

# A Historical Perspective on the Identification of Cell Types in Pancreatic Islets of Langerhans by Staining and Histochemical Techniques

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## Summary

Before the middle of the previous century, cell types of the pancreatic islets of Langerhans were identified primarily on the basis of their color reactions with histological dyes. At that time, the chemical basis for the staining properties of islet cells in relation to the identity, chemistry and structure of their hormones was not fully understood. Nevertheless, the definitive islet cell types that secrete glucagon, insulin, and somatostatin (A, B, and D cells, respectively) could reliably be differentiated from each other with staining protocols that involved variations of one or more tinctorial techniques, such as the Mallory-Heidenhain azan trichrome, chromium hematoxylin and phloxine, aldehyde fuchsin, and silver impregnation methods, which were popularly used until supplanted by immunohistochemical techniques. Before antibody-based staining methods, the most bona fide histochemical techniques for the identification of islet B cells were based on the detection of sulfhydryl and disulfide groups of insulin. The application of the classical islet tinctorial staining methods for pathophysiological studies and physiological experiments was fundamental to our understanding of islet architecture and the physiological roles of A and B cells in glucose regulation and diabetes. (*J Histochem Cytochem* 63:543–558, 2015)

## Keywords

diabetes, beta cells, glucagon, immunocytochemistry, immunohistochemistry, insulin, islet cells, pancreas, staining, somatostatin

The islets of Langerhans were discovered in 1869 by Paul Langerhans when he was a medical student at the Friedrich Wilhelm University in Berlin (Fig. 1). A student of the eminent pathologist Rudolf Virchow, Langerhans described the microscopic anatomy of the rabbit pancreas in his M.D. thesis and reported the presence of “...small cells of almost perfect homogeneous content and of a polygonal form, with round nuclei, mostly lying together in pairs or small groups” (English translation) (Sakula 1988). The function of these cells was, of course, unknown to Langerhans (although he suspected that they might be neural in nature) and, except for describing their morphology, he did not give them a name. The term “islets of Langerhans” was introduced in 1893 by Edouard Laguesse, who observed them in the human pancreas and (with remarkable foresight) suggested

that they may produce internal secretions that regulate glycemia (Laguesse 1893).

The present article is a retrospective history of the histological and histochemical staining methods that have been used by anatomists and pathologists over the years to identify hormone-secreting cell types of the islets of Langerhans (hereinafter called islets) and understand their function in glucose homeostasis and the pathophysiology of diabetes

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**Figure 1.** Paul Langerhans 1878.

mellitus (hereinafter called diabetes). The main theme of this account focuses on the cells that secrete the canonical islet hormones—insulin, glucagon, somatostatin, and pancreatic polypeptide—recognizing that other endocrine factors may also be expressed in the islet, and that neural (Ahrén et al. 2007), extracellular matrix (Westermarck and Westermarck 2013), and stromal (Bollyky et al. 2012) elements are also essential components of the functioning islet.

Primarily for convenience, I make an arbitrary distinction between the terms “tinctorial staining” (i.e., histological staining methods that basically reveal microscopic anatomy) and “histochemical staining” (methods that identify chemical constituents of cells and organs). Tinctorial and histochemical staining methods both impart contrast (most often as colors) to islet cells, including their intracellular secretory granules, and are useful for interpreting the microscopic anatomy of islets. Admittedly, tinctorial vs. histochemical is a somewhat arbitrary distinction, as even the classical tinctorial methods for staining different islet cell types are grounded in differences in the chemical properties of the respective hormones (or other components of their cytoplasmic granules); although, these properties were (and, in some cases, still may be) unknown.

## Islet Cells and Diabetes

By the end of the 19<sup>th</sup> century, experimental pathologists and physiologists had hypothesized that the intimate anatomical

relationship of islet cells to a rich capillary network suggested that these cells secrete a substance into the blood to influence carbohydrate metabolism (Laguesse 1893; Diamare 1899; Schäfer 1895), a hypothesis that required evidence of physiological independence of the islets from the exocrine cells of pancreas. Debate centered on the question of whether the islets represented degranulated pancreatic exocrine cells, as it had been observed that pancreatic exocrine cells that were “exhausted” by alkaloid treatment resembled islet cells. Researchers soon discovered that removing the pancreas produced elevated blood sugar and diabetes in experimental animals (von Mering and Minkowski 1890; Minkowski 1893). Pathologists, notably Eugene Opie, found lesions of the islets in pancreases that were removed at autopsy from people who were afflicted with diabetes (Opie 1901a, 1901b), thus making a link between diabetes and a deficiency in an islet secretion (that was later named insulin) that was eventually isolated and used to treat patients with type 1 diabetes two decades later (Bliss 2007).

In the years that followed, we have learned a great deal about the diversity and function of the cells that comprise these miraculous little endocrine organs, which are vital to life, have so many tasks that are essential for metabolic homeostasis, and whose failure can result in diabetes. How we came to understand the identities and functions of the cell types that comprise the islets is a fascinating story of how microscopic anatomy and staining methods complemented the functional analysis of islet cell physiology and the pathophysiology of diabetes in humans and experimental animals. Much of what we know about their function has been learned from microscopic studies of the pancreas using chemicals that “stain” islet endocrine cell types, permitting them to be visually distinguished from each other and from pancreatic exocrine cells, neural tissue, and stromal elements.

## Nomenclature of Islet Cells

Over the years, the naming of islet cell types has followed three different but parallel conventions. Many authors use the Greek letter designations “ $\alpha$  cell” and “ $\beta$  cell” when referring to the cells that produce glucagon and insulin, respectively, whereas others spell out “alpha cell” and “beta cell”, and many papers in the literature use the Roman letter terms “A cell” and “B cell” for these same cell types. Following the latter convention, the islet cell that secretes somatostatin is called the “D cell” (also  $\delta$  or delta cell) and the islet cell that secretes pancreatic polypeptide is called the “F cell” (also called the PP cell). The trend in recent years seems to favor the Greek letter names for islet cells, especially for the cells that secrete glucagon and insulin, although it is understood that the Greek and Roman terms are interchangeable in this context. For uniformity and clarity, the present article uses the Roman letter terms for islet

cell types, regardless of the convention that may have been originally used by authors in the cited articles.

### Early Staining Methods for Islet Cells

Pioneering anatomical studies of islets were performed primarily with histological stains such as hematoxylin and eosin, which permitted observations of changes in islet morphology but had minimal value for the identification or discrimination of islet cell types. The problem was addressed in a classic paper by Lane in 1908: "The principal difficulty thus far in dealing with the Islets of Langerhans has been the want of a definite method by which to distinguish the cells of the islets from the cells of the pancreas itself; for although there is an apparently constant content of islet tissue in the pancreas, and although the areas of islet tissue, in sectioned pancreas, stand out in sharp contrast with the tubules of the pancreas [Note: this means the exocrine acini], the physiological distinctness of one kind of tissue from the other is the very question upon which histologists and pathologists have most disagreed" (Lane 1908).

By the beginning of the 20<sup>th</sup> century, microscopists had recognized two types of islets cells based primarily on morphology but also on staining properties with safranin and gentian violet (Diamare 1899; Laguesse 1895; Schultz 1900; DeWitt 1906). These dyes are the components of the classic Gram stain, which identifies Gram-positive bacteria based on the chemical and physical properties of their cell walls; although, the functional meaning of their staining of nuclei and cytoplasmic granules of islet cells was unknown. However, it seems likely that these two types of cells stained with safranin and gentian violet corresponded to A and B cells (Bloom 1931).

