The Application of Techniques for Specific Staining and Enhancement to the Ultrastructural Study of the Pancreatic Islands of Langerhans

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THE APPLICATION OF TECHNIQUES FOR SPECIFIC
STAINING AND ENHANCEMENT TO THE ULTRASTRUCTURAL
STUDY OF THE PANCREATIC ISLANDS OF LANGERHANS

by

Vern Leonard Shellman

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Vern Leonard Shellman
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INTRODUCTION

Diabetes is a metabolic disorder characterized by an inability to properly regulate glucose metabolism. This results in an elevation of blood glucose (hyperglycemia) and excretion of glucose into the urine (glucosuria). The degree to which these symptoms are expressed is found to vary among individuals. For the most severe cases of untreated diabetes an additional symptom may present itself, that being the appearance of ketone bodies in the blood (ketosis). This occurs as a result of fatty acid degradation to avert starvation.

The utilization of glucose is normally regulated by hormonal secretions of the pancreatic islands of Langerhans. In particular, the hormone insulin plays an important role during this process. What happens when the concentration of glucose in the blood exceeds a pre-determined level (such as after a meal) is that the excess glucose triggers the release of insulin from its site of storage in the beta (B) cells of the pancreatic islets. The insulin then enters the bloodstream and is carried by way of the vascular system to the various tissues of the body where it ultimately comes into contact with individual cells. Upon attaching to specific insulin receptor molecules on the surfaces of cells, insulin is able to regulate the uptake of glucose into the cells. When diabetes is present this sequence of events is disturbed at some point, leading to a build-up of blood glucose.

Although all diabetics have in common the impaired ability to utilize glucose, this can be brought about in very different ways. Listed below are some of the conditions that in theory can lead to the
diabetic state:

1. The absence of B cells. If through trauma or some other cause B cells were lacking or reduced in number, then little or no insulin could be synthesized and glucose metabolism would be impaired.

2. The absence of insulin. If the B cells failed to produce insulin, or produced insufficient amounts of it, the result would be as above.

3. The signal to release insulin not received. This would be an informational error in which the B cells would not recognize that there was excess glucose present in the blood, and would therefore fail to release insulin.

4. Impairment of the release mechanism. In this case the B cells would produce insulin and respond to the presence of glucose in the blood, but due to a defect in the release mechanism no insulin could be released.

5. The release of defective insulin. Here insulin reaching the target cells would be defective and therefore biologically inactive. Such a defect would have to alter the shape of the insulin molecule. If the alteration occurred at the pre-translational level it would have to be genetic in origin; however, it is conceivable that insulin in the blood could be altered chemically and so, too, give rise to an inactive molecule.

6. The destruction of insulin. In the event of autoimmune disease antibody synthesis would be directed against insulin. When insulin would be released into the bloodstream circulating
antibodies directed against insulin would combine with it and remove it from the system.

7. Faulty insulin receptors. Here is a case in which there would actually be nothing wrong with normal pancreatic function or insulin release. Rather, the problem would concern the insulin receptors on the target cells of the body. In some way the number or efficacy of these receptors would become altered so that insulin could no longer trigger glucose uptake. The result would be nearly the same as if the receptors worked properly but no insulin were produced.

These types of diabetes are represented in the flowchart in figure 1 along with some possible means of induction. For the sake of simplicity only main categories are shown with no further attempt at subdivision.

Not all of the possible means of induction that are shown, or even all the causes, have been demonstrated to produce diabetes. I am not aware, for example, that diabetes has ever been definitively shown to be the result of the B cell either not producing or failing to release insulin. On the other hand, a universal cause for diabetes has yet to be found. The important premise is to recognize that any of these causes, by whatever means of induction, would lead to a diagnosis of diabetes mellitus, despite the fact that the cause in each case is very different. For this reason, diabetes might better be considered to be a "syndrome" with varied etiology but similar symptomatology.

Clearly then, diabetes is not a simple disease but one which can be brought about in many different ways. In fact, if we assume that all the possible means of induction which I have indicated here do indeed
Fig. 1. A flowchart indicating the various etiologies of diabetes along with some possible means of induction for each.
Insulin Released

Insulin Reaches Target Cells

Insulin does not Reach Target Cells

Cause:
- Defective Insulin
- Faulty Insulin Receptors
- Insulin Destroyed

Possible Means of Induction:
- Genetic
- Chemical
- Genetic
- Autoimmune
- Autoimmune
- Viral
- Chemical

Fig. 1. Continued
exist, and that the causes given are valid, then there are over twenty
distinct ways in which the disease may become expressed, disregarding
that many of the "causes" shown can be even further subdivided. It is,
perhaps, this multi-faceted aspect of diabetes which, more than any other
reason, accounts for the difficulty that has been encountered in attempting
to understand the disease. With research being carried out independently
by immunologists, biochemists, geneticists, and virologists among others,
it has been facetiously suggested that diabetes is a disease that has
something of interest for everyone. But understanding a complex problem
such as this need not be unnecessarily difficult. At the risk of appear-
ing simplistic it could be said that what is required is to reduce the
problem to its fundamentals by first studying the system through which
insulin is made available. Here a starting point might be realized from
which investigations of the various diabetic types can be made.

