Isolated Pancreatic Islets of the Rat: An Immunohistochemical and Morphometric Study

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ABSTRACT Although there is a recent increase in the use of the isolated pancreatic islets of the rat in the transplantation and functional studies, there has been no detailed quantitative assessment on the size and cellular constituents of islets after the isolation procedure. The present work was undertaken to study the size classes of the isolated islets and the morphometry of their cellular populations.

Islets of the rat pancreas were isolated by using the intraductal collagenase digestion technique, the most commonly used procedure for the isolation of pancreatic islets. Different endocrine cells of the isolated islets were stained by immuno-peroxidase staining techniques. The distribution of the cellular constituents of the isolated islets was similar to that of the intact islets of the normal pancreas; A, D, and PP cells were peripherally arranged around the centrally located B cells. However, morphometric quantitative study showed that the percent volume and percent number of A, D, and PP cells of the isolated islets were lower than those of the corresponding intact ones. Further, the mean true diameter of the isolated islets was lower than that of the intact ones. These data indicate loss of islet cells during the process of isolation. Most of the lost cells were from the periphery of islets. This may provide an explanation for the incomplete metabolic control and recurrence of hyperglycemia encountered after isolated islet transplantation in the treatment of diabetes mellitus. It seems that further refinements of the isolation techniques are necessary to obtain islet tissue with total cellular integrity, before a complete success in transplantation could be achieved.

Key words: Immunohistochemistry, Isolated islets, Morphometry, Pancreas

Moskalewski (1965) was the first to isolate islets from the pancreas of the guinea pig using an enzymatic digestion technique. Lacy and Kostianovsky (1967) modified the technique and used the density gradients to purify isolated rat islets. Further improvement of the technique was made by Sutton et al. (1986) who used the method of intraductal injection of collagenase. This technique facilitated harvesting large number of islets for use in transplantation procedures to treat diabetes (Sutherland et al., 1980; McEvoy and Leung, 1983; Toledo-Pereyra et al., 1984; Alejandro et al., 1986; Dibelius et al., 1986; Hesse et al., 1986; Lake et al., 1988; Tze and Tai, 1988; Huxlin et al., 1990). The isolated islets were also used to evaluate their ability to secrete insulin in vitro in response to glucose load (Lacy et al., 1968; Slavin et al., 1977; Halban et al., 1986; Eizirik et al., 1989; Grodsky, 1989).

The process of isolation inevitably leads to mechanical and enzymatic trauma to the islets themselves (Moskalewski, 1965; Vance et al., 1968; Buchanan and Mawhinney, 1975; Slavin et al., 1977) especially to their peripheral part, which is rich in A, D, and pancreatic polypeptide (PP) cells. The central core of islets, which is formed of insulin-producing B cells, was kept almost intact. These changes in the peripheral part of isolated islets presumably affect the hormonal secretory function of B cells. There is growing evidence that glucose-induced insulin release is dependent not only on the integrity and number of the insulin-containing B cells, but also on their interactions with the neighboring B and non-B cells (Pipeleers et al., 1982). In these studies, however, the effect of the isolation procedure on the different cellular populations and the
work, were fixed in buffered neutral formalin and embedded in paraffin with 57°C melting point. Five micron serial sections were cut from each specimen, and groups containing 6–8 sections were selected at 70 μm intervals from each other. Sections from each group were stained immunohistochemically for insulin, glucagon, somatostatin and PP-containing cells. Paraffin blocks were also prepared from different areas of the pancreas of 6 rats, matched for age, sex, and weight to those of the donor rats used for isolation of the islets. Sections were cut and stained in a similar way to that used for the isolated islets, and were used for comparison.

To localize the insulin producing B cells, the indirect immunohistochemical technique was used (Sternerberger, 1979). The primary antibody used was guinea pig anti-swine insulin serum (lot 030), diluted in phosphate-buffered saline (PBS) with 1% normal rabbit serum. Different dilutions were used to obtain the optimal one; this was found to be 1:500 which gave the best staining with the least background and was used for staining the sections thereafter. The secondary antibody used was rabbit anti-guinea pig immunoglobulin conjugated with peroxidase (dilution 1:200).