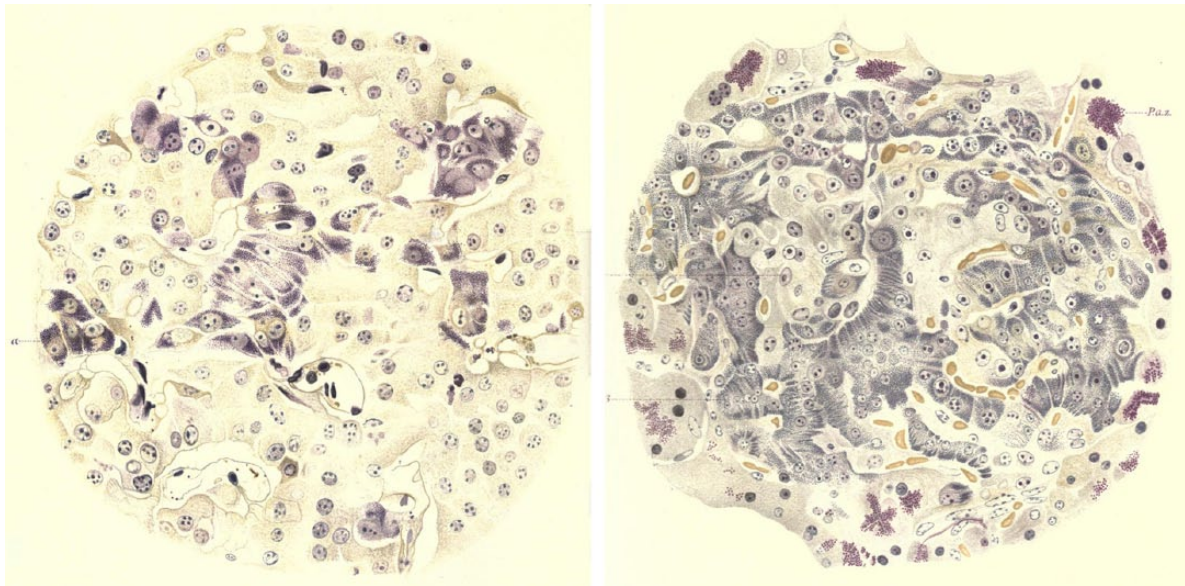
### Beginnings of Islet Histochemistry

The first bona fide histochemical differentiation of islet cell types can probably be attributed to Lane (Lane 1908), who identified two types of granular cells in islets based on their different solubilities in alcohol. Lane derived this conclusion from the staining characteristics of paraffin-embedded sections of guinea pig pancreas after treatment with one of three different fixative solutions: (a) alcohol-chrome-sublimate (an alcoholic solution of potassium dichromate and mercuric chloride), (b) aqueous chrome-sublimate (an aqueous solution of potassium dichromate and mercuric chloride), and (c) 70% alcohol. [Note: although sometimes not specified in early papers, "alcohol" usually meant ethanol.] The sections were stained with Bensley's neutral gentian violet, an alcoholic solution that stains the cytoplasmic granules of exocrine and islet cells a deep violet, and Orange G, an azo dye that stains the cytoplasm orange (Bensley 1914).

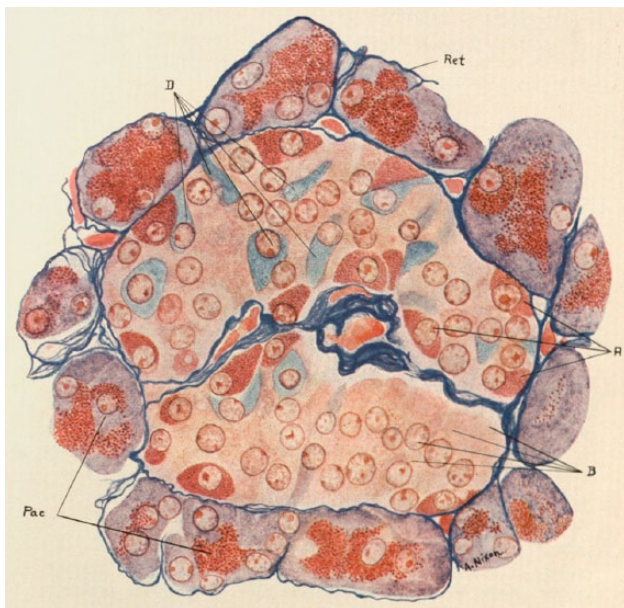
Lane's key observation was that fixation in alcohol-chrome-sublimate resulted in the staining of a morphologically different population of islet cells than was stained following fixation in aqueous-chrome-sublimate. Lane inferred that the guinea pig islet contained two chemically distinct types of cells, a type with granules that contain a chemical that is fixed (precipitated) with alcohol and a type that contains granules containing a chemical that is fixed with the aqueous-chrome-sublimate solution. The former Lane called A cells and the latter he named beta cells (Fig. 2) (later changed to "B cells" by Bensley 1911, 1914), which we now know secrete glucagon and insulin, respectively. Prophetically, Lane suggested that the islets "...in all probability have the function of producing a twofold substance which, poured into the blood stream, has an important effect on metabolism." Lane's seminal discovery was that the chemical nature of the granules of these cell types differed and that this difference could be distinguished by fixation and staining, arguing that the respective cells had separate functions and that both were chemically distinct from each other and from exocrine cell zymogen granules. Lane's finding that A and B cells can be histochemically distinguished by alcohol fixation is consistent with modern immunohistochemical staining observations that islet B cells immunostain with insulin antibodies very poorly in pancreas fixed in ethanol, whereas A cells immunostain robustly with glucagon antibodies after fixation in ethanol (Baskin 2014), and fits with the well-known fact that ethanol extracts insulin from the pancreas.

The papers on islet staining from this early era provide fascinating reading into the scientific mindset of the authors. The preparation of tissue and the staining solutions as well as the protocols are meticulously detailed. The hand-drawn illustrations are works of intricate detail and art, revealing that these investigators were superb microscopists and observers (see Figs. 2, 3). The descriptive narratives are often written in the first person, giving the accounts a sense of immediacy and the reader a feeling of listening to the author. Sadly, such diction and tone are rare in today's publishing environment.

Despite the elegant work of Lane, for the next 30 years, there was little consensus on the identification of islet cell types and their functions. Likewise, it was still unknown whether the postulated cell types that had been identified in guinea pig and human pancreas were representative of other species. Whereas the work of Lane showed that it was possible to differentiate A and B cells using chrome sublimate staining and solubility in alcohol, the technique also left some islet cells unstained, especially in guinea pig islets. Bensley called these unstained cells "clear" or "C cells" and concluded that they represented undifferentiated islet cells (Bensley 1914). Later, Bloom recognized three types of granular cells in human islets that had been fixed



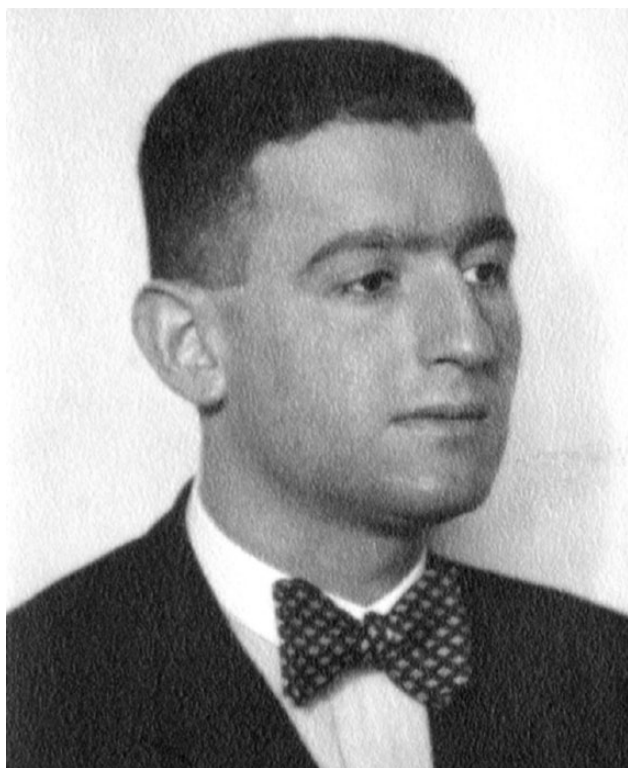
**Figure 2.** Hand-drawn figures from “The cytological characters of the areas of Langerhans,” by Lane, *American Journal of Anatomy*, Volume 7, Issue 3, Pages 409–422, 10 November 1907, illustrating guinea pig islets stained with Bensley’s neutral gentian following different fixations. Panel A represents an islet from a pancreas fixed in 70% alcohol. The islet A cells have a violet-stained cytoplasm, whereas the islet B cells are essentially unstained. Panel B represents an islet from a pancreas fixed in aqueous chrome-sublimite (no alcohol) and shows islet B cells filled with minute granules that stained violet, whereas the islet A cells are stained light orange. In panel B, some pancreatic acinar cells with clumps of violet-stained granules are depicted at the edge of the islet. Copyright John Wiley and Sons. Published with permission.