Such a starting point might be thought to be found in the pancre-
atic B cell. Looking again at figure 1 it will be seen that with the
exception of the last two categories the B cell figures prominently in
the manifestation of diabetes. Whether diabetes occurs as the result of
the B cells failing to synthesize or release insulin or synthesizing de-
fective insulin, it would appear that the B cell is a likely common de-
nominator for the disease. An understanding of the normal workings of
the B cell would, then, be useful in determining what abnormal events
may be responsible when the B cells fail to perform their assigned func-
tion. Indeed, a great deal of attention has been concentrated on the
B cell in recent years with the result that it is probably the best under-
stood cell of all those involved in glucose utilization. However, in
studying the B cell and the important role it plays among the cells of the islet it has come to be suspected that the environment external to the B cell has as great an influence upon B cell performance as does the intracellular environment. That is to say, production or release of insulin by the B cell may be influenced in turn by other cells, specifically other islet cells. If that is so, then B cells may play only a relatively small part in insulin release compared to what previously has been thought to be the case. Possibly the B cell is only an effector cell of the larger cell system responsible for regulating glucose uptake by the body. If true, then the study of insulin release should be carried out with the idea in mind that it is the islet as a whole that is the functional unit of insulin availability and not just the B cell. To concentrate solely on the B cell under those circumstances would be to miss the overall view.

With that in mind, the study of islet anatomy and cell interaction with regard to diabetes would seem to gain some importance. Perhaps the key to understanding the problem lies in learning what organization exists at the islet level before looking too closely at isolated cell types.

The pancreatic islands of Langerhans are roughly spherical clusters of cells which together are morphologically distinct from the surrounding acinar tissue of the pancreas. Functionally, they represent the endocrine portion of this organ. The density of islets within a pancreas varies from one area of the pancreas to another and varies overall in the
case of disease; diabetic animals frequently exhibit fewer islets per pancreas than do normal animals. Usually only in healthy animals is the optimal mean number of islets found, which in the case of the Chinese hamster is on the order of one to two thousand islets per pancreas. The number of islet cells composing an islet is also found to vary, both with the type of animal and even among islets from the same pancreas. In the case of the Chinese hamster again, the number of cells in a single islet can be as high as \( \times 4,000 \).

Within the islet there are found to exist three basic cell types as seen with the electron microscope. They are the alpha (A) cell, the beta (B) cell, and delta (D) cell. The B cell has already been mentioned briefly with regard to insulin synthesis. While there have been reports of more than three cell types by some investigators, the extra cells would seem either to be artifactual or, if real, are found only in a few select species (1, 2, 3, 4). Therefore, the present discussion will be limited to only the three cell types mentioned, as they are found to be common among all the mammalian systems that have so far been investigated (5). Further, all descriptions that follow of islets and islet cells are with specific reference to the Chinese hamster, Cricetulus griseus.

Since the study of islet cell interaction does require being able to differentiate between the three cell types, a brief ultrastructural description is provided for each. These descriptions are for normal individuals.

**Beta Cell**

The B cells occupy the central region of the islet and comprise
roughly 80% of total islet volume (6). Nuclei of the B cells are round to ovoid and contain a fairly large amount of chromatin. The chromatin is unevenly distributed, and is often found marginating the nuclear membrane at regular intervals. The endoplasmic reticulum is of the rough lamellar type and generally inconspicuous. Free ribosomes can be found in the cytoplasm. Golgi bodies are present, though small, and the Golgi complex is usually situated near the nucleus. In cells with decreased numbers of secretory granules the Golgi apparatus is found to be larger, along with a more plentiful endoplasmic reticulum (7). Mitochondria are more numerous than in the alpha and delta cells and are small and slender in shape. The mitochondrial matrix is fairly dense with moderate numbers of well-defined cristae. Lysosomal-like bodies are also frequently encountered in the cytoplasm. Bounded by a membrane, they contain irregularly rounded electron dense areas of particulate matter, as well as electron lucent vacuolated areas that are round in shape. Particularly conspicuous within the B cell are the secretory granules. These granules are produced by budding from the endoplasmic reticulum, and contain pro-insulin (8). They appear as round or, rarely, rod-shaped granules of variable electron density. They are enclosed by single-layered membranes somewhat larger than the granules so that, owing to the moderate electron density of the intervening material, they are seen to be surrounded individually by a bright halo. Occasionally, two granules can be found within a single membrane. Empty granule sacs are only rarely found. In B cells that are located adjacent to capillary structures there is often a polarization of the cell, with more granules being found on the capillary side of the cell and fewer granules found on the side opposite (9).
Alpha Cell

Alpha cells, in contrast to B cells, are found only at the periphery of the islet and are fewer in number than the B cells. With regard to the nucleus, cytoplasm, mitochondria, and Golgi complex A cells do not differ markedly from the B cells just described. Lysosome-like bodies are present but appear amorphous and uniformly electron dense, without the vacuolization frequently found in the B cell. The endoplasmic reticulum occurs as the rough vesicular type and is more prominent than in the B cell. Ribosomes are to be found free in the cytoplasm. As with the B cell, A cells are most easily distinguished by their secretory granules. Containing glucagon, these granules are of greater electron density than the granules found in B cells. They are also slightly larger than B granules and show only slight variation in size and electron density. The granules are closely bounded by a single-layered membrane so that the halo effect seen in the B granules is not apparent. Bar-shaped granules are not seen in A cells.

