STUDIES ON THE METHODS OF STAINING THE ISLET CELLS OF THE PANCREAS

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Cytological studies of the islets of Langerhans of the pancreas have required the use of many and varied histological techniques. Each method has had its advantages and disadvantages, but the most serious deficiencies have been the inexact chromatic specificity and the lack of correlation between the various methods. The relatively small number of cellular structures differentially

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The present study was undertaken with a view to reassessing the various technical methods employed in the past, with the hope of being able to select a small group of techniques the application of which would reveal the minute details of the cytological structure of the islets of Langerhans with uniformity and precision. In the course of this work, which extended over a period of four years, most of the techniques of fixation and staining that have been described and recommended in the literature of the last 60 years were carefully carried out on the pancreatic tissues of man and a variety of animal species. The various species to the tissues of which the histological techniques were applied included large numbers of rabbits of various ages, and considerable numbers of the common laboratory anti domestic mammalian species. Smaller numbers of birds, reptiles, and fishes were also studied.

As a result of this exhaustive survey, several methods described in the literature have been modified and combined to provide a relatively small group of simple techniques which, if carefully applied, yield the desired results with consistency. These methods are suitable for the detailed cytological study of the embryonic or the adult pancreas and the normal or the pathological pancreas. It is to be emphasized, however, that the techniques which it is the purpose of this paper to describe will yield completely satisfactory results only if care is exercised at every stage of the method, since the best of staining procedures cannot retrieve the damage of poor fixation or incomplete dehydration. For this reason, the technical methods suitable for the study of adult or embryonic pancreatic tissues are presented in detail.

METHODS

Embryos are killed by immersing the open uterus in the fixative. A few minutes after immersion a small perforation should be made in the amniotic sac with scissors. This allows the embryo to come into contact with the fixative while retaining its placental attachment. The attached placenta is most useful for manipulating the embryo. Newborn animals are killed by decapitation. Adult animals may be killed by air embolism, a blow on the nape of the neck, or by intravenously injected pentobarbital sodium. Each one of these methods has its obvious advantages and disadvantages. Air embolism should be avoided when killing pregnant rabbits because the air bubbles pass into the embryonic sac and render the subsequent treatment of the embryos difficult.

Samples of pancreas should be removed immediately after death. Embryos younger than 15 days since mating of their progenitors should be fixed and processed in toto. The pancreas of embryos between 15 and 20 days old are dissected as soon as the surfaces of the embryos are hard enough to permit complete horizontal section of the abdomen, or in about one-half to one hour after fixation begins. As the age of the embryo increases, the abdomen is opened as soon as possible, in order to expose the pancreas to the fixative. The pancreas is removed together with the neighboring organs and placed in fresh fixative. Pancreases of newborn and adult animals must be dissected and removed with extreme gentleness. Specimens from animals such as the rabbit with a diffuse pancreas are fixed in large pies, while those from animals with a compact pancreas (the dog) should be trimmed in the fresh state into blocks about 3 mm thick.

ROUTINE PROCEDURES FOR THE STUDY OF THE GENERAL CYTOLOGICAL ASPECT OF THE PANCREATIC ISLETS

1. Fix tissue for 8 to 16 hours in Zenker-formol. Zenker’s solution is made up as follows:
   - Mercury bichloride.......................................................... 5.0gm.
   - Potassium bichromate.................................................... 2.5gm.
Sodium sulfate ........................................... 1.0gm.
Distilled water ........................................... 100 cc.

To 80 cc. of the above solution 20 cc. of 40% formaldehyde (formalin) neutralized with sodium carbonate or marble chips. The formalin must be neutral when tested with litmus paper, and it should added just before use. It is better that the tissues be placed in the dark while they are fixed in this mixture.

2. Wash fixed tissues in running water for 24 hours or more.

2. Bloom.\textsuperscript{1m} Gomori.\textsuperscript{1p}

3. (a) Dehydrate the tissues from animals 1 week of age or older as follows:
95% alcohol containing 0.5% jodine ...................... 24 hours
Dehydrated alcohol twice ............................. 8 hours
Dehydrated alcohol used once ....................... 16 hours
Dehydrated alcohol .................................... 8 hours
Shake bottles at least four times a day.