**Figure 3.** A drawing by Bloom from “A new type of granular cell in the islets of Langerhans of man,” from *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology*, 1931, illustrating a human islet stained with the Mallory-Heidenhain azan trichrome technique. A cells (A) are stained reddish brown, D cells (D) are blue, and B cells (B) are stained faintly orange. The figure shows pancreatic acinar cells (Pac) and reticular fibers (Ret). Copyright John Wiley and Sons. Published with permission.

in Zenker-formol (mercuric chloride, potassium dichromate, sodium sulfate, and formalin) (also called Helly’s fixative). When the islets were stained with the Mallory-Heidenhain azan trichrome technique, Bloom observed A and B cells as described by Lane and also a third granulated cell type in human islets that he called the D cell, but he was not able to identify a clear (C) cell in human islets (Bloom 1931) (Fig. 3).

In general, these tinctorial staining methods produced variable results on the islets of different species and it was difficult to correlate specific cell types with specific functions. Interestingly, in 1937, Thomas reported a study of islet staining from the tail of the pancreas of 41 different mammalian species representing ten orders and 25 families (including bats, ring-tailed coati, flying squirrel, camel, and jaguar). He claimed that Lane’s fixation and staining method produced inferior and inconsistent results as compared with other techniques. Thomas used more than seven different staining techniques in his study, although the most useful was said to be the Mallory-Heidenhain azan trichrome method. He reported that the staining reaction of all the mammalian islet species studied was essentially similar to the description that had already been established for the human pancreas (Bloom 1931). He also identified three types of granular cells (called A, B, and D cells) on the basis of granularity and differential coloration of cytoplasmic granules in all 41



**Figure 4.** George Gomori. Image courtesy Special Collections Research Center, University of Chicago Library. Published with permission.

species, although Bensley's unstained C cell was found only in islets of guinea pig and opossum.

### The Gomori Age

Before the 1930s, the stains used to study islet A and B cells gave excellent cellular differentiation on the islets of guinea pig pancreas but relatively poor and irregular results on the islets of other species including humans. The Mallory-Heidenhain azan trichrome method, a useful stain for islet A cells, unfortunately stained islet B cells rather poorly (Bloom 1931). There was a strong need for robust staining methods to identify islet B cells in particular to complement the growing field of islet pathophysiology in animal models as well as for human diabetes. Watershed events of this era were the publication of two methods for staining islet B cells by George Gomori, a professor of medicine at the University of Chicago (Fig. 4).

#### *Chromium Hematoxylin-phloxine Method*

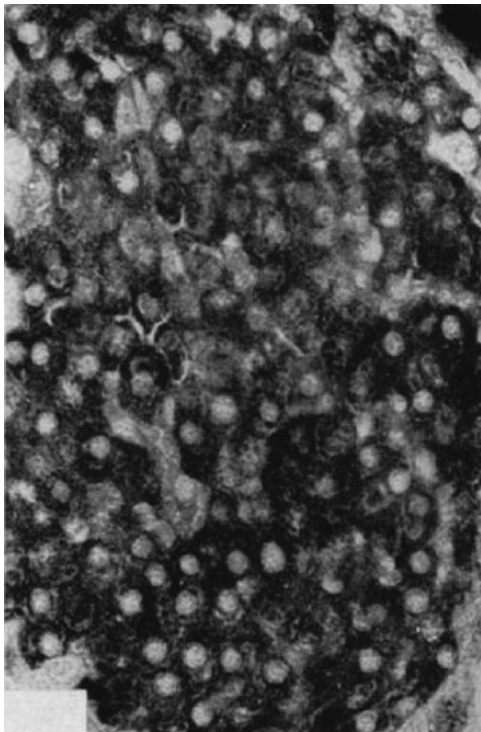
In 1939, Gomori published a staining protocol that sharply distinguished B cells from other islet cell types in human pancreases that were fixed in Bouin's fluid (picric acid, formalin, acetic acid) (Gomori 1939). Gomori refined this

technique in his 1941 paper, based on 70 normal and pathological human pancreases (Gomori 1941). Following the protocol of the Mallory-Heidenhain azan trichrome method, the sections were oxidized with potassium permanganate, decolorized with sodium bisulfite, and then stained in an acidic potassium dichromate hematoxylin solution. The reaction was monitored with a microscope until the desired staining was obtained; then the sections were differentiated in acid alcohol. The counterstain was phloxine, a common dye that renders the cytoplasm a colorful reddish hue. When properly applied, this technique—known as the chromium hematoxylin-phloxine method—stained the islet B cells an intensely deep blue and the A cells bright red. The D cells stained capriciously and variably pink-to-red in hue and were difficult to distinguish from islet A cells on the basis of color. In contrast, with the Mallory-Heidenhain azan trichrome stain, D cell granules could be stained deep blue, contrasting with red A cell granules; but the B cell granules (and thus the B cells) appeared unstained. Thus, by using both the Mallory-Heidenhain azan trichrome and the chromium hematoxylin-phloxine stains on adjacent sections, an investigator could get crude (but reproducible) differential counts of A, B, and D cells from pancreas sections and, moreover, observe how the numbers and appearances of these cell types were associated with pathophysiological changes in islet function in humans and experimentally in animal models.

The chromium hematoxylin-phloxine method (which became known as the first “Gomori stain”), paired with the Mallory-Heidenhain azan trichrome stain, was a standard approach for identifying islet A, B, and D cells for several decades. It was almost always used following formalin fixation, generally Bouin's fixative or Zenker-formol. Although it stains islet B cells with an intensity that is in some measure proportional to insulin content, the chromium hematoxylin-phloxine method is not biochemically specific for insulin. Moreover, the chromium hematoxylin-phloxine technique also stains many extrapancreatic cell types and was widely used, for example, to stain zymogen granules red in pancreatic exocrine cells and adenohipophyseal cell types (prolactin and growth hormone cells stained red, thyrotropes and gonadotropes stained blue). When applied to islets, the resulting blue stain was considered to be specific for B cells.

#### *Aldehyde Fuchsin Method*

In 1950, Gomori published an aldehyde fuchsin staining technique for elastic fibers that also stains cytoplasmic granules of islet B cells as well as a variety of other endocrine cell types, including adenohipophyseal basophils, neurohipophyseal neurosecretory cells, mast cells, and gastric chief cells (Gomori 1950). This protocol, known as the “Gomori aldehyde fuchsin stain”, is capable of producing

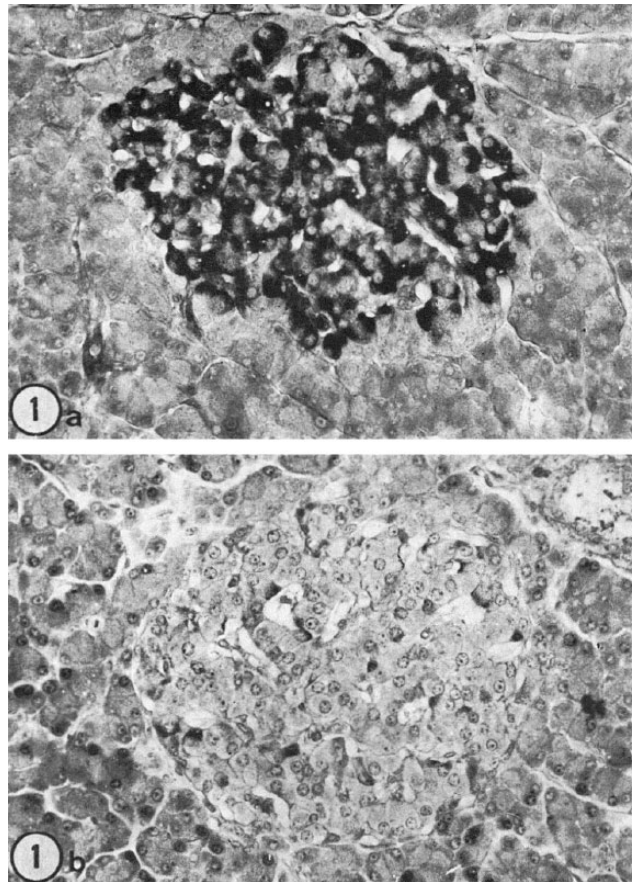


**Figure 5.** Rat islet stained with Gomori's aldehyde fuchsin after oxidation in periodic acid. Image from "A comparison of the staining affinities of aldehyde fuchsin and the Schiff reagent," by Scott and Clayton, *Journal of Histochemistry and Cytochemistry*, 1953. Copyright Histochemical Society. Published with permission.

intense staining of islet B cells (Fig. 5). However, the technique produced variable staining results among different investigators and species. The problem of inconsistent results was attributed to the mode of preparation, storage, and stability of the aldehyde fuchsin solution, which was prepared from basic fuchsin and allowed to "age" before use, and also to the variety of fixative solutions that were used by different investigators (Cameron and Steele 1959; Mowry et al. 1980; Mowry and Kent 1988). Despite these difficulties, the method had appeal partly because the intensity of islet B cell staining with Gomori's aldehyde fuchsin appeared to be roughly related to islet insulin content (Fig. 6) and other islet cell types were unstained. Gomori's aldehyde fuchsin method was widely used in various formulations (Scott 1952; Scott and Clayton 1953; Cameron and Steele 1959; Mowry 1983) until immunohistochemical methods supplanted routine use of tinctorial B cell stains.