The avidin-biotin complex (ABC) technique was used to localize glucagon, somatostatin and PP-producing cells as described previously (Hsu et al., 1981). The primary antibodies used were rabbit anti-porcine glucagon serum (lot 011), rabbit anti-human somatostatin serum (lot 050d), rabbit anti-human PP serum (lot 109e). They were tried in dilutions ranging from 1:10,000 to 1:100 in PBS with 1% normal swine serum. It was found that the optimal dilutions of the anti-glucagon, anti-somatostatin, and anti-PP sera were 1:500, 1:500, and 1:5,000, respectively. These dilutions were used for all the staining procedures thereafter. The secondary antibody used was biotinylated swine anti-rabbit immunoglobulin (dilution 1:200). Sera and antisera were obtained from Dako Corporation (Carpenteria, CA). Sections were incubated in the primary antibodies for overnight in a humidity chamber, at 4°C. The chromogen substrate used was 3,3-Diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) and the sections were counterstained with Harris’ hematoxylin to facilitate the nuclear identification.

Specificity control for the immunohistochemical staining included omitting the antibody sera, which were replaced by non-immune sera, and absorption of the primary antisera with the purified antigens.

Morphometric Analysis

Morphometric study was carried out on the immunohistochemically stained sections. Twelve to twenty islet profiles were chosen at random from each slide, with a total of 130–180 from each specimen. They were morphometrically analyzed at a magnification of 1,000 ×. Sections of intact islets, taken from different parts of 6 normal pancreases, were similarly analyzed. Standard morphometric methods were used to estimate the volume density (Vv) and the percent number (Nn) for each cell type (Weibel, 1963) and to calculate the mean true diameter (D) of the isolated and the intact islets (Williams, 1977).

The volume densities (Vv) of B, A, D, and PP cells per islet tissue were calculated by the point counting method of Weibel (1963). With a calibrated grid inserted into the ocular lens of the microscope, the total number of the intersections over the cytoplasm and the nucleus of the specified cell type (Pc) as well as over the whole islet (Pi) were determined. The percent ratio of the number of intersections of the specific cell type per total islet intersections Ppc = (Pc/Pi) × 100 was expressed as the volume density of this cell type for the islet quantitated (Vpc = Ppc).

The numerical percent of each specific cell type (Nnc) per total islet cells was calculated using the nucleus as the counting base. The nuclei of the stained and unstained cells per islet profile were counted. The ratio of specific cell nuclei to the total islet nuclei, was expressed as the percent number of that specific cell per islet cells Nnc = (Nc/Ni) × 100. Calculations for B, A, D, and PP cells were done in adjacent or semi-adjacent sections stained specifically for these types of cells.

The profile diameters (D) of the islets were calculated from the equation D = \( \frac{N d_1 + d_2 + \ldots + d_n}{N} \), where N represents the total profiles measured and d1, d2, . . . , dN represent the profile diameters.

The results of the morphometric study were presented as the arithmetic mean ± standard error of mean. Student’s t-test for non-paired observations was used for statistical evaluation of the data.

RESULTS

The freshly prepared isolated islets are recognized under the dissecting microscope by their characteristic well-defined, rounded or oval shape, showing high opacity and milky white color. They are also recognized under the phase-contrast microscope, as well-defined rounded or oval bodies with a faint green tinge, in contrast to the acinar tissue which appeared irregular in shape and showed a lighter tinge. The series of experiments which were performed to obtain the optimal conditions for isolation of the islets, showed that the highest yield of islets, (342.7 ± 15.8 islets/rat pancreas) was obtained by the use of collagenase at concentration of 2 mg/ml (Table 1) and incubation time of 22 min (Fig. 1). Poor yields of isolated islets were obtained with incubation periods less or greater than 22 min and with collagenase concentration of 1 or 3 mg/ml. Higher concentrations of insulin (318.45 ± 11.3 mU/g protein) and C-peptide (176.43 ± 16.44 pmol/g protein) were also obtained from samples of islets isolated with collagenase at concentration of 2 mg/ml and incubation for 22 min (Fig. 2); the latter were selected at the optimal digestion conditions. The purity of the isolated islet preparations was 20–40% after separation with the discontinuous Ficoll gradient. Most of the impuri-
TABLE 2. The concentration of adenine nucleotides in the isolated pancreatic islets compared with that of the freeze-clamped intact pancreatic tissue¹