Delta Cell

Delta cells are limited to the islet periphery and are positioned roughly between the outermost A cells and the underlying B cells. They are the least numerous of the three islet cell types. Most D cell organelles are similar to those described for the alpha and beta cells. Golgi bodies are frequently prominent and the endoplasmic reticulum is mainly of the rough lamellar type. Free ribosomes are found in the cytoplasm. Like alpha and beta cells, D cells have characteristic granules which, in the D cell, contain somatostatin. The D granules are generally round but have a tendency toward polymorphism. The granule size on the average
is smaller than that of A or B granules, but amorphic D granules may occasionally be longer. The D granule is less electron dense than the A granule and is rather closely surrounded by its single-layered membrane.

While it is beyond the intended scope of this paper to provide a complete discussion of the kinds of changes which occur within the islet during the diabetic state, it should nevertheless be mentioned that changes are to be found. For example, the B cell may show signs of degranulation, expansion of the endoplasmic reticulum, and enlargement of the Golgi apparatus (7). Also, the diabetic B cell is frequently found to exhibit large deposits of glycogen within the cytoplasm (10, 11, 12, 13). The A cell expresses fewer signs of pathology but it, too, may possess cytoplasmic glycogen deposits and also a form of destruction of cytoplasmic granules by lysosomal digestion, termed alpha-granulolysis (13, 14). In severe diabetic cases the islet may be partially depleted of B cells with a concomittant relative increase in numbers of alpha and delta cells (7, 12, 15, 16). Delta cells have rarely been shown to exhibit pathology. These examples should not be considered complete but do represent some of the salient pathological features.

As stated, the three islet cell types each have their own particular domain within the islet. The alpha cells occur in a layer at the periphery where they effectively form an interface between islet cells and the surrounding exocrine tissue. The delta cells are directly below the alpha cells or interspersed slightly among them, while the beta cells compose the core and make up the bulk of the islet. This arrangement has been found to be common among most of the animal systems so far studied.
(17, 18). Considering the ubiquity of the pattern, and the fact that specific cellular organization is generally found to serve a purpose, one might believe it is utilitarian and not coincidental. The spherical symmetry of the arrangement further suggests that whatever significance this organization has it is directed toward the islet and not at the surrounding acinar tissue. It has in fact been suggested that this arrangement might possibly be a functional one, the purpose of which would be to provide the mechanical framework for a network regulating insulin release in which all three islet cell types would participate (18, 19). This view is supported by various studies that have pointed at the possibility of interaction among the different cells within an islet.

One way in which islet cells might interrelate is by the use of hormones. Somatostatin, for example, is known to be produced by D cells (20, 21, 22) and to also be a strong inhibitor of both insulin secretion by the B cells and glucagon secretion by the A cells (23, 24, 25, 26). It may be that the D cells, being in a strategic position between the A and B cells, would be able to influence the islet response to glucose levels by antagonizing the release of these hormones. Glucagon and insulin may in turn have regulatory effects, such as the inhibition of glucagon secretion by insulin (27). At present our understanding of hormonal control within the islet is insufficient to be able to say that it plays a part in insulin release. The fact that there are such effects at all, however, suggests that they may have importance beyond what is already known.

Other evidence of cell-to-cell communication within islets is found in the types of intercellular junctions. Junction specializations
in the form of tight and gap junctions between islet cells have been demonstrated in the rat (28) and also in humans (29). Such junctions have been found between A cells and A cells, B cells and B cells, and A cells and B cells; presumably D cells have not been studied. While tight junctions are thought to be used by the cell as a means of channelling extracellular fluids and also to a lesser extent cell anchoring (29), gap junctions are thought to act as portals for intercellular communication. Gap junctions have been demonstrated in tissues other than pancreas to be permeable to ions and small molecules (up to M.W. 500) (30, 31). It is possible that by this means metabolic signals could be transferred between islet cells and thus play a part in islet regulation (32). It has recently been shown that the dye fluorescein, when injected into a single islet cell, will be transported to adjacent cells (33). Furthermore, the number of cells surrounding the injected cell to which the dye is transferred is found to increase significantly in the presence of glucose. Whether or not gap junctions are involved in this type of intercellular transport is still unknown. Another study, however, has shown that there can be a correlation between the number and size of gap junctions and B cell secretory activity (34).

Finally, the presence of nerve fibers surrounding and to a lesser extent penetrating islets may suggest a third form of communication between islet cells. These non-myelinated nerve fibers, most with cholinergic nerve endings, have been reported both in the Chinese hamster (7, 12) and the Djungarian hamster (35). The fibers largely occur in the loose connective tissue surrounding the islet but are also found to penetrate the interior where they contact the three major cell types (35). Why
islet cells would require neural tissue to intercommunicate is not immediately obvious, however at least one possible explanation is suggested by the geometry of the situation. For an islet sixty cells in circumference (twenty cells in diameter) and a chemical message transfer rate between two cells of one second (33), the total message time for one peripheral islet cell to contact another on the other side of the islet would be thirty seconds. That is disregarding that by the time the chemical messenger reached the other side of the islet it might well be too dilute to effect a response, so that it would have to be resynthesized en route, adding to the message transfer time accordingly. Likewise, any feedback from the receiving cell would take equally long so that it could be tens of minutes before the whole islet were synchronous in its response. The message transfer time could be shortened tremendously by use of nerve fibers by the islet cells. Messages could then be sent with essentially no time lag from one end of the islet to the other. It could also be done without the need for an excessive number of nerve fibers. As long as a small number of cells were in immediate contact by use of neurons the rest could be relayed the message chemically by the receiving cells, cutting the message transfer time down to only a fraction of that required if it were sent entirely by chemical. Nerve fiber attachment "pads" have in fact been observed at intervals on the surfaces of islets (36).