### *Chemistry of Aldehyde Fuchsin Staining of Islet B Cells*

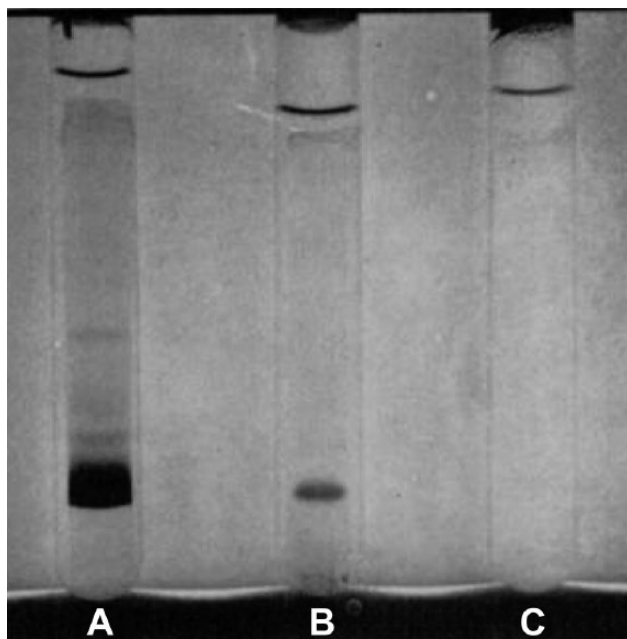
The chemical basis for the method was assumed to be the reaction of aldehyde fuchsin with insulin after prior oxidation by  $\text{KMnO}_4$  or periodic acid (Scott 1952). Following the elucidation of the chemical structure of insulin, Scott and Clayton (1953) hypothesized that insulin is oxidized at



**Figure 6.** Rat islets stained with Gomori's aldehyde fuchsin method. Top panel is an islet from a control rat, showing intensely stained granulated B cells. Bottom panel shows an islet from rat that was treated with a sulfonylurea to stimulate insulin secretion, showing depletion of staining. Image from "A portrait of the pancreatic B cell," by Orci, *Diabetologia*, 1974. Published with kind permission kind permission from Springer Science and Business Media.

disulfide bonds to sulfonic acid groups, which act as decolorized Schiff reagents. In the presence of aldehyde, the uncolored Schiff reagent changes to a magenta color, thus presumably staining insulin in B cell granules (Bangle 1954, 1956; Bangle and Alford 1954).

The feasibility of this mechanism was tested by biochemical studies on the reaction of aldehyde fuchsin with insulin by Kvistberg et al. (1966), who analyzed the staining of beef zinc insulin in polyacrylamide gels following disc electrophoresis. They prepared aldehyde fuchsin according to Gomori's recipe and aged it for 3 days before use (the "age" of aldehyde fuchsin solutions was found to affect its staining properties, although the reasons for this were not understood). The gels were oxidized with  $\text{KMnO}_4$  and  $\text{H}_2\text{SO}_4$  before staining (control gels were unoxidized), and then stained in the aldehyde fuchsin solution, and subjected to destaining to remove unreacted dye. This was essentially the same protocol that was used on pancreas tissue sections. The authors observed that aldehyde fuchsin stained insulin in the gels only if they had been oxidized



**Figure 7.** Reproduction of Figure 1 from “Staining of insulin with aldehyde fuchsin,” by Kvistberg, Lester, and Lazarow, *Journal of Histochemistry and Cytochemistry*, 1966, demonstrating staining of gels after disk electrophoresis. (A) Beef insulin stained with a dye for protein (aniline blue black) immediately after gel electrophoresis. (B) Beef insulin stained with Gomori’s aldehyde fuchsin after gel was oxidized with  $\text{KMnO}_4\text{-H}_2\text{SO}_4$ . The aldehyde fuchsin stained the same band that contains insulin in gel A. (C) Beef insulin that was stained with aldehyde fuchsin without prior  $\text{KMnO}_4\text{-H}_2\text{SO}_4$  oxidation, showing a lack of stained insulin band in the absence of oxidation. Copyright Histochemical Society. Published with permission.

after electrophoresis (Fig. 7). The authors confirmed these results by eluting insulin from unstained gels with acid alcohol and assaying for insulin by immunoassay. This study, by Kvistberg et al. (1966), is a classic demonstration of the application of biochemical techniques to understand the chemical basis of histochemical staining specificity of a dye molecule for insulin in islet B cells. Later, Greenwell et al. (1983) performed a controlled analysis on the effects of fixation and oxidation on the ability of aldehyde fuchsin to stain insulin, proinsulin, and other proteins in polyacrylamide gels. These latter investigators confirmed that the oxidation treatment was necessary to obtain positive aldehyde fuchsin staining but also concluded that this staining reaction was not related to the cysteine content of the proteins, thus raising unresolved doubt about whether either insulin or proinsulin is actually responsible for aldehyde fuchsin staining of islet B cells.

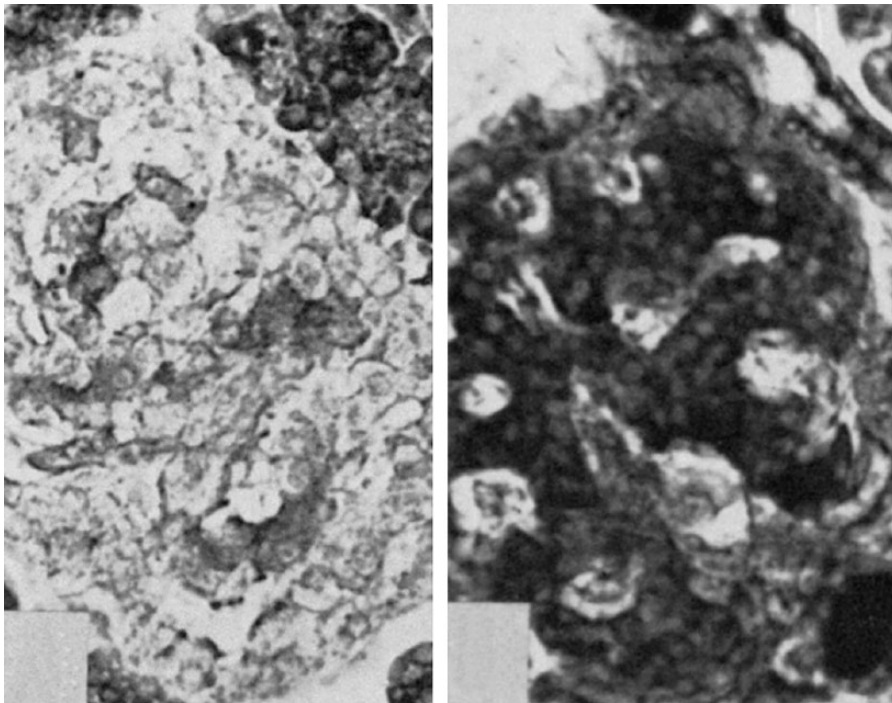
### Barnett and Seligman Technique

The efforts to base islet B cell staining on the chemical structure of insulin benefitted from developments in colorimetric methods for detecting protein-bound sulfhydryl groups