(b) Dehydrate tissues from embryos and animals younger than 1 week of age according to the following schedule
Alcohol 40% ............................................... 2 hours
Alcohol 50% ............................................... 2 hours
Alcohol 60% ............................................... 2 hours
Alcohol 70% ............................................... 2 hours
Alcohol 80% ............................................... 3 hours
Alcohol 90% ............................................... 3 hours (with 0.25% iodine)
Alcohol 95% ............................................... 10 hours (with 0.25% iodine)
Dehydrated alcohol .................................... 3 hours
Dehydrated alcohol .................................... 5 hours

4. (a) Clear tissues from animals 1 week of age or older with toluol in the following manner:
Toluol used twice ...................................... 8 hours
Toluol used once ....................................... 16 hours
Toluol, pure ............................................. 8 hours
Toluol, pure ............................................. 8 hours

(b) Clear tissues from embryos and tissues from animals younger than 1 week of age according to the following scheme:
Toluol-dehydrated alcohol 1:1 ........................ 2 hours
Toluol-dehydrated alcohol 3:1 ...................... 2 hours
Toluol used twice ...................................... 3 hours
Toluol used once ...................................... 10 hours
Toluol, pure .............................................. 2 hours
Toluol, pure .............................................. 4 hours
5. (a) Embed tissues from animals older than 1 week in a mixture of paraffin (melting point, 50-52 C.) and raw, thoroughly dry beeswax (yellow wax, U. S. P.) (9: 1) in paraffin ovens at 54-56 C. as follows:
Paraffin oven No. 1.......................................................... 8 hours
Paraffin oven No. 2.......................................................... 16 hours
Paraffin oven No. 3.......................................................... 1.5 days
It has been found that the adequate elimination of toluol from the tissue requires the use of three ovens.
(b) Place embryos and tissues from animals younger than 1 week in a mixture of toluol and paraffin (1: 1) for two hours and then treat as adult tissues.

6. Block in ice water, using metal boats.
7. Cut at 2.5-5 or 7.5 microns (u) as desired.
8. Mount sections by the gelatin method and dry at 45 C. in an oven containing some formol vapor.

MASSON TRICROME STAIN APPLIED AS A GENERAL CYTOLOGICAL AND DIFFERENTIAL STAIN FOR THE PANCREAS. 3a

1. Hydrate
2. Treat with iodine-alcohol 1% for 10 to 30 minutes.
3. Rinse with distilled water.
4. Treat with sodium hyposulfite 5% solution for 1 minute.
5. Wash in tap water for 5 to 15 minutes.

6. Rinse well with distilled water.

7. Treat with iron-alum (iron and potassium aluminum sulfate) 5% for 5 minutes to 10 hours at 52-56 C.

8. Rinse cautiously in distilled water.

9. Stain in Regaud’s hematoxylin for 5 minutes to 10 hours at 52-56 C

10. Rinse with 95% alcohol until no more color comes out.

11. Differentiate at 52—56 C. in picric-alcohol solution (2 parts of a saturated solution of picric acid {trinitrophenol} in 95% ethyl alcohol diluted with 1 part of 95% alcohol) until chromatin is sharply differentiated. Control this step under the microscope by rinsing with 95% alcohol. There is some advantage in slightly overdifferentiating the nuclei.

12. Wash in running water until no more picric acid is present, 20 to 40 minutes or more according to the thickness of the section.

13. Stain at least one hour with a ponceau-fuchsin mixture, comprising 4 parts of 1% ponceau,1 part of 1% acid fuchsin,\(^4\) and 1 part of 1% fuchsin S,\(^5\) all diluted 1:10 in 1% acetic acid water. Twelve hours of staining produces excellent results.

14. Rinse briefly in 1% aqueous acetic acid.

15. Differentiate in a 5% phosphomolybdic acid solution until the delta cells are unstained or pale gray. The time varies, depending on the brand of ponceau, but is approximately 2 to 10 hours at 52-56 C. Check with the microscope, using distilled water.

16. When delta cells and other cyanophilic structures are thoroughly differentiated, rinse well with distilled water.

17. Apply aniline blue made according to Masson’s original method, for 10 to 20 minutes. A subdilution of this stain 5: 100 in 1% acetic acid is more convenient in controlling the staining process. It is applied for 2 to 6 hours while controlling the Process under the microscope until the desired effect is obtained.

