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A METHOD FOR QUANTITATIVE MORPHOLOGIC COMPUTER ANALYSIS
OF TISSUES APPLIED TO THE ISLETS OF
LANGERHANS IN THE AVIAN PANCREAS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Robert Daniel McClish, B.A., B.S., M.A.

* * * * *

The Ohio State University

1973

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INTRODUCTION

In the course of investigating the distribution of the islets of Langerhans, it was found that the distribution of the islets within the exocrine pancreas is not well defined in any mammalian or avian species. The large number of islets, their small size, and apparently random distribution make objective evaluation of experimental results difficult.

The basic goal of this research, therefore, has been to develop a method which will determine the distribution of the islets of Langerhans with enough precision to make it possible to determine the limits of normal islet distribution. It is anticipated that the normal limits can then be used to identify and classify pathological changes which affect islet distribution. This is not a new goal. Bensley in 1911 stated:

... it may be pointed out that we do not know for a single species, nor have we an adequate method for ascertaining, the total number of islets of Langerhans in the pancreas, or the normal range of variation in this respect. Yet we have numerous records of experimental researches which deal with increase or diminution of the number.

The problem has changed very little in the intervening years. Numerous research efforts concerning quantitative increase or decrease in islet tissue are still being produced, and the claims of increase or decrease are still subject to question.
Ledley (1959), in his description of automatic analysis of chromosome karyograms by the computer, states:

Huge masses of material are being generated at an ever-increasing rate, such as photomicrographs of chromosomes relating to genetic diseases, photomicrograph sequences showing the dendritic structure of nerve cells.

... Individual pictures hold a great wealth of precise numerical information, such as morphological and structural characteristics of lengths, areas, volumes, densities ...

... However, the large-scale quantitative analysis of these pictures cannot be approached by manual methods because of the tedium, the extensive time and the need for manual precision.

... The possibility of automatic quantitative analysis of pictures of biological and medical importance promises to open up entirely new kinds of investigation which could not formerly have been attempted (Ledley, 1959).

Automatic computer analysis of chromosome karyograms is appropriate because a technique for separating the chromosomes, placing all of them in one plane and in a proper orientation for individual chromosome identification, already exists in the squash technique. In addition, prior to Ledley's work, it had already been established that certain well defined clinically important abnormalities could be identified by chromosome analysis.

The islets of Langerhans of the pancreas offer a similar, though more complex, situation. It has been accepted since 1922, when Banting and Best demonstrated the effect of pancreatic extract, that a deficiency of certain islet cells or of the hormone they produce, insulin, causes diabetes.
Staining methods have now been developed which definitively identify islets. However, perhaps because of the large number of islets, their three dimensional distribution, and the lack of a technique for visualizing islets as conveniently as the chromosomes of a cell can be visualized by the squash technique, a relationship between islet volume or number and diabetes (short of almost complete absence of islets) has been very difficult to establish.

Since the digital computer can store, retrieve, and manipulate an amount of data large enough to measure and identify the morphological relations of the islets within the pancreas, numerical and volumetric relationships involving a very large amount of data can be evaluated. It is, therefore, reasonable to use the computer in attempting to establish, at the level of detail made possible by computer analysis, whether islet volume and number is an important clinical entity. The islets of the avian pancreas have been chosen for this study because previous morphological work is available which applies to the islets and because data accumulated toward the construction of a computer model of the islets could be useful in research concerning diabetes. To use the computer, it was necessary to develop new techniques for processing histological material (embedding, sectioning, staining, and mounting techniques) which would allow a bridging of the gap which presently exists between morphological relationships visible on histological sections and data that can be processed and stored by a computer. For a method which
bridges the histological section/computer data interface to be practical, it must provide for data collection without an inordinate investment of time or money. It must also yield a permanent record which is readily accessible for comparison or manipulation of data sets.

As a first step, to make the summation and evaluation of data more efficient and less time consuming, a computer program was developed which computes volume from measured islet areas, calculates total islet and total pancreatic volumes, and produces an islet/exocrine and islet/islet ratio for the volume and number of islets in an entire pancreas, pancreatic lobe or sublobe.

To simplify the data collection process and to make the data collected more available for evaluation, a second computer program was designed, and a new method of collecting data was developed. This second program offers the possibility of processing data collected by an optical scanning device. From this data, position, shape, and volume of individual islets can be calculated and can be stored for future comparison with data collected from other tissue specimens. To further speed data collection, the linear scan method of data collection which was used in version one of Program Two was replaced by rectangular and triangular approximation in version two. The processing of data, once collected, is the same in both versions of Program Two.

As a morphological basis for computer analysis, pancreatic gross relationships were observed, and body measurements were recorded for the
pigeon, chicken, and duck. The computer techniques and procedures described will be used to determine mathematically whether islet distribution is or is not random; if specific distribution patterns do exist, their limits of variation will be determined.

MATERIALS AND METHODS

In order to transform morphological relations into data that can be processed by a computer, cartesian coordinates were assigned to equally spaced points on the plane of the histological section. The coordinates of those points which correspond to significant structural outlines were recorded, and computer programs were developed to process the data to yield mathematical models or statistical data (see Appendix E).

The data used in this study has been obtained from celloidin or double embedded serial sections of the pancreas mounted on glass slides or on 35mm mylar strips. Devices used in obtaining data include a mylar millimeter grid, a series of graph paper templates, a grid system superimposed on the microscopic image in a binocular microscope (Plate IV, figs. c and d), and an Itek Mesallina X-Y Measuring Table (Plate V, figs. a, b, c). The data was punched on data cards automatically by the Mesallina Measuring Table (Plate V, fig. d) and by a technician in the other systems. Punched data cards were fed into an IBM 370 computer system and processed by any one of several Fortran programs developed especially for this research, the choice of program being determined by the data desired.
Plate I. The Avian Pancreas.

Fig. a. Ventral lobe of the pigeon pancreas in the duodenal loop. Notice two grooves on the ventral surface.

Fig. b. Ventral lobe of the chicken pancreas in the duodenal loop.

Fig. c. Dorsal lobe of the chicken pancreas. Note the pancreatico-duodenal branch of the celiac artery and the recurrent intestinal branch going to the small intestine.

Fig. d. Rat pancreas in the mesentery. Note its diffuse nature and that the vessels supplying the intestine pass through the pancreas.
To test these materials, the islets of Langerhans of the avian (pigeon) pancreas were chosen because the avian pancreas is a solid, well defined entity located almost entirely within the loop of the duodenum. This contrasts sharply with the more diffuse distribution of the rodent pancreas (see Plate I, fig. d).

The pancreas of eleven barn pigeons, four chickens, and two ducks was investigated grossly. The entire loop of the duodenum containing all lobes of the pancreas and the spleen was removed and placed on an aluminum frame especially designed to prevent curling or twisting. The specimens were fixed in 10% formalin and embedded in celloidin (see Appendix A). Four pigeon pancreases were investigated to determine islet distribution. As the sections were cut, they were removed from the knife by a mylar strip or an acetate or mylar tape 1 mil (.025mm) thick (see Appendix B). Strips or tapes containing sections were numbered consecutively and were stained immediately or stored until ready for staining. Acetate tapes were mounted on mylar strips 35mm wide and 5 feet long, using Mayer's albumin (see Appendix C). The mylar strips containing the histological sections were placed on standard 35mm film developing reels, and sections were stained 10 to several hundred at a time. A number of standard pancreatic stains were used including: Gomori's chrome alum hematoxylin fuchsin (CAHF), chrome alum hematoxylin phloxin (CAHP) and aldehyde fuchsin, and the PAS trichrome of Lazarus (Appendix F). Stained sections were covered with Krylon crystal clear
enamel, Acrylyd acrylic enamel, or Hanna-Tile epoxy resin (Appendix D) and stored in standard 35mm Patterson negative files, on 35mm movie film reels, in negative storage boxes; or were transferred to 1x3 inch glass slides and stored in slide boxes (see Plate III, figs. a and b). Islets on each section or on every tenth or twentieth section were then measured using grids mounted beneath the slide and in the ocular image of the section. The coordinates for the position and outline of each islet were recorded on computer data cards which were processed by one of two Fortran programs (See Appendix E).

In addition to the data obtained from Programs One and Two, a series of models were constructed of the pancreas. To aid in evaluation of islet data, gross morphology of the pancreas and duodenum (including pancreatic length before and after fixation) and the following physical characteristics were recorded for each animal: body shape and coloration (color photographs), age, sex, and body measurements (body weight, length, volume, and wingspan).

OBSERVATIONS

Gross Observations

The avian pancreas (pigeon, duck, and chicken) is composed of at least two distinct lobes, a ventral and a dorsal (Plates I and II).
Plate II. The Pigeon Pancreas.

Fig. a. Freshly removed pigeon pancreas in the duodenal loop. Note portions of liver and gizzard also removed (Pigeon #7). Ventral lobe.

Fig. b. Dorsal lobe of pigeon pancreas and duodenal loop embedded in celloidin block. Note spleen near left border of picture (Pigeon #7).

Fig. c. Ventral lobe of pigeon pancreas (Pigeon #6).

Fig. d. Dorsal lobe of same pigeon pancreas as shown in fig. c. Embedded in celloidin. Note the dorsal lobe does not extend as far into duodenal loop as the ventral lobe (fig. c).
The dorsal lobe of the pigeon pancreas is approximately 3 1/2 cm long and can be subdivided into several sublobes and a filamentous spleen segment which extends cranially from the left border.

The ventral lobe of the pigeon pancreas fits between the ascending and descending limbs of the duodenum. The lateral walls of this lobe are attached by connective tissue to the duodenal limbs and are concave where they contact the duodenal walls. The result is an "I" beam ventral lobe cross section. The average length of the ventral lobe is 4 cm. This lobe of the pigeon pancreas is characterized by two grooves on its ventral aspect (Roth, 1968).

The lobes of the duck pancreas resemble those of the pigeon in shape and arrangement. The dorsal lobe is approximately 10 cm long and can be divided into several sublobes including a cranial extension, the spleen segment. The ventral lobe is 8 cm long and has a compact "I" beam structure similar to that of the pigeon. The ventral lobe of the chicken pancreas is V shaped, long (12 cm), and filamentous. Two filamentous extensions proceed from the body of the dorsal lobe toward the spleen. Mikami and Ono (1962) divide the chicken pancreas into four lobes (dorsal, third, ventral, and splenic).

**Technique**

**Specimen Preparation**

It was found that dehydration in acetone/alcohol following formalin fixation caused less shrinkage than dehydration by the standard
ethyl alcohol series. Dehydration was aided by constant stirring. To prevent damage to the specimens by the rotating magnet of the magnetic stirring device, and also to cause a more effective circulation of fluid, the top of a plastic bottle (Plate VI, fig. c) was placed over the magnet in the center of a large cylindrical sealed jar. The resulting current shown in fig. c (cited above) yields a more uniform and more rapid fixation, dehydration and celloidin impregnation.

The embedding jar is composed of a large upper storage compartment and a smaller lower embedding compartment. The original 5% celloidin solution fills both compartments, but as evaporation proceeds the concentrated solution is directed by the slanting back plate of the storage compartment into the embedding area. The jar is so constructed that the original 5 or 8% celloidin solution is concentrated into one-third or one-fourth the original volume, thus resulting in a final concentration of between 16 and 30%. The celloidin blocks produced by this method are clear and solid. They section easily with the terpineol method and can be stained with standard staining techniques. If larger 10μ sections or thin (2μ or 5μ) sections of any size are desired, a tape support microtome must be used. It may be advisable to infiltrate the mounted celloidin block with paraffin (double embedding). This allows the block to be cut on a tape support microtome without worrying about drying and wrinkling of the sections (See Appendix B).
The Tape Support Microtome

The tape support microtome, described in Appendix B, makes it possible to cut extremely large thin sections. For this system, block size has little to do with section thickness. Serial sections 5 μ thick have been cut from a block 2cm wide by 15cm long.