(Barnett and Seligman 1952b; Barnett 1953) and disulfide groups (Barnett and Seligman 1952a, 1954) in tissue sections. Barnett and Seligman, recognizing that insulin is rich in disulfide owing to its 12% cysteine content (Sanger and Tuppy 1951a, 1951b), used purified crystalline insulin in experiments to develop a histochemical method for staining insulin and used physiological experiments to demonstrate its validity (Barnett et al. 1955). Barnett and Seligman fixed pancreases from rabbits, albino rats, mice, dogs, toadfish, and humans in formalin-based solutions, including Bouin’s fixative, and embedded tissues in paraffin. Gomori’s aldehyde fuchsin method was used to confirm that the histochemical staining for sulfhydryl and disulfide groups indeed identified islet B cells. In carefully controlled experiments that included measurements of plasma glycemia and altered pancreatic insulin content after cytotoxic destruction of islet B cells with alloxan, they observed parallel changes in islet B cell staining for sulfhydryl/disulfide groups and islet insulin secretion. Moreover, islet B cell staining was abolished when the pancreases were fixed in acid ethanol, which extracted insulin from the pancreas (Barnett and Seligman 1954) (Fig. 8). Although not uniquely specific for insulin per se (as cysteine sulfhydryl groups are present in many proteins), the Barnett and Seligman histochemical method was considered specific for B cells within islets (supported by the later finding that glucagon lacks disulfide bonds). Its widespread adoption for islet studies was nevertheless hindered by its technical difficulty compared with the more conventional Gomori aldehyde fuchsin method, which remained the method of choice for most investigators until tinctorial methods were eclipsed by immunohistochemical methods for identifying islet cell types.

### Pseudoisocyanin Methods

An alternate histochemical technique for staining islet B cells, based on the metachromatic reaction of oxidized insulin with pseudoisocyanin, was described by Coalson (1966). In this protocol, the three cysteine disulfide bonds of insulin are oxidized to sulfonic acid groups, which are subsequently detected with pseudoisocyanin dyes, using a method published by Schiebler and Schiessler (1959). The reaction requires the sections to be oxidized in strong sulfuric acid and potassium permanganate, which also considerably damages tissue structure. Coalson demonstrated that the resulting deep purple metachromatic staining was localized to islet B cells. Despite its apparent specificity for B cells in the islets, the pseudoisocyanin staining method failed to attract much use. This may have been because, although the staining it produced was comparable to that obtained with Gomori’s aldehyde fuchsin method (Fig. 9), the Gomori aldehyde fuchsin protocol was already in common use and, more significantly, the metachromatic staining produced by pseudoisocyanin was unstable and usually faded overnight.



**Figure 8.** Staining of islet B cells in paraffin sections of rat pancreas for sulfhydryl and disulfide groups. Left panel shows absence of staining in a rat islet after extraction of insulin by fixation in acid ethanol. Right panel shows positively stained B cells after fixation in Zenker's fluid (mercuric chloride, potassium dichromate, sodium sulfate, acetic acid). Image from "Histochemical demonstration of sulfhydryl and disulfide groups of protein," by Barnett and Seligman, *Journal of the National Cancer Institute*, 1954.

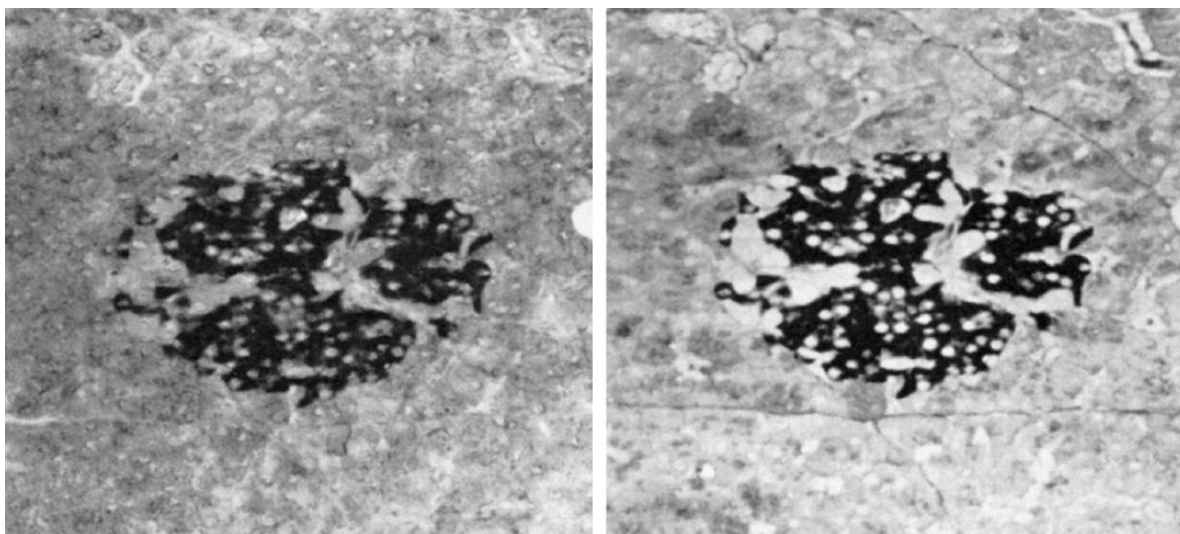
### Islet "Third Cells"

By the end of the 1950s, and before the adoption of immunohistochemical staining techniques, islet A and B cells had been established as functionally distinct cell types secreting glucagon and insulin, respectively. It was also established that islet A and B cells could be identified microscopically by specific tinctorial staining methods, and true histochemical stains, especially for islet B cells, were incipient. However, cells that were distinguishable from A and B cells by virtue of different staining and morphological characteristics had also been described in islets of various vertebrate species (Bensley 1911; Bowie 1925; Bloom 1931; Mosca 1957), leading to the question of whether islets contained a functional "third cell type". The term "third cell type" was applied to any epithelioid islet cell that did not show the staining characteristics of A or B cells with classic tinctorial techniques. A variety of terms were used to name these non-A and non-B islet cells, such as gamma cells (Bowie 1925), C cells (Bensley 1911), D cells (Bloom 1931), and delta (or  $\delta$  cells (Schweisthal et al. 1981). In general, islet "third cell" types were chromophobic (i.e., had clear cytoplasm or stained weakly with conventional tinctorial methods), presumably because of their sparse granule content, as seen with conventional brightfield light microscopy. However, these cells could, in some cases, be stained by certain tinctorial methods such as the Mallory-Heidenhain azan trichrome stain (e.g., Thomas 1937) and others (Epple

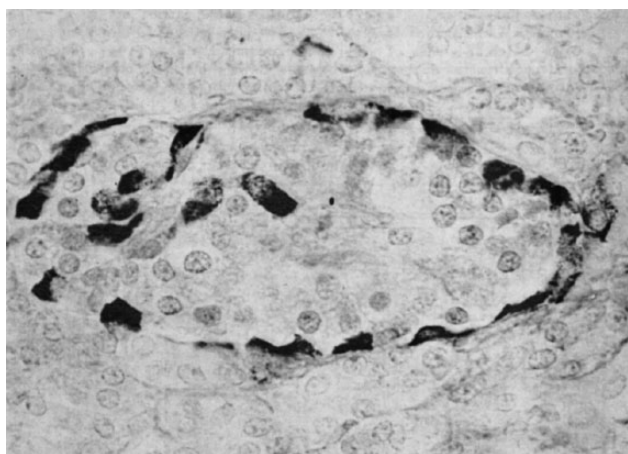
1967; McGadey 1979; Schweisthal et al. 1981) as well as by silver impregnation techniques (Hellerstrom and Hellman 1960; Hellman and Hellerstrom 1961; Grimelius 1968a; Fujita 1968). Bowie's gamma cell was identified in fish islets and is probably not equivalent to the mammalian D cell, which has also been called a gamma cell (Mosca 1957); although, the latter term has also been used for the islet pancreatic polypeptide (F or PP) cell (Malaisse-Lagae et al. 1977). On the basis of staining reactions, the "third cell type" in islets of mammals as well as fish was interpreted by many early investigators as a functional stage of either A or B cells. This literature can be confusing in retrospect because of the uncertainty about the homology of "third cell types", given the different names used by different investigators and in different species, and because the term is no longer in use.