Any of these three proposed means of islet cell communication might be found to exist within the islet. Either alone or in combination they could provide the communications network necessary for the islet to respond efficiently to exogenous glucose levels. As different as these forms of communication are they are not, however, mutually exclusive and
might be found to function together. By way of summary, a hypothetical model of islet regulation including all three means of communication is given in the following paragraph.

The response of the islet to blood glucose levels is regulated by peripheral alpha and delta cells which control the release of insulin by the central B cells. In order that the response be uniform over the islet, when one peripheral islet cell makes a response, that message is relayed by means of nerve cells to similar peripheral cells at periodic intervals over the surface of the islet, which then respond in the same way and at essentially the same time. The response is in the form of released hormones and/or other chemical messengers and these are transmitted to surrounding cells by means of gap junctions between cells. Feedback information from the B cells or from within the peripheral layer would occur in the same way but in the reverse order.

Although this sequence of events is purely hypothetical, the evidence suggests that something similar clearly takes place within the islet. As argued earlier, the understanding of regulation within the islet must be considered very important to the elucidation of islet dysfunction, such as is present in the majority of diabetic cases. To study islet cell regulation will necessitate looking closely at how cells are physically related to each other as well as how cells appear to function individually. Research in this area will require the use of specific techniques, some of which are already available, but also many more that have yet to be developed. The research portion of this thesis deals with the development of such techniques.

The methods employed in this study were chosen to emphasize four different aspects of islet cell ultrastructure and involved the application of six different procedures. They were: enhancement for microtubules (two procedures), visualization of the cell membrane by the use of ruthenium red, visualization of the intercellular space by the use of
lanthanum tracer, and the localization of calcium within islet cells by precipitation (two procedures).

Enhancement for microtubules was chosen because of the role microtubules possibly play during insulin release from the B cells (37). Two hypotheses have been put forward to date to explain how this may occur. The first (38, 39) proposes that there is an influx of calcium in the B cell upon stimulation by glucose, and that this calcium acts on the microtubules causing them to contract. With one end of a microtubule attached to the limiting membrane of a B granule and the other attached to the plasma membrane there would be a net movement of the B granule to the cell surface. The second hypothesis maintains that the microtubule acts as a conduit to conduct insulin to the plasma membrane (40).

Microtubules are supposed to fuse with the B-granules which then discharge their contents through the hollow portion of the tubule. Neither of these hypotheses has so far been proven and doubt has been cast on microtubule involvement by at least one study that finds no association of B-granules with microtubules outside of what would be expected for chance encounters within the cell (6). Regardless, it is well documented that interference with the structural integrity of microtubules by various drugs inhibits the release of insulin from B cells (41). This would seem to establish a functional role for microtubules in insulin release. Further studies are needed at this time to ascertain precisely how microtubules are involved, and those studies could be aided by techniques designed to make microtubules more prominent within the cell.

The ruthenium red and lanthanum procedures both provide infor-
mation about the surfaces of cells and how different cells relate to each other. Ruthenium red attaches mainly to polyanions having a high charge density, such as acid polysaccharides which occur in the plasma membrane (42). This results in membranes exposed to ruthenium red becoming electron-dense and therefore highly visible. Due to the procedure employed it is possible with ruthenium red to stain only the outer membrane of intact cells and not the interior. Lanthanum on the other hand fills in the intercellular space between cells to make it opaque to electrons. What is seen then is a very well-defined boundary indicating the border between two cells, something not easily determined by conventional methods. By the use of a lanthanum tracer it is also possible to identify the types of junctions connecting different cells.

Since the discovery that calcium ions enter the B cell when stimulated by glucose there has been an effort to determine how this is involved in insulin release in order to be able to apply that information to the case of diabetes (43, 44). Two basic methods have been employed to localize calcium in tissues and they are the two procedures repeated here. Although they have both been used for localizing calcium in islet cells before, we intended to try a different approach to analysis. Therefore these procedures were included in this study. The first method precipitates calcium by combining it with oxalate ion to form an insoluble salt. The second method employs pyroantimonate ion as the precipitating agent. Both give an electron-dense precipitate where calcium ions are concentrated. The precipitate is best seen in an unstained section, which is required in any case since post-staining with uranyl will dissolve out calcium oxalate (45) and tissues treated with pyroantimonate are incap-
able of being post-stained (46).

When performing calcium precipitation on tissues it is usually necessary to verify by other means that calcium is actually contained in the observed precipitates, since both oxalate and antimonate ions will form precipitates with other ionized elements. A method typically employed for that is X-ray fluorescence. In this method the specimen is put in a scanning transmission electron microscope which is equipped with an X-ray detector. The area of the section containing the precipitate is scanned with high energy electrons that bombard the area. When an atom is struck by one of these electrons an orbital electron may be boosted to a higher level and a more excited state. When that electron then decays back to the ground state it releases a photon, frequently in the X-ray band. That X-ray can be detected by the instrument and its energy measured. Since each bombarded element will give X-rays of a characteristic energy, by observing what energies of X-rays are given off the elemental make-up of the bombarded area can be determined.