A very important advantage of this microtome is the lifting away of the sections from the knife by the tape. As a result, there is no friction between the advancing knife blade and section and no wrinkling of the section. The result is similar to that of floating the cut section on water as in the use of a boat on the glass knife for cutting plastic or as in the water on knife technique of Huber for cutting paraffin (Walls, 1932). Tape support sections do not have to be spread, the water bath is eliminated, and the sections are easily handled, stored, or stained and mounted.

Since the sections are supported by the tape, they are not touched by the hands or by any instrument throughout the entire cutting and mounting process. Cut sections may remain on the original tape, may be mounted in the standard manner on glass slides, or may be mounted on mylar. The use of mylar in place of glass for section support is an intriguing modification well suited to the tape support microtome. The principle of tape support can be applied to both rotary and sliding microtomes and may also have use in cutting electron microscope sections.
Mylar Film for Section Support

Polyester film is extremely stable, and some difficulty in causing sections to adhere was anticipated. Mayer's albumin, however, worked quite well, forming a bond which was sufficiently secure to allow vigorous washing in running tap water. If a thin mylar tape loop is placed on the tape support microtome, it is possible to collect sections directly on mylar and to carry out all subsequent histological procedures on the original tape. The time and labor saved with this procedure is significant, but it has been difficult to obtain the right combination of thin mylar backing and chemically resistant adhesive on the same tape.

The use of mylar film stored in 20 or 36 inch widths on rolls 20 to 100 yards long is more economical than the use of glass slides in the preparation of histological sections. Optically clear large glass slides (larger than 2x3 inches) and especially large glass cover slips (larger than 22x60mm) are very expensive. Strips or sheets of polyester film up to 3 feet square can be cut (see Plate III, figs. a and b) from the roll with no increase in the unit cost. It is therefore possible to produce sheets (8 1/2x11 inches) which fit into a standard notebook and which are large enough to hold histological cross sections of an adult head or heart, etc. The epoxy or acrylic coating can also be spread over the larger surface with no increase in unit cost.

Since large sections are especially easy to cut with the tape support microtome, this method is well suited to the production of large, thin, gross or histologic sections.
Plate III. Mylar Section Strips. Storage and Use.

Fig. a. Some of the apparatus commercially available for storage of 35mm strips. A dust free box containing 3 compartments is shown in upper left corner. It is setting on a notebook designed to store 35mm film negatives which is quite appropriate for storing short section strips (see fig. b.). Longer strips can be stored on 35mm movie film reels which can be stored in metal cans (lower right).

Fig. b. Pigeon pancreas sections on 35mm mylar strips stored in loose leaf notebooks designed for storage of 35mm negatives.

Fig. c. Section strip reel holder and microscope adapter for viewing mylar section strips.

Fig. d. One of the ways mylar strips may be held flat for viewing with a microscope.
Another advantage of film as a section support is the ease of storage. Strips 7 to 9 inches long have been stored in commercially available film negative envelopes and notebooks, in film negative storage boxes, and on 35mm movie film reels (Plate III, figs. a and b). For smaller sections, 8 or 16mm film reels would be useful and economical in terms of materials and space. Mylar strips 200 feet long have been stored on photographic film reels. This is especially useful because such reels can be mounted on any device accepting 35mm film. Many other applications, not appropriate for glass slides, are already available because the technology of the film industry can, in many cases, be applied directly to histological mylar section strips. An individual section mounted on a mylar film strip has been projected for as long as half an hour with no obvious deleterious effects. See Plate III, figs. c and d for an adaptor which enables section film strips to be used on a standard optical microscope.

Section Preservation

If the thickness of the coating material is within the 0.12 to 0.20mm range and is optically homogeneous, all microscopic objectives will form bright, well defined images. Krylon, and Acrylyd enamels and Hanna, and Dura Seal epoxies have yielded permanent, flexible optically homogeneous coatings (Appendix D). Epoxy seems to yield the brightest, most pleasing colors, but this may be due to the very
slight yellow tint of this material—very similar to that of Canada balsam or picolyte. Acrylyd yields a covering which appears to be most "crystal clear." It is not known at present how well the colors will be preserved with time, but no fading has been observed as yet. Krylon has a tendency to become brittle with age, and large areas will separate from the mylar surface if the film is bent rapidly or sharply as in a motion picture projector or optical measuring device. The acrylyd and epoxy coatings have (in most cases) remained very flexible and have neither cracked nor separated from the polyester film surface.

If the coating medium, such as Krylon, is too thin, air will enter the tissue and spread along connective tissue or muscle interfaces, causing portions of the section to appear black or dull. With a Krylon coating, a strip can be returned to xylene to dissolve or partially remove the coating and can be recoated. Needless to say, this is wasteful of time and undesirable. It is therefore important to make certain a covering of the correct uniform thickness is produced the first time.

If the coating is too thick, too thin, or not homogeneous, the high dry objective will not yield a clear, well defined image. Also, a thick coating, especially Krylon, will not dry sufficiently throughout and a thick surface which comes into contact with other portions of the mylar strip will often adhere. Coating areas which adhere will then be ripped off when the strip is unrolled. Adhesion areas have not developed in the catalyzed resin coverings because
they harden irreversibly within a limited time once catalyzed. The most desirable base we have used is 3 or 6 mil (.075 or .150mm) mylar, and the most desirable coating has been acrylic catalyzed enamel.

**Obtaining the Data**

It was found that the time required for transforming shapes or areas into $x,y$ coordinates was the most important factor in determining whether a given coding/processing arrangement was practical. Two rather basic mechanical coding devices were found to be impractical from both a time and an accuracy standpoint (Plate IV, figs. a and b). The optical grid system (Plate IV, figs. c and d) has the most potential for future development, although our system is at present too slow for coding of $x,y$ coordinates of the total pancreas. The optical grid system is preferred because it allows turning from one objective to another; therefore a rapid shift can be made from less to more detailed observation whenever necessary. Since projection onto a screen is not involved, the image is more detailed, and resolution for a given magnification is greater. At present, a method for automatic recording of coordinates with this system is being developed by the author.

The most practical system has employed the Mesallina measuring table. Using this apparatus, data and fiducials can be collected rapidly, and the table and optical system are stable enough to eliminate drift due to vibration or shift in relative positions of lens, specimen, and projection table. The combination of the rapid embedding technique, the tape support microtome, and the mylar strip mounting and staining techniques makes computer model construction of an organ possible.
Program One

It is possible to use the computer as a large calculator. Program One is designed to do this (See Appendix E). Data can be collected manually using millimeter grids or templates from every tenth, twentieth, or hundredth slide in a serial section series (version one). The data may also be collected in a more automated fashion (version two) on a Mesallina measuring table (Plate V, figs. a through d). Data collected from each slide is assumed to be representative of adjacent slides, and estimates of islet/exocrine volume and number ratios can be computed rapidly.

Fig. 1. Graph paper template used to obtain islet section area measurements from projected images.

The method requires little in terms of special equipment and can be made as precise as desired by selecting a series of slides with only
Plate IV. Coordinate Coding Devices.

Fig. a. Disk type coordinate reader. The upper disk gives the x coordinate, the lower disk the y coordinate. Coordinates are read on the disk rims beneath the verticle wire to the right of the microscope. Coordinates must be recorded and punched manually.

Fig. b. Optical beam coordinate reader. The beam arranged along the diagonal in the picture indicates the x coordinate, the beam in the lower right corner indicates the y coordinate. Coordinates must be recorded manually.

Fig. c. Optical coordinate reader. No moving parts. More accurate than the mechanical readers. Position of the two grids is shown, one in the base of the microscope ocular, the other beneath the slide.

Fig. d. Optical coordinate reader. Slide is shown in position over the lower grid. Grid shown on lower left corner of microscope stage is placed in one of microscope oculars and is thus superimposed on lower grid and section images.
Plate V. Mesallina Measuring Table.

Fig. a. In the foreground are the white table and the x and y coordinate wheels (black and at lower left and lower right corners). Midway back is the white scanning device with crosshairs and attached TV camera and lens. In the background, is the CRT (TV) on the left and the digital indicator on the right.

Fig. b. In operation. A section image appears on the table and an image of the cross hairs can be seen on the CRT (cathode ray tube). The arm holding the cross hairs is located near the lower border of the photograph slightly to the right of the mid line.

Fig. c. Mesallina measuring table. Section strips are mounted on reels seen near the top of the photograph.

Fig. d. Automatic card punch which is attached to Mesallina measuring table by a small Honeywell computer.
few slides between measured intervals. The ultimate in this direction is to measure every slide. This is not desirable using this program, however, since beyond a certain point, increased density of measurement will yield no additional data, and islet number counts may actually be distorted by counting the same islet in several adjacent sections.

In general, this system can yield useful non-specific data such as overall islet/islet (or exocrine) volume and number ratios. It is therefore useful for evaluation of rather gross changes which occur in an organ or system.

Fig. 2. To illustrate the method used in measuring islet areas.

Program Two

The computer may be used much more effectively if it is involved in actual data collection. This is due, in part, to the requirement that measurements made by a mechanical or scanning method must be more objective than those made entirely by hand, and in part to the potentially rapid response time. In Program Two (See Appendix E), area measurements are made directly by the computer, using one of the methods diagrammed in Figure 8 (a, b, or c).
Program Two, Version One - Linear Scan

Data for this program is to be gathered by a linear scanning method. All intercepts are recorded for each area as it is encountered before moving on to a new area, for instance:

<table>
<thead>
<tr>
<th>Recorded Points</th>
<th>1,2</th>
<th>3,4</th>
<th>5,6</th>
<th>7,8</th>
<th>9,10</th>
<th>11,12</th>
<th>13,14</th>
</tr>
</thead>
</table>

Fig. 3. Showing method of scan used in coordinates one program. Intercepts 1 through 14 are recorded before the scan continues to the next islet.

The scan line may continue from point 2 or any other chosen point such as shown in fig. 4.

Fig. 4. The scan of each islet is completed before scan of another islet is begun and scan of all islets in area 2 is completed before scan of area 3 is begun.
Each section (fig. 4) is divided into a number of convenient areas. All islets in area one are scanned before moving to area two. All islets in area two (4, 5, 6) are then scanned before moving to area three, and so on. To key a section to the preceding and following sections, three or more fiducials are recorded for each exocrine section. These correlation points are determined by silk threads embedded in, holes punched in, or grooves cut into, the original block.

![Diagram showing method to key sections](image)

Fig. 5. Method used to key one section to the next preceding and the next following sections.

Corresponding islet areas on succeeding sections are added to calculate islet or exocrine volume. Maximum and minimum x and y coordinates are recorded for each islet or exocrine section, and a total islet count is generated.

The data thus generated can be printed out en masse or processed to yield distribution, concentration, or other desired data.
Fig. 6. Model of the pancreas used to develop and test the version one-linear scan program. The large rectangular volume represents the exocrine pancreas. The inner shaded blocks represent islets.

Fig. 7. Sections cut from the model above. Islets are indicated by shading.
It now becomes practical to record fiducials and thus to locate islet masses in relation to their location within the pancreas (see fig. 9).

Since islet location is given in relation to exocrine mass, a three-dimensional reconstruction can be made, and islet volume and number can be determined from the reconstruction (See fig. 10).
Fig. 10. Three consecutive sections are shown. Islet number and volume is computed by comparing each islet on a section (5 and 7) with all islets on the preceding and following sections. When a match is found, islet areas are added and the higher ID number is changed to equal the lower.

Islet concentration or distribution can be represented in terms of percentage of volume near the periphery of the gland or near the central axis, etc. (See fig. 11).

Fig. 11. Graphs showing relative distribution of large and small islets in the ventral lobe of the duck pancreas.

In addition, the three dimensional model can be reproduced visually, using a standard plotting device or a cathode ray tube.
Program Two, Version Two - Area Approximation

This program differs from the linear scanning program only in the manner of initial data coding and processing. Data consists of five digit numbers ordered in \(x,y\) couples. Version two offers a flexibility in data gathering not present in the linear scanning program.