<table>
<thead>
<tr>
<th>Sample</th>
<th>Incubation time (min)</th>
<th>ATP (nmol/mg of protein)</th>
<th>ADP (nmol/mg of protein)</th>
<th>AMP (nmol/mg of protein)</th>
<th>Total adenine nucleotides (nmol/mg of protein)</th>
<th>ATP/AMP ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated islets</td>
<td>0</td>
<td>1.34 ± 0.07</td>
<td>0.331 ± 0.064</td>
<td>0.289 ± 0.043</td>
<td>1.903 ± 0.231</td>
<td>4.74</td>
</tr>
<tr>
<td>Isolated islets</td>
<td>30</td>
<td>1.34 ± 0.09</td>
<td>0.315 ± 0.029</td>
<td>0.341 ± 0.037</td>
<td>1.986 ± 0.099</td>
<td>3.95</td>
</tr>
<tr>
<td>Isolated islets</td>
<td>60</td>
<td>1.28 ± 0.19</td>
<td>0.379 ± 0.027</td>
<td>0.422 ± 0.040</td>
<td>2.083 ± 0.244</td>
<td>3.04</td>
</tr>
<tr>
<td>Freeze-clamped</td>
<td>0</td>
<td>1.67 ± 0.25</td>
<td>0.39 ± 0.17</td>
<td>0.232 ± 0.057</td>
<td>2.30 ± 0.32</td>
<td>7.18</td>
</tr>
</tbody>
</table>

¹Values are presented as means ± SEM, for eight specimens of isolated islets and six specimens of freeze-clamped intact pancreatic tissue.

Fig. 3. Insulin release from islets isolated with 2 mg/ml collagenase and 22 min of incubation, in response to a challenge twice with 15 and 30 mmol/liter glucose, then a final challenge with 15 mmol/liter glucose together with 15 mmol/liter theophylline to elicit a maximal response.

The morphometric study showed that the cellular populations of the isolated islets were quantitatively different from those of the intact islets. The results of the morphometric study are summarized in Table 3. Whereas the volume density and the percent number of the B cells of the isolated islets were significantly higher than those of the intact islets, the volume densities and the percent numbers of the peripherally arranged A, D, and PP cells of the isolated islets were significantly lower than those of the intact islets (Fig. 12).

The mean profile diameter of the isolated islets was significantly lower ($P < 0.001$) than that of the intact islets (Table 3). The profile sections of the islets showed a mean axial ratio of 1.468 in the isolated islets and of 1.345 in the intact islets, indicating that the islets could be treated as spheroids. The size-frequency distribution of the profile diameters of the isolated islets showed an overall shifting of all the size classes toward smaller sizes as compared to the corresponding classes of the intact islets of the normal pancreas (Fig. 13). This indicates loss of islet mass during the isolation procedure which affected all sizes of the islets. The cumulative frequency distribution of the profile diameters of the isolated islets showed that their population contain a range of sizes, justifying the use of the formula of Pullman (Williams, 1977) to calculate the mean true diameters (D) for the isolated and the intact pancreatic islets, which were found to be 104.547 and 196.319 $\mu$m, respectively.

DISCUSSION

Isolation of the islets has become a commonly used procedure to obtain a relatively pure endocrine tissue for transplantation experiments or for functional metabolic studies. In the present work, the islets were isolated from the pancreas of the rat by the intraductal collagenase digestion technique of Sutton et al. (1986). This was selected since it is the most commonly used technique for the isolation of pancreatic islets. Our preparations of the isolated islets proved to be functionally intact and viable. This was assessed by measuring the adenine nucleotide concentrations in the isolated islets, which were not significantly different from those of the intact islets of the freeze-clamped pancreatic tissue. In addition, isolated islets which were incubated in vitro for 30 to 60 min maintained their adenine nucleotide concentrations. Repeated challenge of the isolated incubated islets with 15 and 30 mmol/liter glucose resulted in highly reproducible responses of insulin release which were maintained for up to 5 h. Maximal response was elicited by theophylline, a standard test for measuring the functional integrity of the islets (Lacy et al., 1972; Malaisse, 1973; Henquin and Meissner, 1984).