18. Rinse briefly with distilled water.

19. Differentiate the aniline blue with 1% aqueous phosphomolybdic acid for 10 to 45 seconds.

20. Rinse in 1% acetic acid and place in acetic acid 1% for 5 to 30 minutes until all colors and structures are clearly defined.

21. Dehydrate with dehydrated alcohol (absolute alcohol) (avoid dilute alcohol) by the dropper method or by using three changes of dehydrated alcohol for 2 to 5 seconds, and 1 minute, respectively.
22. Clear with three changes of toluol for 5 minutes each. Rinse with fresh toluol before mounting in Canada balsam or permount,* dry on a hot plate at 52-58 C. for 24 hours. Use No. 0 or 1 cover slips and cover slip weights.

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**EXPLANATION OF FIGURE 1**

*A,* photomicrograph of part of a pancreatic islet front a rabbit 1 year old, stained by the trichrome technique. Alpha cells (red-brown) appear granular and almost black. Their Golgi apparatus can be seen as a negative image. Beta cells (pink) appear gray and are faintly granular. They constitute the greater part of this islet. Their Golgi net also appears as a negative image. Delta cells (sky-blue) appear a faint or pale gray color. They are agranular and may be seen clustered around the group of alpha cells located at the lower left part of the islet. The acinar tissue shows a minimal amount of retraction, and cell borders are indistinct. Zymogen granules are coarse and dark. This section is cut at 2.5 µ. X 1,200.

*B,* photomicrograph of an islet from the same block of tissue shown in *A,* stained by the Gomori technique. Alpha cells (red) and beta cells (steel blue) are almost indistinguishable in the photograph, although some of the alpha cells are darker gray. A cluster of delta cells (pale gray-pink) may be seen clearly at the lower left corner of the islet. Pale areas in these cells. That photograph as vacuoles represent the macular zone. The section is 2.5 µs in thickness. x 1,200.

**RESULTS.**—*WHEN THE SECTIONS ARE THIN (2.5 ) THE GRANULES OF THE ALPHA CELL ARE SEEN INDIVIDUALLY. THEY ARE STAINED IN DEEP FUCHSIN, FUCHSIN-BROWN OR BROWN-LILAC. THESE THREE VARIETIES OF GRANULAR STAINING OF THE ALPHA CELL MAY COEXIST IN THE SAME ISLET.* M’MITOCHONDRIA CANNOT BE DIFFERENTIATED FROM ALPHA CELL GRANULES. THE GOLGI APPARATUS APPEARS AS A JUXTANUCLEAR VACUOLE. A CYANOPHILIC MACULAR STRUCTURE OF IRREGULAR SHAPE AND POSITION IS CLEARLY DEMONSTRATED.

**BETA CELL GRANULES STAIN SALMON-PINK. MITOCHONDRIA STAIN RED-BROWN OR FUCHSIN-RED BUT ARE NOT ALWAYS EASY TO DISTINGUISH FROM THE GRANULES. HOWEVER, WHEN THESE STRUCTURES ARE SEGREGATED, THE COLOR DIFFERENCE IS STRIKING. WHEN THE CYTOPLASM CAN BE SEEN BETWEEN GRANULES, IT HAS A PALE GRAYISH TINT. THE GOLGI NET OF THE BETA CELLS APPEARS AS A SERIES OF BRANCHED CLEAR CHANNELS. BETA CELLS ALSO SHOW A CYANOPHILIC STRUCTURE SIMILAR TO THAT OF THE ALPHA CELL. OCCASIONALLY BETA CELLS SHOW, ON ONE EDGE A CUTICULA THAT IS BRILLIANTLY STAINED RED-ORANGE.