**A - RECTANGULAR APPROXIMATION**

\[
\text{area } a = (X_2 - X_1) \times (Y_2 - Y_1)
\]

**FORTRAN PROGRAM:**

```
501 M=3, KKK=4
6 JAREA(N11)=(L(M)-L(M+2))+(L(M+1)-L(M+3))
7 N11=Area(N11)+Area(N11)
```

**B - TRIANGULAR APPROXIMATION**

**FORTRAN PROGRAM:**

```
505 M=15, KKK=8
A=DSORT(((L(M+2))-(L(M)))+(L(M+3)-(L(M+1))))
B=DSORT(((L(M+1))+(L(N+6)))+(L(M+3)-(L(M+7))))
C=DSORT(((L(M+6))-(L(M)))+(L(M+6)-(L(M+7)))
S=S+(A+B+C)
Area(N11)=Area(N11)+DSORT(S*(S-A)*(S-B)*(S-C))
```

Fig. 12. This method of data collection is extremely flexible and can be used to gain either a rapid approximation (represented on the left side) or a detailed description of a section or islet area (represented on the right side of both the rectangular and triangular area calculation diagrams. A - represents method used to calculate area with rectangular areas. B - represents method used to calculate area with triangular areas.
Exocrine areas are calculated (as shown in fig. 12) by using four successive data points to define two triangles. The lengths of all three sides of both triangles are calculated from the distance

\[ P_1P_2 = \sqrt{(X_2-X_1)^2 + (Y_2-Y_1)^2} \]  
(difference formula)

formula and four successive data points [defined by eight coordinates or, in Fortran, by an array of eight numbers, L(1) through L(8)]:

<table>
<thead>
<tr>
<th>Points</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coordinates:</td>
<td>(X₁, Y₁)</td>
<td>(X₂, Y₂)</td>
<td>(X₃, Y₃)</td>
<td>(X₄, Y₄)</td>
</tr>
<tr>
<td>Fortran array:</td>
<td>L(1), L(2), L(3), L(4), L(5), L(6), L(7), L(8)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[
\begin{align*}
    a_1 &= \sqrt{(X_2-X_1)^2 + (Y_2-Y_1)^2} \\
    b_1 &= \sqrt{(X_2-X_4)^2 + (Y_2-Y_4)^2} \\
    c_1 &= \sqrt{(X_4-X_1)^2 + (Y_4-Y_1)^2}
\end{align*}
\]

\[
\begin{align*}
    a_2 &= \sqrt{(X_3-X_4)^2 + (Y_3-Y_4)^2} \\
    b_2 &= \sqrt{(X_2-X_3)^2 + (Y_2-Y_3)^2} \\
    c_2 &= \sqrt{(X_4-X_3)^2 + (Y_4-Y_3)^2}
\end{align*}
\]

Area is calculated from the trigonometric formulas for the semiperimeter (S) and the area (A) of a triangle (three sides given):

\[
\begin{align*}
    S &= 1/2 (a + b + c) \\
    A &= \sqrt{S(S-a)(S-b)(S-c)}
\end{align*}
\]

All triangular areas defined by the data collected are calculated consecutively and added to yield the total exocrine area. (See fig. 12, part A, for the Fortran programming of this procedure).

Islet areas are calculated by rectangular approximation. Islets are
indicated by a series of coordinate couple pairs \([ (X_1, Y_1), (X_2, Y_2) ] \). Each two successive points recorded indicate the location of two angles of the rectangle which are located at opposite ends of one of the diagonals. The length of the rectangle is computed by subtracting \(X_1\) from \(X_2\), and the width is computed by subtracting \(Y_2\) from \(Y_1\). Area is then computed \((L \times W)\), and the partial areas are added to yield total area. Rectangular approximation allows for collection of approximately the same data as collected by triangular approximation but with the measurement of fewer points (only half as many if the same scan lines are used). Since islets are numerous in some areas, the number of points required per islet is important. In addition, some islets consist of only a few cells, and collection of four points for such islets with the Mesallina measuring table is difficult. On this machine, recording of the additional points (triangular approximation) adds little to the accuracy of small islet measurement but doubles the time required to make the measurements.

Results of Computer Programs

Figure 13 contains a computer printout of results obtained from Program One, using previously collected data for the domestic duck (Anas domestica). The total time for processing the data and printing out the results was .2 of a second. The use of this program yields accurate figures quickly and takes much of the tedium out of data processing. In contrast, the original collecting and processing of the data took approximately
two weeks. The present procedure takes two to four days, depending on the number of sections measured and the density of the measured coordinates.

RESULTS - PIGEON

<table>
<thead>
<tr>
<th>TOTAL EXOCRINE VOLUME</th>
<th>TOTAL ISLET VOLUME</th>
<th>ISLET PER CENT OF TOTAL</th>
<th>TOTAL NUMBER OF ISLETS</th>
<th>NUMBER OF TISSUE SECTIONS MEASURED</th>
</tr>
</thead>
<tbody>
<tr>
<td>102.292342</td>
<td>30.53082</td>
<td>0.00377</td>
<td>0.37667</td>
<td>360</td>
</tr>
</tbody>
</table>

INDIVIDUAL SECTION AREAS

| 102.29223 |

RESULTS - DUCK

<table>
<thead>
<tr>
<th>PANCREAS VOLUME</th>
<th>DARK ISLET VOLUME</th>
<th>LIGHT ISLET VOLUME</th>
<th>DARKISLET NUMBER</th>
<th>LIGHT ISLET NUMBER</th>
<th>RATIO A/B</th>
<th>RATIO A/B PANCREAS</th>
<th>RATIOS ISLET</th>
<th>PANCREAS SEGMENT</th>
<th>ANIMAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>716.51</td>
<td>2.85</td>
<td>1.93</td>
<td>441.00</td>
<td>1350.00</td>
<td>1.46</td>
<td>0.39</td>
<td>0.67</td>
<td>DORSAL</td>
<td>6</td>
</tr>
<tr>
<td>420.42</td>
<td>1.56</td>
<td>1.28</td>
<td>164.00</td>
<td>450.00</td>
<td>1.24</td>
<td>0.26</td>
<td>0.66</td>
<td>VENTRAL</td>
<td>6</td>
</tr>
<tr>
<td>99.76</td>
<td>1.31</td>
<td>0.81</td>
<td>92.00</td>
<td>142.00</td>
<td>3.19</td>
<td>0.69</td>
<td>3.19</td>
<td>SPLENIC</td>
<td>6</td>
</tr>
</tbody>
</table>

Fig. 13. Program One results for data obtained from serial sections of the duck and pigeon pancreas. Original data for the duck was obtained from McClish, R. D., 1959. "Number and distribution of the islets (Langerhans) in the pancreas of the domestic duck and some observations on the insular cell types." Unpublished M.A. thesis, The Ohio State University. Data for the pigeon was obtained on a Mesallina measuring table produced by the Itek corporation.

The results obtained for the pigeon are from the dorsal lobe of the pancreas of a male domestic pigeon or rock dove (Columba livia) which weighed 452 gms and had a body volume of 655cc. The dorsal lobe was 31mm long; the ventral lobe was 35mm long.
DISCUSSION

Francotte in 1884 (Gatenby and Beams, 1950) used condensation of steam to cause rapid evaporation of the ether/alcohol solvent. He placed small pieces of tissue in a test tube, covered the material to be embedded with a small amount of celloidin, introduced steam, and corked the test tube. He reported good results using this method.

Later, Walls (1932) described a hot celloidin technique. Because of the pressure developed in the heated closed jars he maintained that the tissue was rapidly and completely infiltrated. The method has not become popular, apparently because of the danger and inconvenience involved in using high pressure.

Bensley and Bensley (1938), Davenport (1960), Koneff and Lyons (1937), Bennett (1940), and McDonald (1964) recommended the use of low viscosity nitrocellulose (L.V.N.) in connection with solvents such as amyl acetate. Because of its low viscosity, L.V.N. can be used in higher concentration, can penetrate more rapidly, and can therefore form a denser block in a shorter time. The denser block is advantageous in that thinner sections can be cut. L.V.N. is much lower in price than celloidin and is not as sensitive to the presence of water. It is not popular because a solution which is inadvertently allowed to evaporate will yield an explosive solid.

Inman (1968) introduced a method for embedding brain tissue using acetone/alcohol as a dehydration medium. He infiltrated the tissue with celloidin at 37°C in a vacuum. Embedding is carried out by any
of the standard methods. This method was found to be quite useful but not very time saving since the large blocks used (1x3 inch or 1x6 inch) hardened so slowly that the initial time saving was of little significance. Therefore a way of speeding up not only infiltration but also block production was searched for. Embedding frames and racks (as described in Appendix A) which fit into a vacuum embedding jar were developed, and the specimen in celloidin was subjected to a vacuum not only to the point of infiltration but beyond, to the point of solidifying. If pieces of celloidin are introduced into the embedding jar just prior to placing it in the embedding oven, the solid celloidin will rob the solidified block of additional solvent and cause a harder block to form more quickly (Walls, 1932).

The Tape Support Microtome

Bush (1952) described an automatic microtome which employs thin base 35mm film 3 mils (.0035 inches or .075mm) thick. The film is caused to adhere to the block surface by a pressure bar, and register is maintained by a considerable film tension. Such a design may be suited for production of large numbers of serial sections in close registration appropriate for use on standard movie equipment. The tape support microtome, in contrast, employs a .9 or 1 mil (.025mm) tape (See Appendix B). Such tape is commonly used in the production of engineering drawings and can be obtained from any engineering supply house. Available tapes have a variety of adhesive surfaces and various degrees of chemical resistance. Since tension on the tape loop is much less than tension on the film necessary for operation of the Bush automatic microtome, thinner tape can be used, and sections 2μ to 200μ can be cut.
A method of mounting celloidin sections in sheets of collodion was being used as early as the 1890's. Portions of the sheets were cut away and distributed to students in histology. Kerr in 1908 (Gatenby and Beams, 1950) described fixation of sections to Kodak film with a drop of an absolute alcohol/ether solution. Rasmussen, in 1932, first reported "an apparatus to minimize labor and time in mounting and studying" serial sections. In 1944, he published an extensive description of this apparatus and described a technique for storing serial neurological sections in cedarwood oil, balsam, or 70% alcohol between two strips of cellophane. He stored the cellophane strips on special reels and viewed the sections with a specially constructed binocular microscope. The system has not become popular, most likely because of its specialized requirements -- thick celloidin sections and a special apparatus for mounting, storing, and viewing.

Minckler (1942) described the use of a continuous roll of cellulose acetate. Sections were embedded in the acetate by soaking them in acetone before placing them in contact with the acetate strip. Sections had to be prestained in the block or on a sheet of glass before mounting. Several obvious problems would be: variation in the depth of embedment of sections into the acetate resulting in a distorted image, especially with the high dry objective; irregularity in the acetate strip due to dissolving of the acetate in areas where too much acetone was used; and the tedious handling necessary during
staining and mounting of the sections. The method consists essentially of dissolving the sections into the acetate film. She states that some wrinkling does occur, but this can be removed with a hot iron.

Richards, Small, and Collyer (1944) reported on the characteristics of plastic cover slips and concluded that plastic could yield satisfactory results if optically homogeneous sheets between 0.12 and 0.20 mm could be obtained. They suggested the use of a high dry objective with an optical correction collar. According to them, the collar should be set at a position corresponding to the thickness of the plastic cover slip.

Vroman (1953) and Ransom (1953) reported the use of Krylon acrylic spray for section preservation. For oil immersion, Vroman recommends the use of non-drying Crown immersion oil because Krylon is soluble in xylene. Regular immersion oil dries on the Krylon and cannot be removed without xylene. King (1958) described the use of Lucite (methyl methacrylate polymer) to cover sections on glass slides. He dissolved the Lucite in xylene and placed the 30-40% solution on the slide with a medicine dropper. However, only Krylon at present is established as a plastic coating which does not cause color fading with time.

Bush (1952) described an automatic microtome which uses 3 mil thin base photographic acetate film. Sections must be prestained, can be cut at 10 sections per minute, and can be shown on a movie camera. Later
(1955), he describes an improved version which uses mylar and allows for the staining of sections in a 35mm developing tank. He described the use of a mounting medium to hold a mylar cover strip over sections mounted on the 35mm mylar film base. This author has found it difficult to cover large strips (5 feet) of mylar mounted sections before the sections became dry, and it was found that the mounting medium had a tendency to flow out from between the mylar strips and cause adjacent strips to adhere to one another. Bush does not discuss the optical qualities of the mylar covering strip. Optically homogeneous mylar suitable for section covering is not easily obtained, although a light spray of epoxy or acrylic resin over the mylar cover has significantly improved its optical properties.