It was the purpose of this thesis to apply these histochemical procedures to pancreatic tissue in order to observe the cellular function and relationship within the islet. It is hoped that the results of this study will be useful to those wishing to investigate the possibility of an islet-based regulatory network.
All animals used in this study were normal and obtained from The Upjohn Company hamster colony. All animals were killed by decapitation. When live pancreatic tissue was required the organ was kept moist during excision with 0.1M phosphate buffer (pH 7.4). To avoid drying artifacts when live tissue was not required, the pancreas was fixed in situ with glutaraldehyde/paraformaldehyde (2.25%/1.25%) in 0.1M cacodylate buffer. All tissue samples were minced to approximately 1 mm³ sized pieces during the first step of each procedure. At the end of each procedure the treated tissues were dehydrated in a graded series of ethanol solutions and embedded either in a low viscosity medium (Polaron NC1012) or Epon 812. To locate islets by light microscopy, sections approximately 1 micron in thickness were cut and stained with alkaline toluidine blue. Thin sections were cut using glass knives on an LKB model 8800A ultramicrotome and photographed using a Siemens 1A transmission electron microscope.

Control

The tissue was minced in 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) in which it was allowed to fix overnight at 4 degrees Celsius. The tissue was then rinsed once with buffer and fixed in 1% buffered osmium tetroxide for 1 hr. at 4 degrees Celsius. After a water rinse the tissue pieces were stained en bloc for 10 minutes with 1% uranyl acetate in 50% ethanol. Following en bloc staining the tissue was dehydrated through alcohols and embedded in low viscosity medium.
Microtubule Enhancement Stain, Method I

This procedure was modified from Yokota and Fahimi, 1978 (17). The tissue was minced in 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) after which it was allowed to fix in the same solution overnight at 4 degrees Celsius. The tissue was then rinsed in buffer and transferred to a solution of 0.1% Triton X in the same buffer for a period of 1 hr. The tissue was then rinsed in buffer and postfixed at room temperature in 1% buffered osmium tetroxide for 1 hr. After a water rinse the tissue pieces were stained en bloc for 10 minutes at room temperature with 1% uranyl acetate in 50% ethanol. Following the en bloc stain the tissue was then dehydrated through alcohols and embedded in low viscosity medium.

Microtubule Enhancement Stain, Method II

This procedure is a modification of that used by Takahashi (18). The tissue was minced in a 3% solution of glutaraldehyde in 0.1M phosphate buffer (pH 7.4), containing 2% of tannic acid. After 3 hrs. in this solution at 4 degrees Celsius the tissue was rinsed in buffer and postfixed at room temperature in 1% buffered osmium tetroxide for 1 hr. Following postfixation the tissue was dehydrated through alcohols and embedded in Epon 812.

Ruthenium Red Stain

This procedure is modified after Hayat (19). The pancreas was minced in a 3% solution of glutaraldehyde buffered with 0.1M cacodylate buffer and containing 0.05% ruthenium red. After fixing overnight in this solution at 4 degrees Celsius the tissue was rinsed with cacodylate buffer.
buffer and postfixed for 1 hr. in 1% buffered osmium tetroxide which also contained 0.05% ruthenium red. The tissue was then given a water rinse, dehydrated through alcohols, and embedded in low viscosity embedding medium. (Note: fixative solutions containing ruthenium red must be made up immediately prior to use.)

Lanthanum Procedure

This procedure is modified after Neaves (50). The tissue was minced in 3% glutaraldehyde buffered with 0.1M cacodylic acid (pH 7.4) and containing 1% lanthanum hydroxide (see reference for preparation). The tissue was soaked overnight in this solution at 4 degrees Celsius. The tissue was next rinsed in buffer containing 1% lanthanum hydroxide then held in the same overnight at 4 degrees Celsius. Postfixation was for 8 hrs. at 4 degrees Celsius using 1% buffered osmium tetroxide containing 1% lanthanum hydroxide. After a water rinse the tissue pieces were then stained en bloc for 10 minutes with 1% uranyl acetate in 50% ethanol at room temperature. Following en bloc staining the tissue was dehydrated through alcohols and embedded in low viscosity embedding medium.

Calcium Precipitation, Oxalic Acid Procedure

This procedure was taken from Daimon et al., 1972 (51). The live tissue was minced in an unbuffered solution containing 140 mM potassium oxalate and 140 mM potassium chloride at a pH of 7.4. The minced tissue was incubated for 10 minutes in this solution at 37 degrees Celsius. Following incubation the tissue pieces were fixed at 4 degrees Celsius for 2 hrs. with 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) which
contained 40 mM potassium oxalate. The tissue was then washed in buffer containing 40 mM potassium oxalate and postfixed for 1 hr. at 4 degrees Celsius in 1% buffered osmium tetroxide, also with 40 mM potassium oxalate. Following postfixation the tissue was dehydrated through alcohols and embedded in Epon 812.