**THE DELTA CELLS DO NOT POSSESS GRANULES BUT HAVE A GLASSY SKY-BLUE CYTOPLASM WITH SMALL ROD-SHAPED RED MITOCHONDRIA. THE GOLGI NET OF THE DELTA CELL APPEARS AS A CLEAR CANAL THAT IS MODERATELY BRANCHED. A CYANOPHILIC MACULAR STRUCTURE IS ALSO PRESENT, AND IT CAN BE DISTINGUISHED FROM THE REST OF THE BLUE CYTOPLASM BY ITS HIGHER DEGREE OF REFRACTILITY.**

**DUCTULAR EPITHELIUM, CENTROACINAR CELLS, GOBLET CELLS, AND THE CELLS OF THE SEROUS GLANDS ALL HAVE THEIR CYTOPLASM COLORED AN INDIFFERENT PALE BLUE-GRAY, AND SHOW RED MITOCHONDRIA. THE MATERIAL ACCUMULATED IN THE SMALL DUCTS STAINS RED, BUT IN THE LARGER ONES IT APPEARS DIRTY BLUE.**
In addition to typical alpha, beta, and delta cells, the walls of the secretory ducts contain certain cells that stain in different ways, varying from completely unstained “clear cells” to those that are very dark brown.

Acinar cells are lilac at their basal region and possess red mitochondria and red zymogen granules. Many shades of lilac can be seen in the acinar cells. Collagen, mucin, hyalin, and colloid are all stained various shades of blue. Ganglion cells stain brown-gray and show various types of green and brown pigment. Schwann cells are stained red.

Nucleolar chromatin stains deep black and is shown with great delicacy. Nucleoli are red.

In embryos, the results are similar. It is impossible to detail in this paper all of the results obtained with embryonic material, but it may be noted that the differentiation of the various islet cells and the peripheral nervous system is so clear that early immature types of islet cells can be studied in more detail than has ever been possible before.

Gomori’s Chromic Hematoxylin-Ponceau Stain
1. Carry out steps 1 to 5, inclusive, of the trichrome method.
2. Treat with potassium permanganate (Gomori’s original formula) for 1 to 2 minutes.
3. Wash in running tap water for 1 minute.
4. Bleach in potassium meta-bisulfite 5% for 1/2 to 1 minute.
5. Wash in running tap water for 5 minutes.
6. Stain in chromic hematoxylin (Gomori’s original formula) for 15 to 45 minutes at room temperature, or for 5 to 15 minutes at 52-56°C.
7. Rinse with 95% alcohol until no more color washes out.
8. Differentiate in 1% solution of reagent hydrochloric acid in 95% alcohol for 1 minute.
9. Wash in tap water for 5 to 10 minutes.
10. Stain in ponceau-fuchsin (the same staining mixture that is used in the trichrome method) for 15 to 45 minutes.
11. Rinse in 1% acetic acid.
12. Differentiate with phosphomolybdic acid 1% until alpha and beta cells are clear approximately 5 to 30 minutes depending on the ponceau mixture).
13. Rinse with 1% acetic acid and place in 1% acetic acid for 1 minute.
14. Dehydrate, clear, and mount as before.

Results—Alpha granules are deep red. Beta cell granules are steel blue to black; the mitochondria of beta cells are pale red: the cytoplasm of beta cells is pale gray. The cytoplasm of the delta cells is amphophilic, appearing pale gray to gray—orange. Mitochondria of beta and delta cells are pale orange-red. Ductular epithelium, centroacinar cells, goblet cells, and cells of the serous glands all stain an indifferent pale gray to gray-orange. The mucin of goblet cells stain deep steel blue. Basophilic substance in the acinar tissue appears bluish purple, and zymogen granules are red-orange.
In embryonic tissue the method permits a moderately good degree of differentiation of immature islet cells, particularly of the alpha cells, but it is not as useful as the trichrome method.

Procedure For The Demonstration Of The Mitochondria Of The ISLET Cell.
Schridde’s Method)—The procedure is carried out as follows:

1. Fix as follows:
   Muller-formol ................................................................. 2 days
   Muller solution......................................................................2 days
   Osmic acid 2% .................................................................2 days

The formol must be 40% formaldehyde and neutralized as was that used in the preparation of Zenkerformol. Prepare the Muller-formol when it is to be used. Tissues embryos and adult animals are **Fixed** similarly.