Pickett and Sommer (1960) combined the use of polyethylene film (Dupont Cronar) as a section mounting base with the use of Krylon as a preserving cover, following staining on developing reels. They recommended the use of oil and a cover slip over the Krylon for the high dry lens or a lens having an adjustment collar. They felt that Krylon may not be as appropriate for photomicrographs as the standard glass cover slip but stated that otherwise Krylon in combination with Cronar film (DuPont) yields a saving in both time and money. They observed that the Krylon coat becomes somewhat brittle with age (after several months) but felt this is not a serious handicap if the film is not bent sharply. These researchers suggested storage of pathological sections mounted on film by stapling the strips of film to the appropriate patient's record. McCully, et al. (1966) described a
device for viewing 35mm film mounted specimens on a standard microscope. The device stores the film safely enough that it can be sent through the mail repeatedly without damage to the film or its plastic container/microscope adapter.

Due to the present availability of two component epoxy and acrylic resins and paints and the tape support microtome which eliminates the need for a water bath, it is possible to take the good points of several previous methods while avoiding some of their less desirable features. Polyester has been used, making it possible to stain mounted sections. Thin mylar tape has been used to replace the thicker 3 mil acetate used by Bush, making it possible to cut thin sections instead of sections 10μ thick or thicker. Section preservation has been accomplished with flexible, stable coatings which do not become brittle and crack with age. The present study introduced both the use of an airbrush to disperse the material over the section surface and the dipping technique, which is quite a simple and effective method for covering sections (See Appendix C). Presently available materials are stable and flexible enough to render possible the use of these products in standard histological technique. Histological sections mounted on polyester plastics with the new techniques described are preserved on a more stable base and are protected by more durable covering media than standard glass slide preparations. Optical qualities can be controlled rather effectively, and a good technician should be able to prepare sections equal to or better than standard glass slide/glass cover slip preparations.
There have been several lines of quantitative investigation. Three of these lines will be considered: first, area measurement which was begun by Delesse (1847) and Heiberg (1906) involves volume determination based on planimetric measurement of areas; second, islet count which was developed by Bensley (1911) involves the use of supravital dye and yields a total islet count; third, linear scan which was modified from a linear scanning technique Rosiwal (1898) developed for measuring dispersion of minerals in rock has been adapted by Uotila (Uotila and Kannas, 1952), Lazarow and Carpenter (1962), and Hellman and associates (Brolin and Hellman, 1963) for biologic use.

**Area Measurements**

Opie in 1900 (Overholser, 1925), using sections of the pancreas, counted the number of islets in 0.5 square centimeters in the head, body, and tail of the human pancreas. He found the islets of the tail to be 3 1/2 times more numerous than in the head or body.

Dewitt in 1906 (Jaffee, 1951, and Overholser, 1925) studied serial sections of the pancreas of several mammals and of the dove. Dewitt calculated the average number of islets per square millimeter, average islet volume, and percent of islet tissue. Her data was based on observation of serial sections. She stated that islets were more numerous and larger in the central portion of the dove pancreas.
Laguesse in 1906 suggested that the Zellhaufen of Langerhans be called "islets of Langerhans," and in the same year Heiberg (1906) attempted to count the number of islets in an area of 50 square millimeters. By assuming that islets were distributed uniformly throughout the entire pancreas, he calculated the total number of islets of the pancreas. Later, Heiberg developed a different method based on volume measurement (with a planimeter) of drawings taken from selected areas of pancreatic sections. It was found, however, that Heiberg's measurements had to be adjusted because the islets are not uniformly distributed throughout what is called the head, body, and tail of the pancreas (Tejning, 1947).

In 1937, Ogilvie described a method similar to that of Heiberg. He measured the weight of islet tissue and the number of islets in 100 individuals ranging in age from newborn to 64 years. Blocks of tissue were cut from the head, body, and tail of the organ and were fixed and then stained by the Mallory azan method. Fifteen randomly selected fields were projected onto a sheet of paper and the traced islets were cut out. The total percentage of islet tissue in each of the three areas of the pancreas (head, body, and tail) was calculated. Total islet weight was then calculated from this percentage by assuming that islet and exocrine tissue have the same specific gravity. Ogilvie calculated the area of an average islet by assuming all islets were spheres and that the average cross section would cut through the average sphere at the points A and C indicated in the drawing. By measuring radii of randomly selected islets, he calculated an average radius (OB) and multiplied this by \( \frac{4}{3} (AD \times .0096)^3 \) to
get what he considered to be the average islet volume. He then multiplied the specific gravity of pancreatic tissue times the volume of the average islet to get the weight of the average islet and divided this weight into total islet weight to obtain the total number of islets.

The method can be summarized thus:

Area of average island on section = $r^2 = x$

\[ r = AD = \sqrt{\frac{x}{\pi}} \]

\[ R = \text{real radius of average islet (sphere OABC)} \]

\[ \frac{AD \times \tan 30^\circ}{120} = \frac{AD \times 0.096}{120} \]

\[ V = \frac{4}{3} \pi (AD \times 0.096)^3 \]

\[ M = V \times \text{Density} = 4.1 (AD \times 0.0096^3 \times 1.05) \]

\[ N = \frac{\text{Total weight of islet tissue}}{4.1 (AD \times 0.0096^3 \times 1.05)} \]


The correctness of the assumptions in the calculations (outlined above) is important to the relevance of Ogilvie's results. To show that Ogilvie's calculations are correct, it is necessary to give some indication of why his assumptions should be accepted. He did not do this in detail, and some investigators (Tejning, 1947) have asserted that some of his approximations appear to be arbitrary. The scope of his measurements and estimations is, however, very intriguing.

Oakberg (1949) used a modification of Ogilvie's method on 90 chickens and, although he improved the technique somewhat by dividing the gland into seven parts and by actually counting islets in serial sections derived from portions of each of the seven parts, he still did not eliminate many of Ogilvie's original assumptions. Oakberg did note
that alpha islets in the chicken pancreas are not spherical and eliminated calculation of the volume of dark islets based on the assumption that they were spherical.

Tejning in 1947 reported an exhaustive study involving 284 rats divided into four different dietary groups. He eliminated most of the randomness of the previously used methods by serially sectioning (30μ per section) entire rat pancreases embedded in celloidin. Volumetric measurements were made as follows. All islets occurring on every tenth section were drawn on paper, and the total islet area was calculated from the weight of these cut out islet sections. The total volume of islet tissue was calculated from the following formula:

\[ V = \frac{x \text{ number of intermediate sections}}{\text{area of observed islets} \times \text{thickness of sections}} \times \left(\frac{\text{square of the magnification}}{}\right) \]

The number of islets were estimated from formulas based on calculations by Wicksell, developed in 1926. Wicksell concluded that real distribution of islets can be related to apparent distribution observed in the microscope by means of an Abelian integral equation. Tejning not only calculated total number of islets but also divided this total number into 14 subclasses based on islet diameters. It was thus possible for him to indicate distribution of islet tissue in terms of islet size. He concluded that a definite relation exists between total islet volume, numerical distribution of the islets, and volumetric distribution of islets. Hellman (1959) has used this assertion of Tejning in connection with Wicksell's assumptions and conclusions to develop simpler methods of evaluating size and state of the islet organ. The method, however,
has been established as useful only in certain species (rat, mouse, and man—Brolin and Hellman, 1963); it is said not to work when applied to birds. The bird pancreas is composed of two islet types, light islets and dark islets, and these islets are not distributed uniformly throughout the exocrine tissue (Hellerström, 1963).

One of the chief criticisms of Tejning's method has been its unsuitability for use in larger animals or in studies involving more than just a few animals because of the complexity of preparing, measuring, and analyzing so many serial sections. In addition, his method of preparing the pancreas for sectioning destroys most relations except, perhaps, endocrine/exocrine size relations (which is what he has studied). Hellman's scanning and counting techniques (Brolin and Hellman, 1963) apparently overcome these difficulties. He describes the use of a photocell in an automatic scanning microscope and gives data in support of a technique in which only the number of large islets is counted.

Our present method, which involves the calculation of area, volume, or islet location using the computer, is most closely aligned with this line of investigation which starts with Heiberg (1906) in pancreatic measurement and Delesse (1847) (Lincoln and Reitz, 1913) in the determination of rock components.

Program One is based on area measurements of sections selected at regular intervals. The areas of these selected sections are then assumed to represent adjacent sections, and islet and exocrine volume is calculated. The results yield about the same type of information as Carpenter and Lazarow's linear scan method which includes islet/exocrine ratios.
with the additional possibility of obtaining islet concentration data if the coordinate method of obtaining data is used. No specific data concerning a comparison of the time involved in obtaining data using these two methods is available (Lazarow, Carpenter's method versus Program One), but both would involve at least two intercepts per islet. Since their linear scan method involves a predetermined number of scans whereas Program One involves data from each islet, the Lazarow, Carpenter method would be more economical of time and effort on pancreatic sections containing many small uniformly distributed islets (rat), and Program One would be more economical (require fewer data for the same information) on pancreatic sections containing fewer islets (duck).

Program Two yields a different kind of information than the Carpenter, Lazarow linear scan, but the data collection method is very similar to that of Program Two, version one. In Program Two, version one, fiducials are recorded for each section, and islet or exocrine intercepts are recorded in terms of x,y coordinates. This gives the relative position of islets in relation to the exocrine boundary, and the data can be processed by a computer to yield actual size and position relations rather than simply a total component volume ratio which is given by the Lazarow, Carpenter method. Choice between the two methods would probably depend, in most instances, on whether change in islet/exocrine volume ratio was desired (Lazarow, Carpenter) or concentration, distribution, or shift in size and shape was desired (Program Two, versions one and two). Carpenter (1973, personal communication) has suggested that Program Two, version two would be well suited to investigation of the question of where islets originate (from ducts, from
other islets, or from exocrine or centroacinar cells). Program Two introduces new elements to quantitative islet measurement in that the record is stored on computer tapes or cards and is therefore permanent and easily accessible. These programs yield more concrete data than previous quantitative techniques through the introduction of the use of fiducials and x,y coordinate positions for all islet and all exocrine sections.

Islet Count (Supravital Staining) Method

Bensley (1911) developed a method of counting the islets by injecting supravital stains into the aorta of freshly bled guinea pigs. This method, however, did not yield permanent results; the islets had to be counted within 5 to 30 minutes of staining because the supravital stains faded rapidly. Also, the method did not yield volume measurements. Since the size of individual islets varied widely, the correlation between number of islets and endocrine pancreatic volume was not precise. A number of investigators have followed Bensley's lead: Clark (1913), Overholser (1925), Haist and Pugh (1948), Jaffee (1951), and Davidson and Haist (1962).

Only two years after Bensley's original publication, Elbert Clark in the Phillipines published an islet count for the human. Clark (1913) applied Bensley's method to portions of the human pancreas but, according to his own statement, the variations in drying of the tissues and the resulting weight variations caused his experiments to be scarcely more than an attempt to extend Bensley's method to the human.
Overholser (1925) performed a neutral red supravital study, using the albino rat to determine the relation of islet number to age. The age and weight of each animal was recorded, and the pancreas was supravitally stained and divided into duodenal, splenic, and mid-portions. A total count of islets was made on pancreas pieces in normal salt solution under a cover glass. The salt solution was then pressed out and all of the portions in each division were weighed. Overholser concluded that islet number markedly increased during the first 20 days and then remained steady up to 50 days (60gms.). With increasing age, islets continued to decrease slowly in number until 90 to 150 days beyond which the number remained constant. His figures indicate that the number of islets per gram of body weight decreases with growth.