Calcium Precipitation, Antimonate Procedure

This procedure was taken from Daiman et al., 1977 (52). Live pancreatic tissue was minced in 1% unbuffered osmium tetroxide containing 2% potassium antimonate (pH 7.4). The tissue was allowed to fix in the same solution for 3 hrs. at 4 degrees Celsius. The tissue was then dehydrated in graded alcohols and embedded in Epon. A control was needed to ascertain that the precipitation seen would be due to calcium alone. For that, calcium ion was selectively eliminated by chelation with EGTA. The control procedure was to mince the live tissue in Hank's calcium/magnesium-free (CMF) buffer containing 3 mM EGTA, where the minced tissue was allowed to sit for 20 minutes at room temperature. The tissue was then washed at room temperature in three 20 minute changes of Hank's CMF buffer. The tissue was then fixed, dehydrated, and embedded as above.

Calcium Precipitation, Methacrylate Embedding

When performing X-ray micro-analysis on a tissue specimen the procedure usually employed is to make thin sections of the specimen and to carry out the analysis using a scanning transmission electron microscope (STEM) equipped with an X-ray detector. Standard surface scanning electron microscopes (SEM) can also be equipped with X-ray detectors, however, and SEM's so equipped have been available for some time. But there is an inherent problem with using scanning electron microscopy for
X-ray analysis. Just as with SEM imaging, analysis with SEM can only take place at the surface of the specimen. This means that when tissue is to be analyzed, internal structures must be exposed to the outside by breaking, and the tissue must be unembedded in order to be able to image the area being analyzed. These restrictions have limited biologists to using the STEM system when analysis of sub-cellular regions was required. Therefore, as an adjunct to the calcium precipitation procedures included here an attempt was made to develop a method that would enable X-ray micro-analysis to be performed on sectioned tissue using standard scanning electron microscopy.

The method arrived at is based upon using methacrylate embedding medium instead of the epoxy resins normally employed. Methacrylate was chosen because it dissolves readily in several common laboratory solvents. Since it is only the embedding material that is manipulated, the tissue may be treated in any manner desired prior to embedding. The embedded tissue is sectioned in the usual way, but at a thickness between 1 and 2 microns. Sections are heat-fixed to a glass coverslip or silicon wafer (see discussion). The methacrylate is removed by rinsing the sections with 5 drops of amyl acetate. The first drop is allowed to set on the sections briefly for a few seconds before the sections are rinsed quickly with the remaining drops. The coverslip is allowed to air dry, after which it is either shadowcasted from all directions or sputter-coated. The coated sections are then ready for viewing.
RESULTS AND DISCUSSION

Control

For purposes of comparison a micrograph of a conventionally embedded islet is shown in figure 2.

Microtubule Enhancement

Although both methods for microtubule enhancement were repeated several times, neither produced the kinds of results reported in published procedures. No structures positively identifiable as microtubules were found in cells although structures resembling microtubules were observed. Those, however, could not be identified with certainty because of poor cellular preservation and the absence of any enhancement. An exception was found where cells were pulled apart due to osmolarity effects and microtubules were drawn from the cell web into the widened intercellular space. An example of this can be seen in figure 3. There microtubules were found to be enhanced and easily identifiable, though they revealed little information as to their original purpose in the cell. The fact that microtubules in intact cells were not stained could be explained by assuming that islet cells are not as permeable to the staining compounds used in these enhancement procedures as those of other tissues. Hence, microtubules would not be stained unless a cell ruptured, exposing them to the staining compounds.

Another possible explanation for the failure to find microtubules would be that the structural integrity of the islet cells was so badly disrupted during the staining procedures that microtubules could not be recognized. The quality of cellular preservation with either of the
Fig. 2. Control procedure, post-stained section. 21,000 X

Fig. 3. Microtubule enhancement procedure (method II) showing microtubules (arrows). 80,000 X
procedures used was actually very poor. It was discovered when preparing the control tissues used for comparison in this study that fixation procedures which for most tissues worked well gave poor results with pancreatic tissue. Only by paying particular attention to the buffer and fixation times was reliable preservation achieved. In most of the staining procedures used in this study the poor level of preservation is accounted for by inadequate buffers and fixation. While it may be basic to the success of these procedures that they be performed in the manner presented, in the case of the microtubule stains the procedures appear too harsh for a fragile tissue such as the pancreas. The general disorder and organelle swelling found in islet cells treated for microtubule enhancement could in part or fully account for the difficulty in finding microtubules.

Ruthenium Red Stain

Shown in figure 1 is a section of ruthenium red treated tissue which has not been post-stained. The stain follows very closely the surfaces of cell outer membranes. A stain-free area between adjacent cell membranes representing the intercellular space is clearly visible. Notice that the granules within the cells are unstained, as are the membranes delimiting the granules.

Figure 5 shows B granules of a cell whose outer membrane ruptured before or during the staining process. Internal membranes were exposed to the ruthenium red and consequently became stained. The stain is most noticeable in the membranes surrounding the granules. It is noteworthy that the granules themselves have taken up some of the stain, although

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Fig. 4. Ruthenium red procedure showing membrane staining (arrows) of intact cell. 61,000 x

Fig. 5. Ruthenium red procedure showing membrane staining (arrows) of ruptured cell. 61,000 x
inconsistently. As mentioned earlier, variable staining of granules is also commonly seen when using conventional stains. Ruthenium red is known to cause staining by combining with polyanions, which in membranes are represented by molecules with large numbers of ionizable carboxyl, sulfate and phosphate groups (53). While it may not be possible to specifically implicate any of these groups in the staining of B granules, it can be proposed on the basis of the available evidence that the staining granules by ruthenium red depends on the presence of polyanions.