Paraffin sections stained with immunohistochemical techniques showed that the distribution of B, A, D, and PP cells in the isolated islets is not different from that of the intact islets; where A, D, and PP cells are located at the periphery of the central mass of B cells. Similar immunohistochemical localization of B cells of the isolated islets was observed by Groth et al. (1980), Gray et al. (1984), Warnock and Rajotte (1988), Delaby et al. (1989), Kneteman et al. (1989), and Warnock et al. (1989). There has been, to date, no detailed immunohistochemical study showing critical evaluation of other endocrine non-B cells of the isolated islets. The few isolated islets which were detected in the present work containing a relatively large number of PP cells were most probably derived from the lower part of the
Fig. 8. An isolated islet stained for somatostatin. Positively stained D cells are scattered at the periphery of the islet. The section shows a contaminating pancreatic acinus. Immunoperoxidase stain for somatostatin. × 650.

Fig. 9. An intact islet showing D cells with positive reaction (brown staining) to the anti-somatostatin serum. The cells are scattered at the peripheral part of the islet. Immunoperoxidase stain for somatostatin. × 650.

Fig. 10. Two isolated islets stained for PP cells. One islet appears to contain a good number of PP cells, while the other does not. Immunoperoxidase stain for PP. × 650.

Fig. 11. Intact islet from the splenic part of normal rat pancreas. PP-secreting cells appear positively stained with anti-PP serum. They are few in number and scattered at the peripheral part of the islet. Immunoperoxidase stain for PP. × 650.

 Principally the peripheral parts of the islets. This finding is supported by the presence of irregular peripheral region which could be detected in most of the isolated islets. Injury to the peripheral cells of the isolated islets was also reported by Moskalowski (1965), Vance et al. (1968), Buchanan and Mawhinney (1973), and Slavin et al. (1977). Their observations were made, however, without the support of morphometric quantitative data. Our findings agree also with the observations of Trimble et al. (1980), which described fewer A cells in the intraperitoneally transplanted islets as compared to the islets within the normal pancreas. Vance et al. (1968), and Buchanan and Mawhinney (1973) found that the glucagon which is released from the isolated islets of the rat was more variable than insulin. The variable release of glucagon in the isolated islets was attributed to damage of the A cells, which are situated more peripherally than the B cells. Slavin et al. (1977) attributed the injury of the peripheral parts of the islets to the metabolic changes occurring in the islet cells during the isolation procedure. However, this injury could also be due to enzymatic digestion of the peripheral parts of the islets by the collagenase enzyme and mechanical trauma during the process of isolation.

Whether the changes in the normal percentage of the endocrine cells of the peripheral parts of the isolated islets can lead to changes in the hormonal secretory function of the B cells, is not yet known. However, there are various reports indicating the presence of functional cooperation between various endocrine cells
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LITERATURE CITED


of the islet tissue, which plays an important role in regulating the release of insulin in response to glucose. Communication of the cells of the pancreatic islets occurs through gap junctions. These junctions were reported to occur not only between B and B cells and between A and A cells, but also between A, D, PP cells, and adjacent B cells (Orci et al., 1973, 1975; Unger and Orci, 1981; Meda et al., 1983). Pipeleers et al. (1982) found that B cells in the isolated islets of the rat released 30 times more insulin in response to glucose as compared with that released from purified single B cells.

In conclusion, the results of the present work show that the islets isolated by the enzymatic digestion technique have suffered considerable loss of their volume during the isolation procedure. Most of the lost cells were from the peripherally arranged A, D, and PP cells, which led to quantitative changes of the cellular populations of the islets. This might offer an explanation for the incomplete metabolic control which has been a consistent finding in the short-term studies of pancreatic islet transplantation, and the recurrence of hyperglycemia in long-term studies (Steffes et al., 1979; Trimble et al., 1980; Orloff et al., 1987). Evidently, to control diabetes mellitus by transplantation techniques, isolated islet tissue with total cellular integrity seems to be essential. It, therefore, appears that further refinements of the isolation techniques are necessary before a complete success could be achieved.