Haist and Pugh (1948) automated the method of Bensley to include a projected image, a permanent record, and a planimeter on a moving stage which added areas. They stated the method was still technically difficult because of the rapid fading of tissues, but by freezing the tissues could be stored for at least a week. They found that islet volume and number increase with age but decrease in relation to body weight. Since fasting until 30 percent of the body weight was lost caused no change in islet number or volume, they concluded that decrease in insulin with fasting was due to beta cell degranulation rather than destruction. The data obtained with the Haist, Pugh apparatus could easily be fed directly into a computer, but since only islet number and volume can be measured, and since their apparatus already sums this data, there possibly would be no gain in doing so.
Jaffee (1951) used Bensley's method to obtain information concerning islet distribution, size, cell number and ratios in the adult albino rabbit. He stained each pancreas supravitally, removed small pieces from the duodenal, retroperitoneal, and splenic portions and counted all islets in each. The specimen was then dried between filter papers and weighed and the total number of islets in each portion was calculated. A summary of islet measurements that have been made in a wide range of species is included in his discussion. Davidson and Haist (1962) used the Haist, Pugh apparatus to make islet weight studies in rats treated with tolbutamide.

As can be seen from the present review, the method of Bensley still has applications. Both Janus green and neutral red have previously been tried on duck, rat, and guinea pig pancreases in our own laboratory (McClish and Eglitis, 1969) and have proved to be inadequate for computer based research because of lack of enough microscopic detail to allow for accurate quantitative measurements and also because of lack of a permanent record which makes it almost impossible to calculate percentage error. Oakberg (1949) suggested that small islets are easily missed with this technique. This is not to discount Bensley's work, however, which we respect highly and which yielded some very useful and provocative material.

**Linear Scan Method**

Rosiwal in 1898 (Lincoln and Reitz, 1913) devised a linear scan method for determining the percentage composition of rock. He replaced, according to his own analogy, Delesse's leaf of rock with a thread of rock.
Though the method was challenged when it was initially introduced, it has been substantiated both mathematically and experimentally and is now well established as a dependable time saving method of component analysis, Lincoln and Reitz (1913). It consists of recording the lengths of grains (or tissue components) encountered along a series of lines. The percentage volumes of each of the components is calculated by dividing the line length covering each component by the total line length. For macroscopic measurements, Rosiwal suggests stretching a rubber band across the specimen to be measured and recording the intercepts along its upper surface. For microscopic measurements, he suggests ruling lines with semi-transparent ink on the cover glass.

Lincoln and Rietz (1913) developed a mathematical procedure for determining the number of intercepts that must be measured on a given specimen to insure that the error will not exceed 1% and gave mathematical and experimental proof of the usefulness of the Rosiwal procedure. Shand (1916) and Wentworth (1923) developed recording micrometers for speeding and automating the Rosiwal measurements (See fig. 15).
Uotila (1940) (Uotila and Kannas, 1952) published a description of a method for making quantitative measurements of histophysical changes. The method consists of making measurements along 2 straight lines at right angles or along 4 or 5 parallel lines drawn on a projection of the material to be measured.

Carpenter and Lazarow published a description of a "motor driven integrating stage" in 1958 and a more complete characterization of their instruments for automating tissue measurement in 1962. They describe two related instruments, the micrometer component quantitator for making lower power measurements and the electronic component quantitator for making observations under oil immersion. In a paper published in the Journal of Histochemistry and Cytochemistry in 1962, they describe in detail the historical background of their method based on the Rosiwal technique, redevelop the formulas for determining the length of scan necessary for yielding measurements of a given accuracy, and offer experimental verification for their theoretical material.

![Fig.16. Lazarow and Carpenter's micrometer component quantitator. Taken with modification from Lazarow, A. and A-M. Carpenter. 1962. Component quantitation of tissue section. 1. Characterization of the instruments. J. Histochem. Cytochem. 10:325.](image)
The linear scan method has been accepted as a rapid, very useful technique for obtaining tissue volume relationships. It is a practical procedure which has been thoroughly tested and accepted as valid (Lincoln and Reitz, 1913; Uotila and Kannas, 1952; and Carpenter and Lazarow, 1962). However, the goal of the present investigation is to establish distribution and concentration figures for islets of Langerhans. The linear scan yields figures indicating total volume relationships for a whole organ or a preselected portion of an organ. It is not readily adapted to yield data indicating the location of specific volumes and is therefore not suited to an analysis of variation in patterns of distribution.

SUMMARY AND CONCLUSIONS

A system for translating tissue or organ morphology into a series of x, y coordinates which can be stored and manipulated by a computer has been developed. Two programs are described. Program One involves the computer mainly in calculation. It can yield islet/islet and islet/exocrine volume and number ratios. The improvement this program offers over previous quantitative area measuring methods is mainly a speeding of the processing of data due to use of the computer. If data for Program One is collected manually or visually by the use of a grid superimposed on pancreatic sections, data collection is slow and laborious as in most previous quantitative techniques. If, however, the rectangular or triangular approximation technique is used, data collection can be speeded greatly. Program One yields much the same kind of data as yielded by Carpenter and Lazarow's linear scan
method. Versions one and two of Program Two process data, once gathered, in the same way. The data for version one is gathered by a linear scan while data for version two is gathered by rectangular and triangular approximation.

Since the data gathering technique for Program Two, version one, resembles the linear scan method of Lazarow and Carpenter, much of the theory and experimental support for their method can be applied to this program. Program Two can yield more data than a straight linear scan, however, because it includes the recording of fiducials and x,y coordinates which make it possible to construct a three dimensional mathematical model of the pancreas. Program Two has the disadvantage that to obtain the maximum amount of information, consecutive serial sections must be scanned, whereas the Carpenter, Lazarow technique would in few cases require scanning of consecutive sections and would require less dense scanning per section.

To speed up and simplify data collection, rectangular and triangular approximation of areas were substituted in version two for the linear scan of version one of Program Two. Rectangular approximation allows the operator of the scanning device to vary the density of coordinates recorded. For recording the position and size of closely packed small or irregularly shaped islets, many x,y coordinates may be taken in a small area. For recording the size and location of a large or isolated regularly shaped islet, two coordinate pairs may be sufficient. This data collection method is, therefore, flexible and can be modified at the operator's will to yield both extremely gross and extremely detailed data on the same section. When used by an experienced operator, the
rectangular (or triangular) areas method can yield detailed data quickly. Its accuracy, however, is dependent to a greater degree on the skill and understanding of the operator than the linear scan method, and it is not suited for use with an optical scanning device. For such an application, the Program Two linear scan is better suited. Program Two, version two, yields more specific data more quickly but is more difficult to implement in that the computer or technician must distinguish different morphological areas prior to scanning and must change the scanning technique to fit the area.

A new method for rapid embedding of large tissue specimens in celloidin had to be developed for this research. The process is rapid and flexible and yields blocks of a quality similar to that of celloidin techniques taking much longer to process.

A microtome which employs a tape loop for control of section cutting and section manipulation was designed for this research. Extremely large thin sections can be cut readily and no water bath is needed. Whole tapes can be mounted at a time on mylar strips. This results in a speeding up of the processes of mounting, staining, and preserving sections. Use of the tape support microtome makes it possible to cut and process large thin histological sections rapidly with no loss in quality. Mylar, a very stable, glass clear, polyester film has been found suitable for use as a section support, but standard photographic film (acetate) has proven to be impractical as a section base because it is adversely affected by standard histological reagents (such as alcohol). Polyester film has reacted with no chemicals used in our laboratory, has remained clear and flexible throughout the entire
staining and clearing process, and has shown no coloration or deterioration with age. There is, at present, a surprisingly large variety of plastic products suitable for use as mounting and/or covering media. Our observations confirm the usefulness of Krylon as a mounting-covering substitute. Krylon does become brittle with time, and this is a serious drawback because one of the important features of mounting sections on 35mm film is flexibility of the film and its resistance to breakage. Other aerosol sprays and coating films have, therefore, been investigated. The most promising are the two component epoxy and acrylic coatings prepared commercially for auto and floor preservation. Polyurethane liquid plastic appears to have possibilities. A covering that shows promise is clear mylar adhesive tape (1 to 2 mils thick). In combination with an epoxy or acrylic mounting medium, this makes a very pleasing mount. To use the high dry objective, however, it was also necessary to spray the upper surface of the mylar with a light uniform coating of epoxy or acrylic. These coatings have, to date, maintained flexibility and have not caused significant fading.

In summary (histological technique), mylar mounted histological sections can be stained more rapidly and just as effectively as glass mounted sections. They can be preserved quickly and permanently with two component catalyzed epoxy or acrylic plastic coatings. With practice, a uniform epoxy or acrylic coating can be applied which is optically equivalent or nearly equivalent to a standard mounting medium and glass cover slip. The polyester base and epoxy or acrylic cover are more resistant to permanent damage than glass and give promise to be more stable over long periods of time than standard mounting media. The
resulting plastic film containing the sections matches the glass slide in quality and is more flexible in application.

As indicated in the discussion, many investigators have developed techniques for obtaining data concerning islet number, volume, and distribution. The present approach has eliminated some subjectivity in that data is partially collected and is completely processed by a computer. With development of an entirely mechanical optical scanning technique for Program Two, almost all subjectivity would be eliminated. Such a mechanical scanning technique would probably not initially yield data as valuable or detailed as that obtained by a skilled, experienced human observer (Program Two, version two), but the mechanical scanning technique would eliminate the need for a specialist and potentially open the way for semi-automatic clinical evaluation of pathological material.

In summary (quantitative method), rapid tissue processing and quantitative data collecting methods have been designed to make the histological section/computer data interface less formidable than in the past, and two computer programs (with several variations) have been designed to process the data collected.
APPENDIX A

A RAPID CELLOIDIN VACUUM EMBEDDING METHOD
A RAPID CELLOIDIN VACUUM EMBEDDING METHOD

A method for translating histological measurements into data appropriate for computer evaluation has been developed. To do this, it was necessary to develop a method for fixing and embedding large samples of material or whole organs. Such a method must not unduly disturb intra- and extra-organic relations for organs such as the pancreas, but must still allow orientation of a series of organs or tissue specimens so they can be sectioned through approximately the same axis and along the same plane passing through this axis. Celloidin was chosen as the embedding medium because of its transparency, and a vacuum embedding technique which cut normal celloidin embedding time from months or weeks to days was developed.

The fresh pancreas within the duodenal loop is removed from the animal and attached to a U frame (Plate VI, fig. a) with silk thread. The U frame is necessary to prevent twisting and curling of the specimen during fixation, dehydration and embedding.

The specimen is fixed in 10% buffered formalin or Karnovsky's fluid with constant stirring for 12 to 24 hours, washed in tap water, and dehydrated in acetone-alcohol (50/50) for three or four days with a change of dehydrant on the second day. The dehydrated materials
are transferred first to absolute alcohol, then to ether/alcohol (1/1), and finally to 5% celloidin and are allowed to remain in each solution with stirring for 24 hours.

The infiltrated specimens on their U frames are mounted on a rack (see Plate VI, fig. b) which is placed in a large, specially designed embedding jar (Plate VI, fig. d). The rack containing the U frames is placed in the embedding jar which is filled nearly to the top with 5 or 8% celloidin and the jar is closed by a glass closing plate which is held in place by rubber bands (Plate VII, fig. b.). The closing plate used for evaporation embedding is cut so that a small opening remains along the top to allow for escape of evaporating solvent during embedding.

The embedding jar is placed in a vacuum oven (no heat is used). The vacuum is increased gradually so as to maintain a constant but not overly rough boiling. (Inman, 1968).

It is possible to tell by the size and shape of the bubbles formed when to remove the jar from vacuum so that, on compression, the celloidin will form a solid block which contains no bubbles. The celloidin must be of sufficient consistency that it does not flow when chloroform is poured in on top of it. The rack containing the three specimens is loosened from the sides of the jar with a large knife allowing chloroform to flow in between the glass and rack. This hardens the periphery of the celloidin block sufficiently to prevent outflow of the still viscous interior. The rack is then pulled out
and placed in chloroform to harden. When the central area has hardened to the point where it is not easily deformed by squeezing the walls of the metal rack, the sides of the rack are pulled away leaving only the U frames and specimens in the celloidin block.