Lanthanum Procedure

Lanthanum produces its staining effect largely by physical means. All cells are surrounded by an intercellular space which serves as a circulatory system at the cellular level by allowing nutrients to be carried to the individual cells and excretory products to be carried away. When a tissue is introduced into an aqueous suspension of lanthanum hydroxide the lanthanum compound diffuses through the system until it has surrounded all of the cells. When the tissue is later embedded and observed with the electron microscope the intercellular space appears opaque due to the electron opacity of the penetrated lanthanum hydroxide. An example is shown in figure 6. Using this technique it is easy to discriminate between cells. Lanthanum might therefore be used in conjunction with other procedures designed to identify cell types in which it would be important to know what regions of cytoplasm belong to what cells. Identification of cells can be difficult to determine when a tangential section of a cell does not include a piece of the nucleus. With lanthanum the cytoplasmic domains are clearly defined.
Fig. 6. Lanthanum procedure showing lanthanum infiltration (arrows). Post-stained section. 8,600 X

Fig. 7. Lanthanum procedure showing tight and gap junctions (arrows). Post-stained section. 17,200 X
Another use of the lanthanum procedure is in determining types of intercellular junctions existing between cells. Figure 7 shows an enlarged photomicrograph of the intercellular space between two cells. Along the intercellular border, regions can be seen in which the lanthanum has been excluded. These points correspond to tight and gap junctions. While it is possible to tell one type of junction from another using lanthenum tracer it is not easy, and these studies are best performed using freeze-fracture methods. In addition to studying junctions between two cells it may also be possible to learn something from the multi-cellular junction which is also enhanced by lanthanum. An example of a multi-cellular junction can be found in figure 6 where five different cells are seen coming together.

A disadvantage encountered with the use of lanthanum is that there is a limit to the distance over which lanthanum can diffuse within a reasonable period of time. In practice that means that cells more than twenty or so cell units from the periphery of the tissue piece in which they are found will not be reached by lanthanum. Early in this research it was decided that isolated islets could not be expected to represent the islet as it naturally occurs in the pancreas. Therefore, throughout these procedures whole pancreatic tissue was used and the islets were located afterwards. That presented a special problem for the lanthanum procedure since it meant finding islets close to the surface of the tissue piece in which they resided, while finding islets at all was sometimes difficult. Nevertheless, lanthanum permeated islets were found. However, this was with normal hamsters; diabetic individuals can have a far smaller islet density. Therefore, researchers wishing to perform
this procedure on intact and diabetic pancreatic tissue should be aware that locating islets which have been reached by lanthanum may present a significant challenge.

**Calcium Precipitation, Oxalate Procedure**

Although the islets were not stimulated by glucose, electron-dense deposits were nevertheless found using this procedure. Deposits were identified in the nucleus and at various locations in the cytoplasm. The deposits were usually weak and occurred only infrequently. The most prevalent and heaviest type of precipitate was that associated with lysosomal-like bodies. Figure 8 shows such a precipitate. Particularly intense deposits were also found in some cytoplasmic vesicles of lymphocytes. Lysosomal bodies of both alpha and beta cells were seen to exhibit precipitate deposits. Beta granule membranes occasionally appeared enhanced and this was thought likely to be due to a weak calcium precipitate around them. The granules themselves were also slightly enhanced, suggesting a weak precipitate deposited there also.

With this procedure the cells at the periphery of the tissue pieces were always found to be badly swollen and often ruptured. Cells toward the centers of the tissue pieces were more naturally preserved. Therefore, by necessity the cells which were studied were near the center of the tissue piece in which they were found. The question thus arises whether or not the procedure is destructive of pancreatic tissue (see discussion under microtubule enhancement) and if so whether cells that were not destroyed also were not fully exposed to the precipitating agents. What precipitation was found was also weak, suggesting that
Fig. 8. Calcium precipitation (oxalate) procedure showing lysosomal precipitate (arrow). 21,500 X

Fig. 9. Calcium precipitation (antimonate) procedure showing precipitation in granules (arrows) and membranes (double arrows). 17,000 X
heavier precipitates might have occurred had the cells been fully exposed to the oxalate solution. If that were the case then full exposure might also intensify the lightest precipitates such as those around the beta granules.

Calcium Precipitation, Antimonate Procedure

The kinds of precipitates seen with the antimonate procedure were similar but not identical to those seen with the oxalate procedure. Tissue preservation was again poor but not as destructive as in the first procedure. Figure 9 shows two islet cells that were treated with potassium antimonate. As with the oxalate, precipitation can be seen in the nucleus, the cytoplasmic granules, and lysosomal bodies. Some cells exhibit a generalized precipitate throughout the cytoplasm. In all cases the deposits were heavier than those observed with the oxalate procedure. On only two points did the results of the two procedures differ markedly. The first is that whereas with the oxalate method granule membranes could be seen to exhibit a slight amount of precipitate, no such precipitate was seen with the antimonate procedure. In fact, the granule membranes could not be seen even though deposits in mitochondrial membranes were occasionally observed. The granule membranes were intact, however, at the time of precipitation, since in those B cells that exhibited a general cytoplasmic precipitate, a halo representing the absence of precipitate could be discerned surrounding the granules (fig. 9). The second point on which the result of the two procedures differed was that unlike the oxalate, the antimonate procedure showed calcium precipitation to occur at the outer membrane. Between the two cells in figure 9 the membranes
can be seen to be enhanced by such a precipitate. The effect is especially prominent for acinar tissue, an example of which is shown in figure 10. The precipitate is fine and closely follows the membrane, resembling in appearance ruthenium red. Although in theory it should be possible to determine which side of the membrane the precipitate is on, this proved to be difficult with unstained sections. Post-staining for membranes might make the determination easier.