### Argyrophilic Islet Cells

A silver impregnation staining technique for identifying and classifying islet cell types was developed by Scandinavian histochemists in the 1960s (Hellerstrom and Hellman 1960, Hellman and Hellerstrom 1961; Grimelius 1968a, 1968b). The silver staining methods involved deposition of metallic silver over cells that were able to reduce silver nitrate to metallic silver after exposure to a reducing agent such as hydroquinone or formaldehyde. This property is called argyrophilia, and cells that stain with these methods are called



**Figure 9.** Staining of B cells in rat islet with pseudoisocyanin. Left panel shows a paraffin-embedded section stained with pseudoisocyanin, with intense staining of B cells. Right panel shows the same islet as that in the left panel after the pseudoisocyanin stain was removed from the section, and depicts B cells after the section was restained with Gomori's aldehyde fuchsin. Image from "Pseudoisocyanin Staining of Insulin and Specificity of Empirical Islet Cell Stains," by Coulson, *Stain Technology*, 1966. Copyright Informa Healthcare. Published with permission.



**Figure 10.** Islet from a human pancreas stained with the silver nitrate technique, showing black (argyrophilic) islet cells against unstained background. Image from "The argyrophil reaction in islet cells of adult human pancreas: studies with a new silver nitrate procedure," by Grimelius, *Acta Societatis Medicorum Upsaliensis*, 1968. Copyright Informa Healthcare. Published with permission.

argyrophilic cells. In the islets, argyrophilic cells appear intensely black against a yellow background that includes unstained B cells (Fig. 10). Grimelius (1968a, 1968b) identified two cell types that could be distinguished on the basis of their argyrophilic staining properties in formalin-fixed paraffin sections of human autopsy specimens: One type had an elongated shape and stained intensely black with the argyrophilic reaction; another more numerous cell type showed lighter argyrophilic staining and shared tinctorial staining

properties with A cells. Grimelius called them  $\alpha_1$  cells and  $\alpha_2$  cells, respectively, using terminology that Hellman et al. (1962) had proposed for dog islets. The selectivity of the technique was dependent on formaldehyde fixation, as fixation in ethanol- $H_2S$  caused all islet cells to stain black (Grimelius 1968b). Wilander and Westermark (1976) later used electron microscopy to demonstrate that the distinctive cytoplasmic granules of A and D cells exhibited correspondingly different patterns of silver deposition, confirming that the argyrophilic  $\alpha_2$  cells were equivalent to A cells and the argyrophilic  $\alpha_1$  cells were D cells. The D cell was eventually validated as a unique islet cell type that secretes somatostatin by electron microscopy and immunohistochemical staining (Luft et al. 1974; Orci et al. 1975; Erlandsen et al. 1976).

The silver staining properties of  $\alpha_1$  cells and  $\alpha_2$  cells were interpreted to mean that these cells were also capable of secreting biogenic amines (Falck and Hellman 1963; Cegrell 1968). This was based in part on the similarity of  $\alpha_1$  cell and  $\alpha_2$  cell argyrophilic staining properties to those of intestinal argyrophilic cells that were thought to secrete biogenic amines. This hypothesis was extended by Pearse (Pearse 1969; Pearse and Polak 1971) to include the pancreatic islets in his APUD (amine precursor uptake and decarboxylation) cell concept. According to this hypothesis, APUD cells shared the capability of synthesizing biogenic amines and were proposed to have a common developmental origin from neural crest ectoderm. The APUD concept included enteroendocrine argyrophilic cells that secreted peptides as well as many other neuroendocrine cell types that are not argyrophilic. Thus, both islet A cells (i.e., the argyrophilic  $\alpha_2$  cells described by Grimelius) and islet B cells (that are

not argyrophilic) were considered to have the potential of secreting biogenic amines by Pearse. The islet D cell (then called the  $\alpha_1$  cell) was also considered to be an APUD cell by Pearse based on its argyrophilic properties as well as reports that it contained the enteroendocrine hormone gastrin (this was before somatostatin had been discovered). It's now accepted that the APUD concept is invalid at least to the extent that it included islet B cells, which appear not be derived from neuroectoderm (Andrew 1976; Pictet et al. 1976). Furthermore, the specificity of the argyrophilic staining technique for identifying cells that secrete biogenic amines does not agree well with subsequent pharmacological and physiological studies. For example, although islet B cells do not stain with the classic argyrophilic staining methods, recent evidence suggests that islet B cells secrete GABA, dopamine, and serotonin (Garry et al. 1986, 1988; Ohta et al. 2011; Ustione and Piston 2012). Despite these shortcomings of the interpretations of islet cell argyrophilic staining, it's fair to say in retrospect that the method was very useful for identifying islet A and D cells in the pancreas of many vertebrate species before the advent of immunohistochemistry.

## Zinc and Cobalt Techniques

Islet B cells contain a high level of zinc, which plays a key role in the biosynthesis, granular storage, and secretion of insulin. Autoradiographic studies with radioactive zinc showed that living islet B cells preferentially accumulate zinc compared with other islet cells (Wagner et al. 1981). This property proved useful for islet vital staining techniques (Okamoto 1942; McNary 1954). Lukowiak developed a zinc-sensitive fluorescent probe based on Newport Green to selectively label living islet B cells in human islet cell preparations for imaging by confocal microscopy (Lukowiak et al. 2001) and fluorescent activated cell sorting (Liew et al. 2008). The zinc-chelating molecule dithizone selectively stains B cells because of their elevated zinc content. It is used to identify B cells in clusters of developing embryonic pancreatic stem cells (Shiroi et al. 2002; Hefei et al. 2015) and also to identify islets during their isolation from whole pancreas for *in vitro* studies or transplantation (Gray et al. 1983; Bonner-Weir et al. 2000). Interestingly, Bensley (1911) had demonstrated that pancreatic islets could be stained with aqueous neutral red solutions injected via the aorta after exsanguination. This technique was adapted to stain living islets for transplantation without impairing their viability or insulin secretion by injecting 2% solutions of neutral red into the aorta or *i.v.* (Gray et al. 1983). With this method, the islets of dogs, pigs, and rats can be seen as darkly staining bodies against a pale pink background with a stereomicroscope and are visible in frozen sections of rat pancreas. Although neutral red enjoys wide use as a nonspecific, vital stain for many cell types, the reason for its specificity for islets following intravascular injection is not known. Another islet-specific

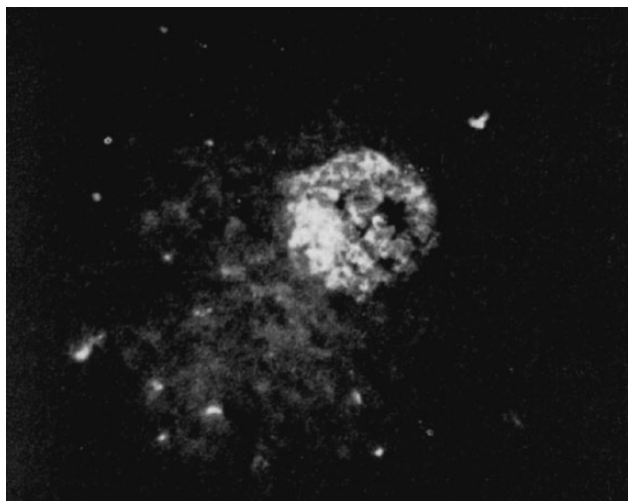
vital stain that has proved useful for islet sorting is a fluorescent dye known as TSQ (Jindal et al. 1993), a toluene sulfonamide that also binds to zinc. More recently, cultured islets have been labeled *in vitro* with dextran-coated superparamagnetic iron oxide (SPIO) nanoparticles and subsequently visualized *in vivo* by magnetic resonance imaging after transplantation into the liver and kidney capsule (Evgenov et al. 2006a, 2006b; Zacharovová et al. 2012). The iron oxide contrasting agents appear to label all islet cell types and the staining can be visualized in tissue sections by light microscopy (Evgenov et al. 2006a).