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### Table 3. The endocrine cell types of the isolated islets compared with those of the intact islets

<table>
<thead>
<tr>
<th>Volume density (percent volume)</th>
<th>Isolated islets</th>
<th>Intact islets</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cells</td>
<td>92.74 ± 0.67 (240)</td>
<td>66.04 ± 0.39 (337)</td>
<td><em>P &lt; 0.001</em></td>
</tr>
<tr>
<td>A cells</td>
<td>4.46 ± 0.52 (331)</td>
<td>19.38 ± 0.71 (232)</td>
<td><em>P &lt; 0.001</em></td>
</tr>
<tr>
<td>D cells</td>
<td>2.18 ± 0.4 (205)</td>
<td>6.09 ± 0.21 (175)</td>
<td><em>P &lt; 0.001</em></td>
</tr>
<tr>
<td>PP cells</td>
<td>0.62 ± 0.18 (331)</td>
<td>8.49 ± 0.49 (292)</td>
<td><em>P &lt; 0.001</em></td>
</tr>
</tbody>
</table>

Percent No.

| B cells                         | 90.75 ± 0.95 (240) | 70.32 ± 0.25 (337) | *P < 0.001* |
| A cells                         | 5.82 ± 0.68 (331) | 18.77 ± 0.71 (232) | *P < 0.001* |
| D cells                         | 2.45 ± 0.42 (205) | 4.23 ± 0.13 (175) | *P < 0.001* |
| PP cells                        | 0.98 ± 0.23 (331) | 6.68 ± 0.58 (292) | *P < 0.001* |

Profile diameter of the islets (μm)

| Isolated islets | 75.41 ± 1.53 (1107) | 133.16 ± 1.37 (1036) | *P < 0.001* |

Data are presented as means ± SEM. Values between brackets represent the No. of the islets examined.

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**Fig. 12.** The volume density (represented as percent volume) and the percent number of B, A, D, and PP cells of islets isolated with 2 mg/ml collagenase and 22 min incubation time, compared with those of the intact islets.

**Fig. 13.** The size frequency distribution of the mean profile diameter of the isolated islets compared with those of the intact islets.
juxtapancreal pancreas, the latter was reported to be rich in PP cells (see Baetens et al., 1979).

The results of the morphometric study indicated that the cellular populations of the isolated islets are quantitatively different from those of the intact islets. The B cells formed 92.74% of the volume and 90.78% of the number of cells in the isolated islets. These were significantly higher than the percent volume and number of the B cells of the intact islets, which were 66.04 and 70.32, respectively. On the contrary, the peripherally arranged A, D, and PP cellular populations of the isolated islets showed significantly lower percent volumes and numbers than those of the intact islets. Our quantitative data of the cellular populations of the intact pancreatic islets are in agreement with those described previously. According to Baetens et al. (1979), B, A, D, and PP cells comprised 82.5, 15, 2, and 0.5% of the volume of the intact dorsal pancreatic islets, whereas, in the ventral pancreas, their percent volumes were 82, 1.3, 2.4, and 14.3, respectively. Hellman (1959), Carpenter and Lazarow (1967), and Dean (1973) have shown that the centrally placed B cells comprise 65–85% of the total volume of the intact pancreatic islets. However, we could not locate in the available literature any quantitative study on the cellular populations of the isolated islets.

The quantitative changes in the cellular populations of the isolated islets were concomitantly accompanied by a dramatic loss of the islet mass. Whereas the mean true diameter of the isolated islets was 104.547 μm, that of the intact islets was 196.319 μm. The loss of the islet mass was uniform and affected all the size classes of the isolated islets, as evidenced by shifting of their size-frequency distribution towards the smaller diameters. The decrease in the percent volumes and numbers of A, D, and PP cells, which are located at the periphery of the islets, indicate that the loss of the islet mass during the isolation procedure had affected prin-
TABLE 1. The total number of isolated islets per rat pancreas and the % purity of the islets obtained with the use of 22 min incubation time and 1, 2, and 3 mg/ml collagenase