This method, which results in the almost instant formation of a block, requires close monitoring and may result (if the correct end point is passed) only in the formation of a series of hardened bubbles. Therefore, if it is not desirable to monitor the process closely, the jar should be removed from vacuum before the critical solidifying point is reached. The closing plate is then slipped upward slightly to close the jar, and the closed jar is allowed to set overnight. The next morning the celloidin will have reached a consistency suitable for hardening with chloroform. If it has not hardened, it should be allowed to stand until sufficient hardening has occurred. The closer to hardening the block is when decompressed, the more rapid the final hardening process will be and the more dense the final block will be.
Plate VI. Preparation for Celloidin Embedding.

Fig. a. Pancreas and duodenal loop mounted on U frame with silk thread.

Fig. b. Rack for holding U frames in vacuum embedding jar. Note wings on U frame which fit into slots on rack.

Fig. c. Fixation and dehydration apparatus. Top of plastic jar shields magnet and directs fluid current.

Fig. d. Vacuum embedding jar with two glass closing plates and rubber bands for holding the plates in place. Note that one plate is shorter. This allows a space to remain at the top of the jar opening so that evaporation can occur.
Plate VII. Celloidin Embedding.

Fig. a. Vacuum embedding oven and two vacuum water pumps used. No heat was used in the embedding process.

Fig. b. Vacuum embedding jar after removing from the vacuum and prior to opening. Note celloidin has solidified.

Fig. c. Closing plate has been removed. Chloroform is now poured in to harden the celloidin prior to removal of the rack.

Fig. d. Removing the finished celloidin block from the U frame.
APPENDIX B

THE TAPE SUPPORT MICROTONOME
THE TAPE SUPPORT MICROTMME

For the present research, it has been necessary to obtain serial sections 5 or 10μ thick, from blocks as large as 2cm wide and 7cm long. For large serial sections, 15 to 20μ thick, celloidin is a satisfactory embedding medium. The traditional celloidin procedure is, however, slow because each section must be numbered and stained individually, and the thinner celloidin sections are easily damaged if handled roughly. This procedure has been modified by using a tape support and either celloidin or double embedded specimens. Double embedding makes it possible to cut the sections dry, but celloidin sections stain more rapidly and yield more pleasing histological sections. A Reichert sliding block microtome was modified to carry a tape loop as shown in Plate VIII. The circumference and width of the tape can be varied to accommodate large or small sections or to collect a greater or lesser number of sections. The loop is placed on the microtome by slipping it over the protruding free ends of the stationary guides after the gravity tension bar has been moved forward (See Plate VIII). It is brought under tension by moving the gravity tension bar back again against the loop. The protective paper strip is then removed and the adhesive surface is allowed to come in contact with the block surface. If a tacky adhesive is used, simple contact
between tape and block surface causes adhesion sufficient to lift the cut sections up and away from the knife. To cut a section, the knife is moved forward across the block surface at a constant slow speed. Adhesion of the tape to the uncut block prevents motion of the tape causing it to roll like the wheel of a bicycle in contact with the pavement. The rolling motion of the tape loop caused by adhesion to the block surface and advancement of the tape guide bars within the loop causes the tape to lift the cut section up and away from the advancing knife at an easily controlled, predetermined rate. Since it is connected to both the block and the knife, the tape loop acts as a feedback mechanism for maintaining a constant relationship between the moving knife edge, the lifting away of the cut section, and the stationary block. As the knife nears the end of its path through the block, the area of adhesion between block and tape becomes less and a point is reached where the force required to rotate the tape is greater than the remaining small surfaces can maintain. If no precautions are taken, the tape is ripped from the block surface along with the cut section which is still partially attached to the block. This results in folding or maceration of a small triangular area of the portion of the section not yet cut. To avoid this, a small support anvil to which the tape is attached has been added. The tape/anvil surface adhesion area is large enough to maintain tape rotation throughout the cutting motion.
Plate VIII. The Tape Support Microtome. Mounting Tape.

Fig. a. Unmodified Reichert sliding block microtome.

Fig. b. Tape support microtome.

Fig. c. Mounting the tape. Gravity tension bar is in the forward position and tape is being slipped over the upper stationary support bar.

Fig. d. Closeup of mounted tape loop.
Plate IX. The Tape Support Microtome. Cutting.

Fig. a. Method of manipulating gravity bar to maintain proper tape tension throughout the entire cutting cycle.

Fig. b. Removing tape from the support anvil just prior to moving block back to start a new cutting cycle.

Fig. c. Closeup of knife, block, and tape in the middle of a cutting cycle. The section is being lifted away from knife by the tape. Since no contact occurs between the knife blade and blade section surface, and the section is supported by the tape during cutting, the section does not accordion and therefore does not have to be spread on a water bath.
APPENDIX C

STAINING AND PRESERVING MYLAR MOUNTED SECTIONS
STAINING AND PRESERVING MYLAR MOUNTED SECTIONS

The standard 1x3 or 2x3 inch glass slide and cover slip has served well in the storage and preservation of histological sections. Present microscopic optical systems are designed for the standard glass slide and cover slip, and the combination of microscope, cover slip, glass slide and substage condenser can yield microphotographs of consistently high quality. The glass slide does have certain disadvantages such as easy breakage (therefore poor shipping or transport qualities) and a tendency for the mounting medium to dry or, at higher temperatures, to flow. This makes it difficult to store slides compactly in direct contact with one another or to project slides easily. A system which corrects or improves the storage, transport, and stability characteristics of the glass slide must, at the same time, meet the requirement of being easily adapted to present standard microscopic optical systems. All standard oil, low, scanning, and especially high dry, microscopic objectives must yield visually and photographically adequate images. The mylar tape section support system described can meet these requirements.

Standard clear mylar 1, 2, 3, or 6 mils thick in rolls 36 inches wide and 20, 50 or 100 yards long has been used. The roll is cut into 35mm strips on a specially designed film strip cutter (Plate X, figs. a and b). The mylar strips can be used full length (60, 150, 300 ft).
if reel storage is desired. In most cases they were cut into five
foot lengths. The five foot length fits standard film developing
reels and tanks and makes staining and processing easier. Sections
of any desired thickness (a range of 2 to 40μm were tested) can be used.
To mount sections, a mylar strip is coated with Mayer's albumin and
the sections are placed on the strip in whatever order is preferred.
If sections have been cut on a tape support microtome (see Appendix B),
a tape of sections 10 to 20 inches long is mounted as a unit and rolled
with a photographic print roller to squeeze out bubbles or excess
albumin (Plate X, figs. c and d). Albumin holds sections on the
mylar strip as effectively as on glass slides. If rapid preparation
of section strips is desired, the freshly mounted moist strips are
placed in a 60°C oven for several hours; otherwise they may be
allowed to set overnight at room temperature. A 5 foot length of mylar
tape is threaded onto a 35mm film developing reel. The reel is placed
in a developing tank, and staining solutions are poured into and out
of the tank at appropriate intervals. Stained sections are dehydrated
in alcohols and cleared in xylene. The cleared sections and tapes are
then covered with a protective plastic coating. This has been done in
two ways.

In the first procedure, the section strips are stretched on an adjus-
table aluminum frame by attaching lead film-developing-weights to
each end of the strip (Plate XI. fig. c). After the strips are stretched
on the frame, they are spray coated with a thin catalyzed layer of acry-
lic or epoxy resin before the sections dry. The most effective method
of application has been to "drop" large droplets onto the surface using
an air pressure as low as 10 to 20 lbs/in\(^2\) and a wide open nozzle. The low air pressure causes a minimum amount of disturbance at the slide surface, and the wide open nozzle allows delivery of a maximum amount of mounting/covering medium in a minimum amount of time. High air pressures (40 lbs/in\(^2\)) can actually blow a section loose from the mylar base or dry the section out before delivering enough material to cover it. In addition, the fine droplets (produced by high pressure spray) dry before flowing into and molding with other droplets to form a continuous surface. High pressure spraying, therefore, produces a semiopaque surface with poor optical properties and distorted or discolored sections. When properly applied, large droplets of preservative coating are dropped onto the section surface with no turbulence, and adjacent droplets congeal quickly to form a thick, optically homogeneous covering embedment of the proper thickness (approximately 0.180mm or 6-7mils).

The second procedure is much simpler but allows for less control. Cleared tapes can be transferred, while still on the developing reel, from xylene directly into a solution of the mounting/covering solution such as Krylon, Acrylyd acrylic enamel or Plasti-Glast epoxy (See Appendix D). When diffusion currents are no longer visible, or after remaining in a closed tank overnight, the reel is removed from the tank, and the mylar strip is unwound and hung up to dry with a film-developing-weight attached to the dependent end.
of the mylar strip (just as with a freshly developed film strip). (See Plate XI, fig. e). An even more convenient method is to take the strip from xylene, remove it from the reel, and, holding each end, simply run the strip through the coating solution. Some control of the dried film thickness can be obtained by diluting the original infiltrating medium to a different degree with either xylene or a thinner appropriate to the coating material.
Plate X. Mylar Section Strips. Preparation.

Fig. a. Closeup of knife arrangement on mylar strip cutter.

Fig. b. Mylar strips being cut on strip cutter. Original roll of mylar is in upper right corner.

Fig. c. Mounting acetate section tapes on mylar strips. The backing on acetate strip (lower left corner) is removed and sections are collected on the tape (second strip from left). The acetate tape with its sections is then mounted on a mylar strip (next to the roller on your left) with Mayer's albumin (next to the roller on your right).

Fig. d. Rolling the mounted section tape to remove air and excess albumin. Note unrolled portion on left is opaque, while properly rolled area on right is clear or translucent.
Plate XI. Staining Mylar Mounted Sections.

Fig. a. Mylar section strip on stainless steel film developing reel.

Fig. b. Apparatus for staining mylar section strips.

Fig. c. Apparatus for application of the coating medium used to preserve stained strip mounted sections.

Fig. d. Kindermann agitator.

Fig. e. Coated section strips hung to dry.
APPENDIX D

PRESERVATION OF MYLAR MOUNTED HISTOLOGICAL SECTIONS -
ALTERNATIVES TO THE GLASS COVER SLIP
A number of materials have been reported as possible replacements for glass cover slips. Several acrylic resins (Krylon and Lucite) in spray cans have been reported to be acceptable and inexpensive. Oversize glass cover slips are expensive and fragile. Krylon is an acceptable alternative to the use of large cover slips. Williams Acrylyd acrylic enamel and Hanna's Hanna-tile epoxy, both two component systems, are even better because they remain flexible and appear to give a more protective coating. They have not caused discoloration of the stains to date but more time is required for a conclusive judgement of their color preservation.

Sections were cut on a tape support microtome (Appendix B) and were mounted and stained on mylar film (Appendices C and F). Short (7 inch) and long (5 foot) sections of film were covered with a wide variety of clear coatings. The following coatings were used:

- Duro-plastic E·pox·E glue, 2 component system

Woodhill Chemical Sales Corp., Cleveland, Ohio 44128
Hanna-Tile epoxy coating, 2 component system
   Hanna Chemical Coatings Corp., Columbus, Ohio 43211.
4 in 1 Alkyd Epoxy Clear Finish. Paint Products Division of U.S. Refining Co., Cleveland, Ohio 44114.
Sherwin Williams Acrylyd Acrylic Enamel, 2 component system;
   The Sherwin-Williams Co., Cleveland, Ohio 44101.
Sears Clear Acrylic 30 65228. Sears, Roebuck and Co.,
   Chicago, Ill.
Tuffilm Spray. No. 543. M. Grumbacher, Inc.
   New York, N.Y. 10001.
Prestofix Workable Fixative. Presto Products Corp.
   Chicago, Ill.
2 mil clear adhesive mylar Cleer-Adheer. Distributed by
   Dayton Plastics Inc., Dayton, Ohio.
Durafame polyurethane liquid plastic clear high gloss
Hanna spraying lacquer. Hanna Chemical Coatings Corp.
   Columbus, Ohio 43211.
Spectrum Spray Paint. SS-160 Clear Acrylic. Division of
   Yenkin Majestic Paint Corp., Columbus, Ohio 43219.