Cobalt was shown to be concentrated in pancreatic islet tissue of a marine teleost fish by Sture Flakmer (Falkmer et al. 1964) using a histochemical technique that involved *in situ* precipitation of metals as insoluble sulfides. Similar to zinc, cobalt has the property of crystallizing with insulin and it forms complexes with sulfhydryl groups such as cysteine and glutathione. These experiments were facilitated by the fact that teleost islets are gathered into discrete lobes called Brockmann bodies. Several days after injection of cobalt chloride into the living fish, the islet lobes were processed for histochemical detection of cobalt. This involved exposing the islets to hydrogen sulfide gas prior to fixation and embedding. The resulting metal sulfides were then demonstrated with a silver-sulfide reaction that produced a black precipitate over the islet cells. A comparison with aldehyde fuchsin staining showed that mostly B cells were identified with this technique; the islet  $\alpha_1$  cells (equivalent of the argyrophilic D cells) were also stained, whereas the islet  $\alpha_2$  cells (equivalent of A cells) did not. Besides not being easily adapted to routine physiological and pathological tissue, the cobalt technique also had questionable specificity, as others also reported that cobalt stained A cells in fish (Mosca 1957).

## Emergence of Immunohistochemical Techniques for Islets

### *Immunofluorescence Methods*

Shortly after the immunofluorescence histochemical technique was introduced by Coons and Kaplan (1950), Lacy and Davies (1957) published a method for staining B cells in rat pancreatic islets with fluorescein-labeled anti-insulin immune serum that was produced in guinea pigs (Fig. 11). They used small pieces of mouse and beef pancreas that were frozen in liquid nitrogen and freeze-dried, followed by embedding and sectioning in wax. Papers describing the immunofluorescent localization of insulin (Lacy and Davies 1959) and glucagon (Baum et al. 1962) in the islets of humans and other mammalian species soon followed. Nevertheless, the use of immunofluorescent techniques for staining islet cells was slow to be widely adopted. Few investigators had the expertise to produce specific antibodies or had access to the fluorescence microscopes needed for this method. Indeed, as late



**Figure 11.** Original publication of immunofluorescent staining of islet B cells for insulin. Image from “Preliminary Studies on the Demonstration of Insulin in the Islets by the Fluorescent Antibody Technic,” by Lacy and Davies, *Diabetes*, 1957. Copyright American Diabetes Association. Published with permission.

as 1966, Coalson stated in reference to the Lacy and Davies fluorescent antibody technique for identifying B cells, “... fluorescent antibody procedures are too complicated for routine use by many laboratories” (Coalson 1966).

### Immunoperoxidase Methods

The development of enzyme-labeled antibody techniques for immunohistochemistry in the early 1970s revolutionized islet biology. The methods were relatively simple and reliable, and, perhaps foremost, the results could be visualized in a conventional brightfield light microscope. The commercial availability of labeled secondary antibodies, especially soluble peroxidase-antiperoxidase immunoglobulin G (known as the PAP complex) developed by Sternberger and colleagues (1970), brought high quality, reproducible immunohistochemistry within the reach of all islet investigators. Islet B cells could be stained reliably and reproducibly using guinea pig anti-insulin serum (Fig. 12) that was readily available at that time because these antibodies were commonly used for insulin radioimmunoassays. Consequently, reports identifying insulin (Misugi et al. 1970), glucagon (Hegre et al. 1976; Erlandsen 1980), somatostatin (Orci et al. 1975; Goldsmith et al. 1975; Erlandsen et al. 1976; Hegre et al. 1976), and pancreatic polypeptide (Hegre et al. 1976; Greider et al. 1978) based on immunoperoxidase staining, both by light and electron microscopy, soon appeared in the literature, thus confirming the endocrine nature and morphology of the respective islet cell types expressing these hormones (Fig. 13). Armed with hormone-specific antibodies and both immunofluorescent and immunoperoxidase detection methods, islet

investigators no longer had to rely on capricious tinctorial histological stains and relatively nonspecific histochemical methods to identify islet cell types.

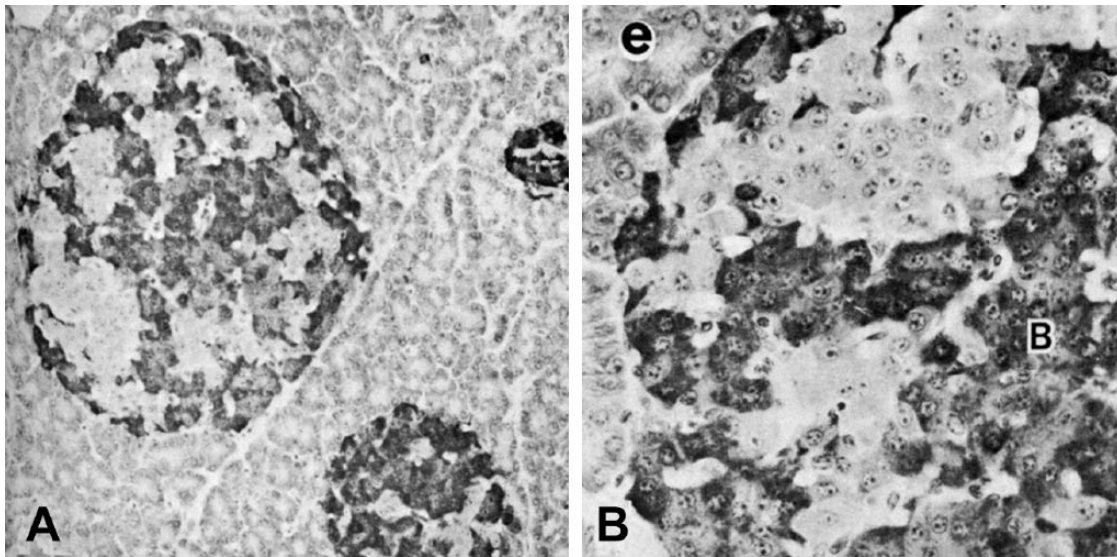
Nevertheless, novel tinctorial methods for staining islet cells continued to appear occasionally. Kito and Hosoda (1977) described a triple labeling tinctorial protocol that incorporated argyrophilic, aldehyde fuchsin, and lead-hematoxylin stains in a single tissue section. McGadey (1979) developed a method for exquisitely staining A, B, and D cells in the same islet with a combination of alcian blue, chrome hematoxylin, acid fuchsin, and aurantia dyes. Even as late as 1982, the dye orcein was shown to specifically stain B cells in human pancreas (Callea and Desmet 1982), but these esoteric tinctorial methods never gained wide use because of the advantages and simplicity of immunohistochemistry for identifying islet cell types.

### Electron Microscopy

Researchers had described the ultrastructural morphology of islet cells since the early days of electron microscopy (Lacy 1957; Williamson and Lacy 1959; Meyer and Bencosme 1965). These and later studies established the morphological features of islet cell types, including the structure and appearance of their cytoplasmic secretory granules (Orci 1974). Immunohistochemistry, particularly in combination with ultrastructural morphology provided by transmission electron microscopy, enabled the definitive identification of islet cell types expressing peptides and other molecules that could not easily be differentiated with classical tinctorial staining methods. Identification of islet cell types by electron microscopy was aided by techniques for correlating the analysis of identified individual cells at both the light microscopic and ultrastructural levels and especially by the application of immunoperoxidase techniques for electron microscopic studies of islets (Pelletier 1977; Erlandsen 1980). Notably, the pancreatic polypeptide secreting F cell (also called the PP or gamma cell), generally appears chromophobic because its secretory granules are not revealed with the classical islet staining methods, whereas the secretory granules in F cells can be readily stained by immunohistochemistry with antibodies to pancreatic polypeptide and visualized by electron microscopy (Greider et al. 1978; Baskin et al. 1984). More recently, a cell that has been called the epsilon cell, has been identified at the periphery of human and rat islets using antibodies to ghrelin (Wierup et al. 2004; Andralojc et al. 2009); although, its status as a distinct islet cell type remains to be firmly established.