<table>
<thead>
<tr>
<th>Collagenase concentration (mg/ml)</th>
<th>No. of rats</th>
<th>No. of islets per rat (mean ± SEM)</th>
<th>% purity of the islets (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>140.7 ± 11.7</td>
<td>72 ± 1.8</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>342.7 ± 15.8*</td>
<td>82.6 ± 1.9**</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>132.3 ± 8.6</td>
<td>70 ± 5</td>
</tr>
</tbody>
</table>

*No. of the isolated islets at 2 mg vs. number at 1 or 3 mg/ml, P < 0.001.
**% purity at 2 mg vs. % purity at 1 or 3 mg, P < 0.01 and P < 0.05, respectively.

Fig. 1. The number of isolated islets per rat pancreas at different incubation times with 3 mg/ml collagenase. Values at the bottom of each column represent the number of the rats used for isolation of the islets.

A response of the islets to theophylline, which led to the release of 318.3 ± 60 μU insulin/hr/mg protein (Fig. 3).

The immunohistochemical staining of the isolated islets showed four populations of cells which were distinct from each other not only in their specificity for each primary antibody, but also in their respective number and morphological distribution. The majority of the islet cells were B cells and they reacted positively to anti-insulin serum. The reaction of the isolated islets to the anti-insulin serum was generally weaker than that of the intact islets of the normal pancreas (Fig. 4, compared with Fig. 5). In many isolated islets, particularly the large ones, the cells differed greatly in their degree of reaction; the cells at the periphery of the islets were almost well granulated, whereas the cells in the center were somewhat degranulated (Fig. 4).

The A cells reacted positively with anti-glucagon serum. They were distributed at the periphery of the isolated islets (Fig. 6), similar to their distribution in the intact islets (Fig. 7). A cells of the isolated islets appeared, however, less well organized than in the intact islets. They did not form a complete mantle around the B cells, but were rather arranged in clusters scattered at the periphery of the isolated islets. It was also noticed that some of the peripheral cells seemed injured and the blood vessels on the periphery of the islets were torn.

The D cells stained positively with anti-somatostatin serum. They were somewhat variable in number and were dispersed at the periphery of the isolated islets (Fig. 8), whereas in the intact islets they were located either at the periphery of the islet or dispersed in an intermediate position between the A and B cells (Fig. 9). The immunohistochemical reactivity of the D cells of the isolated islets was weaker than that of the intact pancreatic islets.

The PP cells stained positively with antiserum to pancreatic polypeptide. The cells were arranged singly or in clusters at the periphery of the isolated islets (Fig. 10) similar to their arrangement in the intact pancre-
size of the pancreatic islets, was not quantitatively documented.

In the present work the main objective was to study immunohistochemically the distribution of the cellular constituents of the functionally viable isolated pancreatic islets of the rat together with morphometric evaluation of the different cell types and the size classes of the isolated islets. Intact islets of normal pancreases were similarly studied and were used for comparison.

MATERIALS AND METHODS
Isolation of the Pancreatic Islets

The pancreatic islets were isolated from adult male Lewis rats using the ductal perfusion method of Sutton et al. (1986) which is based on the original collagenase digestion technique of Lacy and Kostianovsky (1967).

The rats were anaesthetized with ether inhalation, and the common bile duct was cannulated with a fine polyethylene tube (PE-50) immediately after incision of the abdomen. The duct was then ligated near the duodenum, and the rat was bled to death. The pancreas was infused with 7 ml of cold, freshly prepared, collagenase (Sigma type V (lot 101H6511) in medium M199. The pancreas was quickly excised and incubated at 37°C. Different concentrations of collagenase (1, 2, and 3 mg/ml) and different digestion times (16, 18, 20, 22, 24, 26, and 28 min) were used to obtain the optimal digestion conditions for this batch of collagenase. The action of collagenase was stopped by adding cold medium M199.