2. Wash in running water for 24 hours.

3. Employ the same dehydrating and embedding techniques used previously. Do not add any iodine to any of the alcohols.

Staining With Iron Hematoxylin.—
1. Hydrate.
2. Treat with iron—alum 5% for 6 to 10 hours at 52-56 C., or for 1 to 2 days at room temperature.
3. Treat with Regaud hematoxylin for 6 to 10 hours at 52-56 C. or for 1 to 2 days at room temperature.
4. Rinse briefly with distilled water.
5. Differentiate with iron—alum 1% until mitochondria are distinct (approximately 5 to 60 minutes).
6. Rinse well with distilled water. , F
7. Wash in three changes of distilled water for 60 minutes each.
8. Dehydrate, clear, and mount as before.

Results.—Mitochondria and zymogen granules are black. The granules of beta cells are gray. The results are excellent in older embryos and in younger animals.
PROCEDURE FOR THE DEMONSTRATION OF THE GOLGI APPARATUS OF THE ISLET CELLS

Silver Method of Aoyama_The procedure follows:
1. Fix for 5 to 7 hours in a solution of
   Cadmium chloride.................................................................................................................. 1 gm.
   Distilled water ......................................................................................................................... 85 cc.
   Neutral formol (.40% formaldehyde) ......................................................................................15 cc. (add the formol before using).

2. Rinse three times in distilled water quickly and transfer to a solution of AgNO3 2%,
   in the dark for 9 to 12 hours, it is advisable to shake the specimen a few times during this procedure.

3. Rinse twice in distilled water quickly in the dark and the transfer to a freshly prepared solution of Hydroquinone......................................................... 1gm
Sodium Sulfite................................................................. 0.15gm
Distilled water.................................................................. 85cc.
Neutral formol (40% formaldehyde)......................................15 cc.
Leave in the solution for 12-24 hours, and then wash three times with distilled water for 10 minutes in each wash.

4. Employ the same dehydrating and embedding techniques used previously. Do not add any iodine to any of the alcohols.
Staining.—Staining is done as follows

1. Hydrate.

2. Treat with gold chloride, 1 gin, in 500 cc. of water, for 3/ to 1 hour. Check under the microscope until tolling is completed (Golgi apparatus is black).

3. Rinse in distilled water briefly.

4. Treat with sodium hyposulfite 5% for 5 to 15 minutes.
5. Wash in tap water for 15 minutes.

6. Counterstain with Masson trichrome or Gomori’s hematoxylin.

7. Dehydrate, clear, and mount as before.

The Mallory—Heidenhain azocarmine method can also be used for counterstaining. Counterstaining with chromotrope hematoxylin can be performed without any modification. Counterstaining with the trichrome method can be done without modification if started at Step 13. The results, however, are not as warm as when the following method is used. After Step 5 of the Aoyama procedure to Step 6 to 9 of the regular trichrome procedure and then differentiate the nuclei as follows:

1. Rinse with distilled water until no more stain comes away.
2. Differentiate the nuclei with 1% iron—alum. The slides must be shaken continuously in order to obtain an even differentiation. Control the reaction under the microscope with the use of distilled water.
3. Rinse with distilled water and wash in running water for 15—20 minutes.
4. Dehydrate, clear, and mount as before.

Results.—The Golgi apparatus is black. The cells are stained in the usual manner of the counterstain, but because of formaldehyde fixation the cytoplasmic details are not as well

Expanation of figure 2

A, phototunctograph of a mitochondrial preparation of a pancreatic islet from a rabbit at birth. The mitochondria appear as fine black filaments. The granules of the alpha cells also stain black, and it is not possible to distinguish their mitochondria in these preparations. Such a black alpha cell may be seen at the apex of the islet.

Coarse spherical black zymogen granules and filamenteous mitochondria are seen in the acinar tissue.
The section is 2.5 in thickness. X<1,200.