Calcium Precipitation, Methacrylate Embedding

A scanning electron micrograph of a leached methacrylate section is shown in figure 11. Although it is impossible with this method to achieve the same degree of resolution as with a transmission electron microscope, major organelles are easily identified. Visible here are nuclei, endoplasmic reticulum, and cytoplasmic granules. Mitochondria are difficult to discern. This particular specimen was shadowed with chromium, but any shadowing metal can be used.

It was not resolved in this study whether or not calcium deposition in such sections is detectable by X-ray fluorescence in an SEM. But assuming that the mass of the precipitate is large enough or that X-ray data can be collected for very long periods of time, it should present no problem. The only limitation to using microprobe analysis on such sections is that they be thin enough that the area to be analyzed does not include extraneous material, such as might lie above or below the area in an overly thick section.

A problem that is encountered when performing this type of analysis is that of background X-rays. These X-rays come from the coating.
Fig. 10. Calcium precipitation (antimonate) procedure showing membrane precipitate (arrows). 7,300 X

Fig. 11. Scanning electron micrograph of leached methacrylate section. Nuclei (arrows) can be seen. 2,000 X
material and substrate which are bombarded by electrons along with the target and produce X-rays that may interfere with the analysis. The interference can be minimized by choosing materials which fluoresce at energies far from those of interest. For example, chromium gives two X-ray peaks at a beam energy of 10 keV but they are far enough from the peaks which correspond to calcium that they present no problem. Glass such as that used for coverslips, on the other hand, produces a very dirty background which when analyzing for calcium is entirely unsuitable. Therefore, as a substrate material pure silicon was chosen. At nominal beam energies it produces but a single background peak that is far removed from those that identify calcium.

Observations

It happens when performing investigations of this sort that invariably there are observed results or phenomena that originally had not been expected from the design of the experiments involved. They are commonly dismissed as artifacts, mere curiosities, or the result of negligence; but occasionally one may intime a hidden fact of nature. Science is replete with inadvertant discoveries.

In the course of this study three unexpected observations were made that, if presented to the proper mind, might suggest practical applications. They are presented below with the sincere hope that someone will be able to make use of them.

The first is depicted in figure 12. These are collagen fibers as they appear after treatment with potassium antimonate. Precipitate can be seen to be deposited at regular intervals along the length of the
Fig. 12. Collagen fibers (antimonate procedure) exhibiting precipitate (arrows). 38,500 X

Fig. 13. Doubled membranes (arrows) from microtubule procedure, method II. 86,000 X
fibers. This was not the calcium precipitation control and no comparison was made with the control, so that the ions giving rise to the precipitate could be other than calcium. If not entirely unexpected from the known structure of collagen, the observation at least serves to give some idea as to the sensitivity of the technique.

Figure 13 shows an unexpected observation discovered when looking for microtubule enhancement by method II. Shown are beta granules. The curious thing about these granules is that instead of each being bounded by a single-layered membrane the membranes surrounding the granules appear to be double-layered. Figure 14 shows a different field of the same preparation in which not only beta granule membranes appear doubled, but also the endoplasmic reticulum and mitochondrial membranes. This is most likely an artifact and not the result of abnormal membrane synthesis by the cell. Possibly this procedure causes hydration and swelling of single-layered membranes, while staining of the hydrophilic layers gives the membrane the appearance of being double.

The last observation I would like to mention was another encountered as a result of the antimonate procedure for calcium precipitation. The micrograph of interest is shown in figure 15. Close scrutiny will reveal the presence of enhanced microtubules/microfilaments around the nucleus and in the cytoplasm. Enhancement appeared to be restricted to islet cells since microtubules were not observed in the acinar tissue. The method of enhancement can only be guessed at, but may involve calcium. Calcium is known to be required for microtubule stability, and it may be through precipitation taking place at the microtubule surface that enhancement is brought about. Considering the difficulty encountered
Fig. 14. Doubled membranes (arrows) from microtubule procedure, method II. 103,000 X

Fig. 15. Enhanced microtubules (arrows) from antimonate procedure. 21,000 X
with islets using other procedures designed specifically for micro-
tubule enhancement, it might be worth revising this procedure to em-
phasize that aspect.
BIBLIOGRAPHY


2. Munger, B.L., Caramai, F., Lacy, P.E.: The ultrastructural basis for the identification of cell types in the pancreatic islets. II. Rabbit, dog and opossum. Z. Zellforsch. 67, 776-798 (1965)


BIBLIOGRAPHY (Continued)


36. Soret, M.G.: Personal communication.

37. Kern, H.F.: Fine structural distribution of microtubules in pan-


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