with glucagon-like activity and the "A-cells with crystalline granules" on the other. Although shape and size of secretory granules cannot be claimed as an absolute criterion for defining a cell type, detailed comparative studies (Klein, 1975; Klein and Lange, 1977) have clearly demonstrated that the hexagonally-shaped dense cores occurred, in *Xiphophorus helleri,* always and only in the "A-cells with crystalline granules", regardless of the fixative employed. Similarly, the "A-cells with round granules" consistently displayed amorphous granules with a circular dense core (Figs. 6, 8). These observations and the precise and constant localization of the cell types under consideration lead us to conclude that the PP-cells are the same as the "A-cells with round granules" and we therefore give a complete description of each cell type (see Table 1).

A. Glucagon-secreting Cells. Secretory granules with hexagonally-shaped dense cores occurred frequently in these cells. As described elsewhere (Klein, 1975; Klein and Lange, 1977), this core could be surrounded by a fine granular material or by a clear halo. However, the fine granular material was never observed when the fixation was carried out without osmium. Crystallographic investigations have demonstrated that these geometrical cores are rhombic dodecahedral crystals (Lange and Klein, 1974), which have now been further explored by crystallographic techniques (Lange, 1979). Similar crystalline granules have been observed in the

Table 1. PP-cells and A-cells (glucagon-secreting cells) in the islet of *Xiphophorus helleri*

	PP-cells	A-cells (glucagon)
Localization	peripheral	more central
Immunostaining with anti-PP serum pre-absorbed with glucagon	+	–
Immunostaining with anti-glucagon serum pre-absorbed with PP	–	+
Adams technique (DMAB-$NaNO_2$)	+	–
Grimelius silver impregnation	–	+
Secretory granules	amorphous spherical dense core	crystalline polyhedral dense core

islets of a few other teleosts: *Fugu rubripes* (Kobayashi et al., 1976; Lange, 1979), *Gadus callarias* (= *G. morhua*) (Thomas, 1970; Lange et al., 1974; Lange, 1977), *Ictalurus catus* (Brinn, 1973), *Ictalurus nebulosus* (Bencosme et al., 1965), *Limanda herzensteini* (Kobayashi et al., 1975), *Limanda limanda* (Thomas, 1975). Although it has not been demonstrated that these crystals are "teleost glucagon", there are strong arguments on behalf of this hypothesis (see Klein, 1977, and Lange, 1979). It is of interest that glucagon has been isolated from the white carp islets and rhombic dodecahedral crystals obtained in vitro (Fang et al., 1979).

B. PP-Cells. PP-cells have been demonstrated by immunohistochemistry in the pancreatic islet of various teleosts (Stefan et al., 1978; Van Noorden and Patent, 1978). However, these investigations were carried out on paraffin-embedded material and in no case has correlative light and electron microscopy been presented. The ultrastructure of PP-cells in teleosts in general cannot therefore be discussed. Very little is known so far about the secretion of pancreatic polypeptide in teleosts. However, it seems very likely that the cells demonstrated with PP antiserum (properly controlled) are PP-secreting cells. The presence of PP-like immunoreactivity in teleosts has been verified by radioimmunoassay of pancreatic islet extracts in *Cottus scorpius* (see Van Noorden and Falkmer, 1979).

Distributed in the peripheral area of the *Xiphophorus* islet, PP-cells and glucagon-secreting cells seemed to be in close association. This topographical proximity has also been noted in *Cottus scorpius* (Stefan et al., 1978). In this species, the two principal islets differed in their content of PP-cells, which were numerous in

the "pyloric islet" but completely lacking in the "splenic islet". In mammals, differential distribution of PP-cells and glucagon cells in various parts of the pancreas has been clearly established (Orci et al., 1976; Orci and Perrelet, 1979). "PP-islets" and "glucagon-islets" were shown to occur in the ventral pancreas (head) and the dorsal pancreas (body and tail), respectively. Study of the endocrine pancreas of *Xiphophorus helleri* cannot be of aid in this respect, since, in this species, a unique islet contains all the endocrine pancreatic tissue.

II. Specificity of Immunoreaction

A. Demonstration of Glucagon-like Activity. The positive reaction to anti-glucagon serum, which was observed in 1972 in the two categories of cells, PP-cells and glucagon cells, led to both types being classified as "A-cells" (or A2-cells) (Klein and Lange, 1972, 1974). The same reaction occurred with the anti-glucagon serum used in the present study. The explanation for these results is that when the glucagon antisera were prepared, the existence of PP was not suspected, since it was first reported in 1971 (Kimmel et al., 1971). It is therefore probable that the glucagon antigens used for immunization were contaminated with PP. In an in vitro test (Enzyme-Linked Immunosorbent Assay: ELISA, see Voller et al., 1976) slight cross-reactivity with BPP was observed with the antiserum employed in this study, and, at the dilutions used, this presumably gave rise to the double staining. The staining of the PP-cells was, however, completely removed by absorption of the antiserum with PP prior to staining. When a more recently produced anti-glucagon serum was used, glucagon cells exclusively were stained.

B. Demonstration of PP-like Activity. This problem also applied to the APP antiserum that reacted with both PP- and glucagon cells in the *Xiphophorus* islet. Using antisera from the same source, similar results were reported in the chick (Rawdon and Andrew, 1979) and in other teleosts as well as *Xiphophorus helleri* (Van Noorden and Patent, 1978). Staining of B-cells was noted in addition. Since the A- and B-cell staining could be removed by absorption of the antibody with glucagon and insulin before staining, it was assumed that traces of insulin and glucagon must have been present in the PP extract of chicken pancreas originally used for immunization. Antibodies to insulin and glucagon would not interfere in a PP-radioimmunoassay system.

In the present study we did not observe B-cells staining in the *Xiphophorus* islet. As our fixatives were different from those used by Van Noorden and Patent (1978), the influence of fixation on histochemical reactions is again emphasized. Rawdon and Andrew (1979), using chick pancreas, were able to remove the contaminating glucagon antibody by using high dilutions of the antiserum. We were unable to reproduce this observation because, as in the study by Van Noorden and Patent (1978), we had to use both the APP and the BPP antisera at rather high concentration to obtain successful immunostaining in *Xiphophorus,* which represents a heterologous species. With the BPP antiserum, no problems of cross-reactivity were encountered.

The present study emphasizes once again the well-known problem of defining a cell type. Cell location, staining affinities and shape of the secretory granules cannot be claimed as absolute criteria. The technique of immunohistochemistry combined with electron microscopy appear to constitute a more appropriate approach. If the occurrence of PP-cells in teleost islets were more fully explored in this way, the distinction between PP- and glucagon cells could be clearly established and should yield a definitive answer to the problem of some non-identified islet cells in teleosts (see Klein, 1977).

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Note Added in Proof: Since this article went to press, we have received a communication from Dr Y. Stefan (University of Geneva) and from Prof. S. Falkmer (University of Lund) that they have recently submitted a paper to General and Comparative Endocrinology entitled:

Immunocytochemical and ultrastructural identification of four endocrine cell types in the pancreas of a marine teleost, *Cottus scorpius*. Disparity between the pyloric and splenic principal islets.