The films were applied in one of three ways:
   Spray can (aerosol) (Plate XII, fig. a)
   Wren air brush (Plate XII, figs. b and c)
   Dipping into solution and hanging to dry (Plate XII, fig. d)
OBSERVATIONS

The most effective results were obtained from 2 component systems and from dipping or air brushing. Dipping is more convenient and yields a smoother, more optically satisfactory surface, but air brushing allows more control.

**Sherwin Williams Clear Acrylyd Acrylic Enamel Catalyzed With Polasol**

This is a crystal clear coating which dries to a hard, flexible film with good optical properties. It can be sprayed, dipped, or poured onto the film surface if the film is first stretched tightly in a horizontal position or caused to adhere to a flat horizontal surface (such as the sink top).

**Hanna-Tile Epoxy Coating**

This is an epoxy coating which covers well and dries hard and clear. If diluted, it can be poured on. It will spread, if properly diluted, to a uniform thickness with a smooth surface. Colors are vivid and well preserved. This material works especially well with a 5 mil mylar cover slip -- forms an extremely tough, protective cover. Hanna-tile compares favorably with the acrylic two component system previously described.

**Duro-plastic E:pox·E Clear Epoxy Glue**

This forms a viscous coat which must be placed on the mylar-section surface by spreading. Because of its thick consistency, the glue has a tendency to disturb section relations and does not spread easily or uniformly. Colors appear enhanced due to a slight yellowish-amber
tint. Covered strips may be placed in the oven to speed up hardening but care must be taken to avoid placing strips in a paraffin oven as paraffin fumes cause clouding of the epoxy. This material is well suited to production of glass slides with an epoxy cover slip-embedding media combination.

Krylon Crystal Clear Acrylic Lacquer
Krylon has been used to preserve histologic sections since 1952-53. It is rather well established that colors do not fade, but the Krylon film becomes somewhat brittle. Since Krylon is soluble in xylene, it supposedly can be removed in the same way as standard mounting media, and the section can be restained or recovered. These past observations have been confirmed, but complete removal of Krylon from a section was found to be difficult.

In addition, two different ways of placing Krylon film over a specimen were developed. The previous report indicated that Krylon in standard aerosol cans was quite convenient and economical. However, thin films of Krylon tend to allow air to enter the specimen and, in addition, tend to preclude use of the high dry objective. Krylon was therefore purchased in bulk form and sprayed on (essentially dropped on) by using little air at low pressure (10 to 20 lbs) with a large fluid volume (Appendix C). This resulted in spattering a thick layer of the Krylon onto each specimen surface. The Krylon spread out uniformly over the surface before a surface film began to form and was set solidly enough to withstand handling within 45 minutes or an hour. To make a more effective transfer from xylene to Krylon, a xylene/Krylon bath (1/1) was introduced following xylene clearing and
prior to Krylon covering. This led to a new idea. The reel was transferred from the Krylon/xylene mixture to a pure Krylon mixture. The section strip was then removed and hung up to dry, allowing excess Krylon to drip onto a paper towel on the floor. This procedure produced a very smooth, uniformly thick coating of the proper thickness to allow use of all microscopic objectives.

Several other acrylic aerosol sprays which resembled Krylon in appearance and composition were also tried:

**Sears Clear Acrylic Quick Drying Coating 30 65228**

This material coated as well as Krylon and appeared quite satisfactory while moist. As drying progressed, the sections were left exposed and turned white. Repeated spraying helped somewhat, but it was extremely difficult to cover the sections satisfactorily. An apparent lack of nonvolatile body makes it necessary to spray frequently, thus covering sections takes too much time to be practical. In addition, the coating is not protective and has a tendency to allow air to enter and cause areas of sections to turn black.

**Spectrum Clear Acrylic Spray Paint**

This is a very economical cover. However, it dried quickly and almost immediately turned white over the section areas, leaving the sections essentially uncovered. This spray is not appropriate for use as a cover for histological sections.

**4 in 1 Alkyd Epoxy Enamel**

This aerosol spray covers quickly and dries with a smooth surface.
It dries slowly (overnight) but in many instances this is not too serious a handicap. A yellowish tint tends to enhance colors. This may be a reasonable alternative to Krylon since it does not seem to become brittle as quickly as Krylon does.

**Durafame Polyurethane Liquid Plastic Clear High Gloss Coating**

This paint is not available in a spray can and must be sprayed on with an airbrush. It may have a slightly yellow cast but covers well, yielding a uniform, somewhat granular surface. It has possibilities.

**Hanna Spraying Lacquer**

Since Hanna Spraying Lacquer is not available in spray cans, it must be airbrushed. It is too thin to dip but applies easily with an airbrush and covers well with the low air pressure spatter technique previously described (Appendix C) for Krylon. However, it has a yellow cast and becomes brittle on drying; it cracks easily if bent and lifts away from the mylar film in sheets. It is not useful as a coating material.

**Mylar (1 or 2 mil) Adhesive Sheet — Cleer-Adheer**

It is possible to remove the mylar strip containing sections from xylene and to cover it immediately with a mylar film coated with clear adhesive (Cleer-Adheer) which prevents drying of the section. This has definite possibilities, but in practice large areas were adversely affected and dried partially, turning white, or were distorted by pressure applied in sealing the cover. In addition, unless
the mylar was coated with a light spray of acrylic or epoxy enamel, the high dry objective could not be used effectively.

A more effective procedure is to spray the section lightly with acrylic enamel (must be a 2 system catalyzed type as a solvent cannot escape once it is covered with mylar and hardening will not occur) and to cover the sprayed section immediately with adhesive mylar. It was still necessary to coat the mylar cover slip with a light spray of epoxy if the high dry objective was to be used.

**Grumbacher Tuffilm**

This aerosol spray yields a clear coating which does not crack and which can be adapted to use with a high dry objective. It spreads well over mylar and seems to remain flexible. It has possibilities but has not been tested sufficiently to draw any strong conclusions.

**Presto fix**

Presto fix does not readily adhere to mylar. It tends to form small spheres which roll off the strip or collect in lower areas. On drying, when the film is bent, the coating separates from the mylar surface. This material is not appropriate for histological application.
Plate XII. Preservation of Sections.

Fig. a. Some of the materials available for attaching and preserving sections.

Fig. b. Apparatus for coating short section strips.

Fig. c. Wren air brush, pressure regulator and water trap, and coated strips.

Fig. d. Method used to dry dipped section strips.
Appendix E

COMPUTER PROGRAMS FOR PROCESSING ANATOMICAL DATA
Program One - Version Two
Program Two - Version One

**SECTION COORDINATES**

1. Initialize sections.
2. Read section coordinates.
3. Store section coordinates in a list.
4. Calculate section area.
5. Establish original identification number.
6. Identify and check section numbers.
7. Identify sections on adjacent slides.
8. Establish original maxima and minima and calculate islet section area.
9. Calculate islet coordinates.
10. Establish original identification number.
11. Correlate section data.

**COORDINATION OF ISLET SECTIONS ON ADJACENT SLIDES**

1. Identify islet sections.
2. Establish section number.
3. Verify section number.
4. Identify sections on adjacent slides.

**SECTION AREA**

1. Calculate section area.
2. Establish original identification number.
3. Verify section area.
4. Identify sections on adjacent slides.

**ESTABLISHING ORIGINAL MAXIMA AND MINIMA AND CALCULATION OF ISLET SECTION AREA**

1. Establish original identification number.
2. Calculate section area.
3. Identify sections on adjacent slides.

**ISLET COORDINATES**

1. Calculate islet coordinates.
2. Establish original identification number.
3. Verify islet coordinates.
4. Identify sections on adjacent slides.

**SECTION NUMBER**

1. Calculate section number.
2. Verify section number.
3. Identify sections on adjacent slides.

**COORDINATE OF ISLET SECTIONS ON ADJACENT SLIDES**

1. Identify islet sections.
2. Establish section number.
3. Verify section number.
4. Identify sections on adjacent slides.

**SECTION DATA**

1. Calculate section data.
2. Verify section data.
3. Identify sections on adjacent slides.

**SECTION IDENTIFICATION NUMBER**

1. Establish section identification number.
2. Verify section identification number.
3. Identify sections on adjacent slides.

**ISLET SECTION DATA**

1. Calculate islet section data.
2. Verify islet section data.
3. Identify sections on adjacent slides.

**ISLET COORDINATES**

1. Calculate islet coordinates.
2. Establish original identification number.
3. Verify islet coordinates.
4. Identify sections on adjacent slides.
CONSTRUCTION OF ISLET ARRAY: ELIMINATION OF EXTRA SECTION NUMBERS

SETTING FIRST DATA SET EQUAL TO FIRST ORIGINAL DATA SET

CONSTRUCTION OF ISLET ARRAY FROM NUMBER 2ND ON

REDUCING K AND I VALUES TO CORRECT VALUE AFTER LAST INCREMENT

FINAL PRINTOUT OF ISLET DATA

EXTRACINE AREA, AND NUMBER OF ISLETS ON SECTION

FINAL PRINTOUT OF ISLET NUMBER, SECTION NUMBER, NUMBER OF SLIDES

TOTAL ISLET NUMBER PLUS SECTION AND LEVEL NUMBERS
C FORMAT STATEMENTS

0106 C

0107 1 FORMAT(11,F10.6,F11.0)      
0108 
0109 FORMAT(2X,F4.0)         
0110 
0111 FORMAT(2X,F4.0)         
0112 
0113 FORMAT(2X)              
0114 
0115 FORMAT(2X,F4.0)         
0116 
0117 FORMAT(2X)              
0118 
0119 FORMAT(2X,F4.0)         
0120 
0121 FORMAT(2X)              
0122 
0123 FORMAT(2X,F4.0)         
0124 
0125 FORMAT(2X)              
0126 
0127 FORMAT(2X,F4.0)         
0128 
0129 FORMAT(2X)              
0130 
0131 FORMAT(2X,F4.0)         
0132 
0133 FORMAT(2X)              
0134 
0135 FORMAT(2X,F4.0)         
0136 
0137 FORMAT(2X)              
0138 
0139 FORMAT(2X,F4.0)         
0140 
0141 FORMAT(2X)              
0142 
0143 FORMAT(2X,F4.0)         
0144 
0145 FORMAT(2X)              
0146 
0147 FORMAT(2X,F4.0)         
0148 
0149 FORMAT(2X)              
0150 
0151 FORMAT(2X,F4.0)         
0152 
0153 FORMAT(2X)              
0154 
0155 FORMAT(2X,F4.0)         
0156 
0157 FORMAT(2X)              
0158 
0159 FORMAT(2X,F4.0)         
0160 
0161 FORMAT(2X)              
0162 
0163 FORMAT(2X,F4.0)         
0164 
0165 FORMAT(2X)              
0166 
0167 FORMAT(2X,F4.0)         
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0169 FORMAT(2X)              
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0171 FORMAT(2X,F4.0)         
0172 
0173 FORMAT(2X)              
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0175 FORMAT(2X,F4.0)         
0176 
0177 FORMAT(2X)              
0178 
0179 FORMAT(2X,F4.0)         
0180 
0181 FORMAT(2X)              
0182 
0183 FORMAT(2X,F4.0)         
0184 
0185 FORMAT(2X)              
0186 
0187 FORMAT(2X,F4.0)         
0188 
0189 FORMAT(2X)              
0190 
0191 FORMA1(2X,F4.0)         
0192 
0193 FORMAT(2X)              
0194 
0195 FORMAT(2X,F4.0)         
0196 
0197 FORMAT(2X)              
0198 
0199 FORMAT(2X,F4.0)         
0200 
0201 FORMAT(2X)              
0202 
0203 FORMAT(2X,F4.0)         
0204 
0205 FORMAT(2X)              
0206 
0207 2 FORMAT(2X,F4.0)         
0208 
0209 FORMAT(2X)              
0210 
0211 FORMAT(2X,F4.0)         
0212 
0213 FORMAT(2X)              
0214 
0215 FORMAT(2X,F4.0)         
0216 
0217 FORMAT(2X)              
0218 
0219 FORMAT(2X,F4.0)         
0220 
0221 FORMAT(2X)              
0222 
0223 FORMAT(2X,F4.0)         
0224 
0225 FORMAT(11,F10.6,F11.0)      
0226 
0227 FORMAT(11,F10.6,F11.0)      
0228 
0229 FORMAT(11,F10.6,F11.0)      
0230 
0231 FORMAT(11,F10.6,F11.0)      
0232 
0233 FORMAT(11,F10.6,F11.0)      
0234 
0235 FORMAT(11,F10.6,F11.0)      
0236 
0237 FORMAT(11,F10.6,F11.0)      
0238 
0239 FORMAT(11,F10.6,F11.0)      
0240 
0241 FORMAT(11,F10.6,F11.0)      
0242 
0243 FORMAT(11,F10.6,F11.0)      
0244 
0245 FORMA1(11,F10.6,F11.0)      
0246 END
Program Two — Version Two

For transition, the text is not fully legible due to the image quality. However, it appears to be a FORTRAN program with comments and code for handling sections on adjacent slides.
DATA FOR ISLET SECTION 1J

CONTINUE

END CHECK

CONSTRUCTION OF ISLET ARRAY—ELIMINATION OF EXTRA SECTION NUMBERS

CONSTRUCTION OF K ISLET ARRAY FROM NUMBER TWO ON

REDUCING K AND 1 VALUES TO CORRECT VALUE AFTER LAST INCREMENT

FINAL PRINTOUT OF ISLET DATA

FINAL PRINTOUT OF ISLET NUMBER, SECTION NUMBER, NUMBER OF SLIDES

TOTAL ISLET NUMBER PLUS SECTION AND LEVEL NUMBERS
APPENDIX F

STAINING PROCEDURES
STAINING PROCEDURES

GENERAL PROCEDURES

Fixation: 10% buffered formalin, Bouin's fluid, Zenker formol and
Karnovsky's fluid were used. Most materials were fixed overnight or
for 24 hours in 10% buffered formalin.