The digested pancreatic tissue was disrupted by aspiration through a 14-gauge cannula. The aspirate was filtered through a Nytex filter (mesh size 1,000 μm), collected into a centrifuge tube and the volume was adjusted with cold medium M199 and centrifuged at 400 g for 10 min at 4°C. The supernatant was decanted and the pellet was resuspended in the bottom layer of a discontinuous Ficoll gradient with a density of 1.085. Other Ficoll gradient layers with densities of 1.085, 1.075, and 1.045 were carefully layered on top of the bottom layer respectively, and the tube was then centrifuged at 800 g for 20 min at 4°C. The islets usually collect at the first and the second interface (between the layers with densities of 1.045, 1.075, and 1.085, respectively). The islets were aspirated and placed into another centrifuge tube and washed twice by using cold medium M199; each time these were centrifuged at 400 g for 10 min at 4°C. The pellet of the islets was resuspended in the medium and transferred to a petri dish. The islets were then further purified by hand picking of the non-islet tissue with the help of a binocular dissecting microscope with a black background, illuminated by two horizontal beams of fiberoptic light. The whole process was carried out under a laminar flow hood to avoid contamination of the isolated islets.

The islets isolated from each rat pancreas were counted under the dissecting microscope immediately after purification and the counting was confirmed under phase contrast microscope by a second observer. Using a calibrated grid attached to the phase contrast microscope the purity of the islet preparation was evaluated. The purity was calculated as the percent number of the intersections which overlie islets to the number of the intersections which overlie islet and non-islet tissues.

Functional Evaluation

Aliquots from the islet preparations which were isolated with different concentrations of collagenase and with different digestion times were functionally evaluated by measuring their insulin and C-peptide contents. Insulin was extracted from the isolated islets by acidified ethanol and quantitated by radioimmunoassay procedure using Coat-A-Count kit. C-peptide concentrations were measured by double antibody radioimmunoassay technique using commercially available kit (Diagnostic Products Corporation, Los Angeles, CA). Radioactivity was determined using Beckman gamma counter Model 5500 for 1 min. The results were expressed as units of insulin or C-peptide per g of protein. The protein content of islet preparations was determined by the procedure of Lowry et al. (1951).

The islets isolated with the optimal digestion conditions (22 min incubation time and collagenase concentration of 2 mg/ml) were further functionally evaluated by determining their adenine nucleotide contents. ATP concentration was measured by the method of Lampricht and Trautschold (1974). ADP and AMP concentrations were measured by the method of Jaworek et al. (1974). Samples of freshly prepared isolated islets were incubated in 1 ml of medium M199 containing 5.5 mmol/liter glucose. Incubations were terminated by the addition of 200 μl of HClO₄ (25%, w/v) to the incubation medium and cooling the mixture to 0°C at 0, 30, and 60 min of incubation time. The precipitated proteins were removed by centrifugation at 13,500 g for 2 min. The supernatant was neutralized with KOH containing 0.5 M-triethanolamine, and the KClO₄ was removed by centrifugation at 13,500 g for 5 min. Adenine nucleotide concentrations were measured in neutralized extracts of the islets with a Beckman DU-6 recording spectrophotometer. The concentrations of the nucleotides were expressed as nmol/mg of protein.

Adenine nucleotide concentrations were also measured in freeze-clamped tissues of intact pancreatic islets using the procedure described by Ardawi and Newsholme (1985).

The ability of the isolated islets to secrete insulin was evaluated at the optimal digestion conditions. The isolated islets were incubated in a 0.5 ml syringe barrel containing medium M199 with 1% (w/v) bovine serum albumin (fraction v). The incubation chambers were gassed with O₂/CO₂ (19/1) and incubated at 37°C in a shaking water bath at 50–60 oscillations/min. An equilibration period of 15 min was allowed and then the islets were challenged twice for 30 min each with medium M199 supplemented with 15 and then 30 mmol/liter glucose, separated by a 60 min rest phase with medium M199 only. After a further rest phase of 60 min, a final challenge of 15 mmol/liter glucose together with 15 mmol/liter theophylline were used to elicit a maximal response from the islets (see Henquin and Meissner, 1984). The insulin release in response to challenge was determined and results were expressed as units of insulin released per unit time per mg of protein.

Immunohistochemical Staining

Islet specimens isolated from eight rats, with the optimal digestion conditions described in the present