B, photomicrograph of a pancreatic islet from a rabbit 1 year old, stained by the Anyama method for the Golgi apparatus and counterstained by the trichrome technique. Both alpha cells (red-brown) and Golgi apparatus (black) appear black in the photograph; beta cells (pink) and gray and granular, while delta cells are pale and agranular. In the center of the picture is a crescent of black alpha cells. The crescent is formed by a tangential section through the tips of several alpha cells. No nuclei have been sectioned. Lying in the hollow of the crescent close to the tip of its lower arm is a dark granular alpha cell, and close to its nucleus is a compact homogeneous black mass that represents the Golgi apparatus. Immediately above and to the right of the crescent of alpha cells lies a large pale delta cell in which can be seen a few small black filaments that constitute its Golgi network. The irregular, branched Golgi apparatus of the beta cells can be seen in the many cells present. Its heaviest portion lies close to the nucleus and corresponds to the negative Golgi image seen in ordinary preparations.
The section is 2.5 is in thickness. X 1,200.

defined as if Zenker-formol fixed tissues had been employed. Nevertheless, an exact differentiation of the alpha, beta, and delta cells is possible, and their Golgi apparatus arc demonstrated at the same time,

This procedure has proved to be of great help in the study of the embryonal islet cells.

It is perfectly applicable when pathologic tissues are to be studied.
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P.uFrXN SF;CTJoN
1. Fix in 1% trichloroacetic acid mixture or in formalin 10%.
2. Dehydrate, clear, embed, and cut in the usual manner.
3. Stain with (a) Fontana’s stain (for argentaffin cells, Masson’s modification) or (b) Foot-Laidlow reticulin stain (Masson’s modification*) or (c) Roger’s silver method as modified by Van Campenliout for nerve impregnation.
4. (e) Counterstain with the trichrome technique, differentiating with 1% iron—alum. (b) If nuclear detail is not important, the counterstain may be started at Step 13 of the Masson trichrome stain.

Results.—Argentaffin cells are black, while the remainder of the tissue has the usual differential characteristics of the trichrome method.

CO1MENT
In the course of a study of the staining methods best suited to the cytological investigation of the pancreas, it was found that Masson’s trichrome stain could be developed into an excellent general survey stain that clearly differentiates alpha, beta, and delta cells. It was also found that both the trichrome and the Gomori method could be used to counterstain differentially Golgi preparations done by the Aoyama technique and to counterstain argentaffin preparations done by Roger’s, Foot-Laidlow, or Fontana’s technique. These various methods, together with that of Schridde for mitochondria, proved adequate for the study of the embryonic pancreas as well as for that of the normal and pathological adult pancreas.

The results of the method detailed above are most typical of the rabbit’s pancreas. ‘The human pancreas can be treated in exactly the same manner, with excellent results. I have found it necessary to alter the time schedules of some of the procedures when applying the methods to the pancreases of birds, fish, turtles, and other vertebrates, but it has seldom been necessary to change the concentrations of the reagent and the staining solutions. It may be noted that embryos and lower forms of vertebrate life fix more (Julicy thais adult tissues or tissues from higher forms.

It has been observed that the trichrome inetnnetl will stain certain cells or parts of cells somewhat differently, even in the same islet. The tinctorial variations have been studied in combination and correlation with the various other techniques described above, and it has been found that most of the color variations are specifically associated with easily demonstrable changes in various cellular organelles. This fact has made the trichronic method extremely useful, since it is possible to employ it as a survey stain that detects simultaneously all of the cyttoplasmic elements that may be demonstrated by other methods.

Satisfactory results can be obtained only by following the techniques faithfully in every detail. It has been found that carelessness at one stage of the procedure cannot be compensated for by great care at sonic subsequent stage. As tic’s of minor faults accumulate to constitute a major defect. If the investigator will acquaint himself with the minute of the techniques he will have at his disposal methods of high specificity, great elasticity, and easy reproducibility.

SUMMARY
A systematic investigation of the various methods available for the cytological study of the islets of Langerhans of the pancreas resulted in the modification and combination of a small group which proved to be suitable for the detailed study of the normal, pathological, embryonic, or adult pancreas.

It was found that Masson’s trichrome stain could be developed into an excellent survey stain that not only clearly differentiated alpha, beta, and delta cells but also detected simultaneously all of the cytoplasmic elements that may be demonstrated more clearly in other methods. Both the Masson
trichrome and the Gomori method can be applied successfully to counterstain differentially preparations in which the Golgi apparatus has been shown by the Aoyazna technique or to counterstain argentafbn preparations done by Roger’s, Foot—Laidlaw and Fontana’s techniques. The Schridde method was used to demonstrate mitochondria. These various methods used alone and in combinations allow unusually complete and exact observations to be made on the Pancreas.

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