### Some Cautions

In many cases, it is not clear whether the localization of a novel molecule to an islet cell represents a functionally unique cell type (such as the classical A, B, and D cells), as it is risky to eliminate the possibility of co-expression with



**Figure 12.** Immunoperoxidase staining of insulin in islet B cells (B) of guinea pig pancreas. Other islet cells and exocrine (e) cells are unstained. Image from “Immunocytochemical identification of cells containing insulin, glucagon, somatostatin, and pancreatic polypeptide in the islets of langerhans of the guinea pig pancreas with light and electron microscopy,” by Baskin, Gorray, and Fujimoto, *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology*, 1984. Copyright John Wiley and Sons. Published with permission.

insulin, glucagon, or somatostatin in the absence of carefully controlled studies; this has been established, for example, by the co-expression of insulin and amylin in islet B cells (Lukinius et al. 1989) and of chromogranin A with insulin and glucagon in the bovine pancreas (Ehrhart et al. 1986). In some cases, the expression of markers identified by immunohistochemistry appears to be unique for specific islet cell types. For example, Bonner-Weir’s group reported that the expression of cytokeratin-19, matrix metalloproteinase-2, and surfactant protein-D were expressed selectively in B cells of developing islets and can be considered as markers of newly formed B cells (Aye et al. 2010).

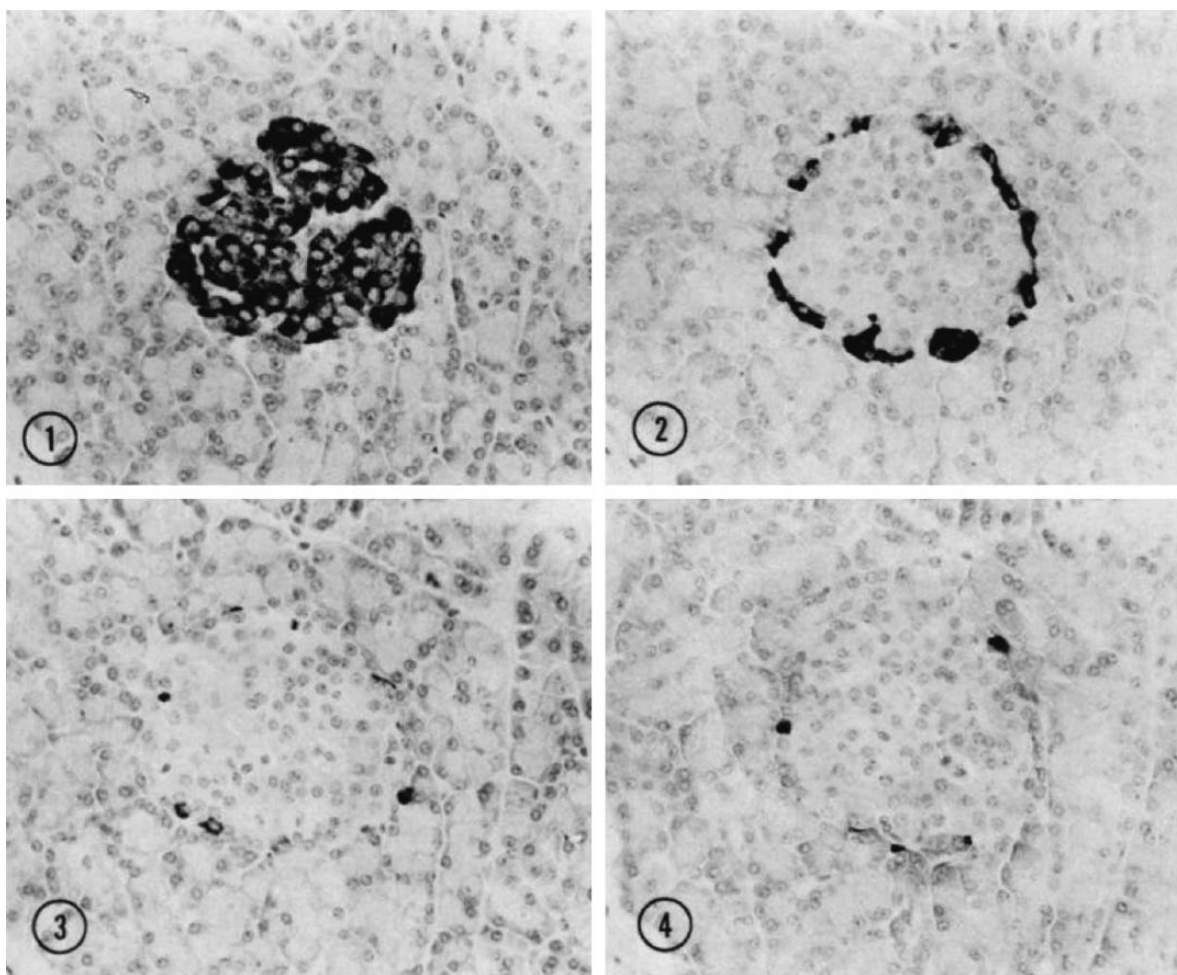
A note of prudence is also appropriate regarding identification of islet cell types based on immunohistochemical staining for antigens other than the classical islet peptide hormones, especially in the absence of valid controls. Islet A cells in particular can show strong nonspecific binding to IgG. Thus positive immunohistochemical staining for non-glucagon antigens can be problematic to conclude as localized in A cells without rigorous validation controls. This phenomenon has been attributed to ionic charge effects, hydrophobic interactions, and complement interactions resulting from basic amino acid residues (and is alleviated by use of poly-L-lysine in the incubation buffer) (Scopsi et al. 1986; Buffa et al. 1979). The importance of rigorous controls for standardizing and validating immunohistochemistry has recently been emphasized (Hewitt et al. 2014).

## Perspective

One could argue, and in principle I would not disagree, that in situ hybridization methods properly belong in this

account, but I have purposely chosen to omit this technique. In situ hybridization, although an extremely powerful histochemical technique for identifying gene expression in islet cells and one that can give us a wealth of information about multiple gene expression and the dynamics of gene responses to physiological mechanisms, nevertheless has not added much fundamentally to our ability to identify islet cell types in a physiological context. This thinking could be accused of being too parochial, as it’s based on a view of islet cells being characterized by the expression of a single hormone (e.g., insulin). Admittedly, this is an endocrinologist’s physiological perspective—islets are classically characterized by cells that secrete the hormones insulin, glucagon, somatostatin, and pancreatic polypeptide. An investigator from another discipline might functionally categorize islet cell types based on their expression of receptors, homeobox genes, transcription factors, immunological markers, or matrix molecules. But I think that an instructive take home message from this historical review is that, while the classic histochemical and tinctorial staining methods were not necessarily specific for identifying islet hormones (i.e., Barnett’s histochemical stains for insulin also stained sulfide groups in other tissues), nevertheless, within the boundaries of an islet, those techniques could reliably identify A, B, and D cells.

Compared to the classical tinctorial and histochemical methods, antibody-based immunohistochemical staining techniques are unquestionably superior, more reliable and reproducible, and have a stoichiometry that can be related to hormone content (albeit, crudely). They also allow us to co-localize other gene products that are expressed with islet hormones under diverse physiological, pathological, and



**Figure 13.** Immunoperoxidase identification of classic islet cell types in rat pancreas. (1) B cells immunostained for insulin. (2) A cells immunostained for glucagon. (3) D cells immunostained for somatostatin. (4) F cells immunostained for pancreatic polypeptide. Image from “Pancreatic islet cell hormones distribution of cell types in the islet and evidence for the presence of somatostatin and gastrin within the D cell, by Erlandsen, Hegre, Parsons, McEvoy, and Elde, *Journal of Histochemistry and Cytochemistry*, 1976. Copyright Histochemical Society. Published with permission.

developmental conditions. While not denying the advantages of antibody-based staining techniques for understanding islet cell biology, it is not a stretch to say that A, B, and D cells could already be reliably identified on the basis of their morphology and classical tinctorial and staining properties before the wide spread use of immunohistochemical techniques. For example, comparing published figures of islets stained with Gomori’s aldehyde fuchsin method and islets immunostained for insulin reveals remarkably similar images that could be easily seen as identical.

In hindsight, it’s clear that the specificity of classical histochemical and tinctorial stains for respective islet cell types was not established solely by histological techniques. The most seminal of those advances involved staining experiments that were conducted in the context of physiological and pathophysiological studies (e.g., animals were treated with alloxan to create diabetes, diabetic animals were administered insulin, and human autopsy tissue from

normal and diabetic subjects were studied and compared with staining results). Thus, advances in islet pathophysiology developed hand in hand with the development of histological and histochemical staining methods for identifying islet cell types and interrogating their functions.

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## Author Contribution

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