Embedding: Fixed tissues were embedded in celloidin or double em­
bedded in celloidin-paraffin.

Sectioning: Sections were cut at 2 to 40µ on a tape support micro­
tome (Appendix B) or on an unmodified Reichert sliding block microtome.

Mounting: Sections cut on a standard sliding microtome are mounted
on 1x3 or 2x3 inch glass slides. It is necessary to cut a large
3 inch section in half to mount it on 1x3 inch slides. Each half must
be mounted on a separate slide. To mount 6 inch sections (chicken or
duck pancreas) on standard slides, they must be cut into thirds. To
avoid cutting, which disrupts morphological relations, we have, for
the most part, mounted the sections intact on strips of mylar film
with Mayer's albumin. For a more detailed description of this process,
see Appendix C.

Removing acetate tape support: It is possible to stain mylar strip
sections without first removing the acetate, but staining may not be
uniform and may even be absent in some areas. It is quite easy to
remove the acetate by the following procedure:
Place strip in absolute alcohol to soften or dissolve the adhesive.
Strip away acetate ribbon. Place in xylol to remove paraffin. Place again in absolute alcohol and ether if celloidin is to be removed.
Rinse in 95% alcohol and transfer to tap or distilled water and then go into the staining sequence.

An alternate method is to thread the mylar section onto a 35mm film developing reel and place it directly into a solution consisting of equal parts of acetone/xylol/absolute alcohol/ether for several hours.

Staining: Staining was carried out in standard stainless steel film developing tanks. Various size tanks are available. Small tanks contain one mylar strip which holds 18 tissue sections 3 inches long. Large tanks hold as many as five stainless steel film developing reels (94 3-inch tissue sections). The larger tanks were only half filled with staining solution and were placed on a Kindermann agitator which continuously rotated the tank while tilting one end up and down.
Celloidin sections stained rapidly, and staining solutions designed for paraffin sections had to be diluted or the staining time had to be cut. Celloidin-paraffin double embedded sections could be stained (except for aldehyde fuchsin) according to standard paraffin staining procedures.

The following stains were used: Gomori's chrome-alum hematoxylin phloxin, Bencosme's chrome-alum hematoxylin fuchsin, Gomori's aldehyde fuchsin trichrome, Lazarus and Volk's periodic acid Schiff trichrome stain, and Thomas's Heidenhain azan stain (Heidenhain's modification of Mallory's aniline blue stain).
Preservation: Those sections mounted on mylar are preserved with films of clear acrylic coatings such as the Borden Company's Krylon Crystal Clear or Sherwin Williams two component system, Acrylon. Epoxy coatings have also been used including Alkyd epoxy enamel made by the U. S. Refining Company, Hanna's epoxy enamel, and the epoxy lacquer and two component epoxy system of the Plasti-Glast Corporation. For more details, see Appendix D.
SPECIFIC STAIN PROCEDURES

Bencosme's Chrome-Alum Hematoxylin Ponceau-Fuchsin

1. Remove acetate tape support (see general procedures section).

2. Refix in Bouin's fluid overnight or for 24 hours. We have found that this additional fixation does in many instances improve staining.

3. Wash with running tap water for 30 minutes to remove the picric acid. This can be done conveniently by a vertical glass tube placed in the center of the reels.

4. Treat one minute in potassium permanganate solution. For thicker sections, treat longer. Prepare no more than 1 hour ahead. (Mix equal parts of aqueous 0.6% potassium permanganate and 0.6% sulfuric acid). If solution becomes clear on standing, discard.

5. Rinse in distilled water. If it is important to be economical, remove the reel from its tank prior to each rinsing. This allows for more complete removal of solution adhering between reel and tank and prevents contamination of staining solutions.

6. Pour 4% aqueous sodium bisulfite into tank and leave for one minute or until color is completely removed.

7. Rinse with running tap water.

8. Pour chrome-alum hematoxylin stain into tank. If Kindermann agitator is used, fill the tank half full. The stain is prepared by mixing equal volumes of 1% aqueous hematoxylin and 3% aqueous chrome alum (chromium potassium sulfate). To each 100 cc of solution, add 0.1 gm of potassium iodate and boil until a deep blue. Ripe immediately. Filter before use. Can be used
until metallic film fails to form on surface or until staining time is too long.

9. Differentiate with 1% hydrochloric acid in 95% alcohol for 1 minute.

10. Rinse with running tap water until section is clear blue.

11. Stain with ponceau-fuchsin for 2 hours. Prepare staining mixture by adding 4 parts 1% ponceau to 1 part 1% acid fuchsin and 1 part 1% fuchsin S. Dilute 1:10 in 1% acetic acid.

12. Rinse with 1% acetic acid.

13. Differentiate with 1% phosphomolybdic acid until light and dark islets are clearly visible.

14. Pour in 1% acetic acid. Leave 5 to 10 minutes.

15. Dehydrate and clear.

16. Place in Krylon until diffusion stops and hang strip to dry.

Gomori's Chrome-Alum Hematoxylin Phloxine

1-10. Same as preceding.

11. Counterstain with 0.5% aqueous phloxine B for 5 minutes.

12. Rinse with distilled water.

13. Fill tank with 5% aqueous phosphotungstic acid solution for 1 minute.

14. Wash with running tap water for 5 minutes until section regains red color.

15. Differentiate in 95% alcohol. If light and dark islets do not stand out clearly, rinse briefly in 70 or 80% alcohol.

17. Clear in xylol.

18. Place in Krylon and hang to dry.

This version is rapid and gives a good differentiation of light and dark islets.

Gomori's Aldehyde Fuchsin Trichrome (Warren)

1. Remove acetate tape support (see General Procedures section).

2. Stain with aldehyde fuchsin for 30 minutes. Prepare by adding 3cc of paraldehyde and 1cc of concentrated hydrochloric acid to each 200cc of a 0.3% solution of basic fuchsin in 60 or 70% alcohol. Ripen at least 3 days. Store in a tightly sealed screw top plastic container in refrigerator. Use paraldehyde from a freshly opened 2, 5, or 10cc ampul.

3. Remove film reel from tank, pour all excess stain from tank, rinse tank with tap water, reinsert film reel and rinse with 95% alcohol until all excess aldehyde fuchsin is removed from sections.

4. Wash with running tap water. Check to see that light islets are stained.

5. Stain with a trichrome mixture until light and dark islet areas are clearly defined (15-20 minutes). Prepare mixture by adding 0.6gm Chromotrope 2R, 0.3gm light green, 0.8gm phosphotungstic acid and 1.0cc glacial acetic acid to each 100cc of distilled water.

6. Cover with 0.5% aqueous acetic acid for 2 minutes.

7. Differentiate, if necessary, with a solution prepared by adding 0.7gm phosphotungstic acid to each 100cc of 1.0% aqueous acetic
8. Rinse with distilled water.
9. Dehydrate with 95% and absolute alcohol.
10. Clear with xylol.
11. Infiltrate with Krylon until all evidence of diffusion disappears or overnight. Remove film from reel and hang film to dry. A more uniform surface can be formed by periodically reversing the end the tape is suspended from until enough drying has occurred to form a surface film which will not flow.

Results: Light islet granules purple, dark islets red.

Lazarus' Combined Periodic Acid Schiff Trichrome

1. Remove acetate tape support (see General Procedures section) and wash in running tap water for 10 minutes or longer.
2. Oxidize in 0.6% aqueous periodic acid for 20-30 minutes.
3. Stain with Schiff reagent for 5-20 minutes. Prepare by adding 1.0gm basic fuchsina and 1.9gm sodium metabisulfite to 100ml 0.15 N hydrochloric acid. Shake at intervals for 2 hours. Add 500mg Norite and shake for 2 minutes. Filter twice and store in refrigerator (Warren et al., 1966).
4. Rinse with 0.5% aqueous sodium metabisulfite.
5. Wash with running tap water 10 minutes.
6. Rinse with distilled water.
7. Stain with Weigert's acid iron chloride hematoxylin for 10 minutes. Prepare by adding 1 part of 1% hematoxylin in 95% alcohol to 1 part of a mixture made by adding 4cc aqueous 29% ferric chloride
and 1cc concentrated hydrochloric acid (sp. gr. 1.18) to each 95cc of distilled water. The 29% aqueous ferric chloride is prepared by adding 48gm of FeCl$_3$.6H$_2$O to each 100cc of distilled water (Lazarus and Volk, 1962).

8. Rinse with 95% alcohol.

9. Differentiate with 1% hydrochloric acid in 95% alcohol.

10. Wash in running tap water for 10 minutes.

11. Stain with 0.2% ponceau, 0.1% orange G, in 1% acetic acid for 2 hours.

12. Rinse with distilled water.

13. Differentiate with 1% phosphomolybdic acid.

14. Rinse with distilled water.

15. Stain with 1% light green in 1% acetic acid for 5-30 minutes.

16. Rinse in 1% acetic acid.

17. Differentiate in 0.5% phosphomolybdic acid for 1-15 minutes.

18. Cover with 1% acetic acid for 20 minutes.

19. Dehydrate with absolute alcohol and clear in xylol.

20. Infiltrate with Krylon or spray on Plasti-Glast epoxy coating. Hang to dry.

Heidenhain's Modification of Mallory's Aniline Blue
(Heidenhain Azan) (Thomas, 1937; Bloom, 1931; Warren, 1966)

1. Remove acetate tape support (General Procedure section).

2. Pour warm azo carmine into a stainless steel tank or a 400cc pyrex beaker and drop reels containing mylar section strips into the solution. Place in 55°C oven for 1 hour. The 0.2% azo
carmine solution is made from boiled distilled water which has been cooled and filtered. The water is then acidified with enough glacial acetic acid to produce a 1% acetic acid solution.

3. Rinse with distilled water.

4. Differentiate with 0.1% aniline oil in 95% alcohol until dark islets stand out as distinct red areas.

5. Control differentiation with 1% acetic acid in 95% alcohol.

6. Rinse with distilled water.

7. Pour in 5% phosphotungstic acid and leave for 2 hours.

8. Rinse with distilled water.

9. Counterstain for 15 minutes to 24 hours. The solution contains 2.0gm Orange G and 0.5gm Aniline blue in each 100.0cc of distilled water. The 0.5gm of Aniline blue may be replaced by 8.0cc Methyl blue. Dilute with distilled water 1/1 for celloidin sections.

10. Rinse with distilled water.

11. Dehydrate, clear, and coat with Krylon, Acrylyd, or epoxy.
LITERATURE CITED


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