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THE UNIVERSITY OF ALBERTA

FLOW CYTOMETRY AND PURIFICATION OF PANCREATIC ISLET CELLS

By

Gregory Stephen Korbutt

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

EXPERIMENTAL PATHOLOGY

DEPARTMENT OF PATHOLOGY

EDMONTON, ALBERTA

FALL, 1988

ISBN 0-315-45601-9

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: Gregory Stephen Korbutt

TITLE OF THESIS: Flow Cytometry and Purification of Pancreatic Islet Cells

DEGREE: Master of Science

YEAR THIS DEGREE GRANTED: 1988

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled FLOW CYTOMETRY AND PURIFICATION OF PANCREATIC ISLET CELLS submitted by GREGORY STEPHEN KORBUTT in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in EXPERIMENTAL PATHOLOGY.

Supervisor

Date: Sept 12/88

This text is dedicated to

my parents

OREST AND GERTRUDE

for their love and support.

ABSTRACT

The preparation of viable single cells from intact islets into separate purified cell populations, will allow independent in vitro studies of the islet's single endocrine cells. These studies may greatly enhance our knowledge of the endocrine pancreas, ultimately providing a better understanding of diabetes mellitus.

Dissociation protocols were designed to disperse rat, canine and human pancreatic islets into viable single cells. Depending upon the type of islet tissue disaggregated, approximately 950-1500 cells were obtained per intact islet.

Single islet cells were analysed for their volume, low forward and right angle light scatter, and FAD autofluorescence. Rat islet cells produced distinct FALS and volume distribution, with a "near" linear relationship found between these two parameters. Canine and rat islet cells displayed two defined FAD peaks, which were altered when analysed at high glucose concentrations.

Rat and canine islet cells were separated on the basis of FALS activity and FAD autofluorescence into single B cells (>95% purity) and single non-B cells (80-85% A cells). Islet non-B cells (rat) were further purified into single A cells (>95% purity) according to NAD(P)H autofluorescence at 20 mM glucose.

More than 75% of the cells in the initial dissociated islet cell preparation were recovered as single purified cells. Cell composition of each purified fraction was determined by immunocytochemistry and acid extractions to obtain hormonal content.

Purified non-B and B cells from both rat and canine islets were examined for their staining characteristics to dithizone. Canine B cells and rat non-B cells appeared red when incubated with dithizone.

Fluorescent activated cell sorting was demonstrated as a powerful tool for the preparation of single, pure and viable rat and canine pancreatic A and B cells. The availability of pure pancreatic A and B cells is expected to increase our understanding of islet cell physiology.

ACKNOWLEDGEMENTS

I wish to express my gratitude to:

My supervisor, Dr. L.E. McGann, for providing me the opportunity to pursue this project, for guidance and inspiration throughout, and for introducing me to the "art" of flow cytometry;

To my committee members: Dr. R.V. Rajotte, for his support, guidance, and inspiration over the past six years, and to Dr. L Jewell for his time and interest in this project;

To Philomena Korbutt, for her patience, understanding, and assistance throughout this thesis;

To Lynn Linklater, for her invaluable assistance in flow cytometry;

To Dr G.L. Warnock, for always finding the time and patience for my questions;

To Teresa DeGroot, for always being a friend and helping me hanging in there;

To all the members of the Rajotte/McGann labs, both past and present for their assistance, and to Dr. V. Manickavel for explaining the techniques for immunocytochemistry.

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LIST OF ABBREVIATIONS

A	Alpha
B	Beta
D	Delta
PP	Pancreatic Polypeptide
ICC	Immunocytochemistry
EGTA	Ethylene glycol bis (beta-aminoethyl ether)-N,N'-tetraacetic acid
EDTA	(ethylene-dinitrilo) tetraacetic acid
FACS	Fluorescent Activated Cell Sorter(ing)
FAD	Flavine Adenine Dinucleotide
NAD(P)H	Nicotinamide Adenine Dinucleotide Phosphate
PMT	Photomultiplier Tube
FC	Flow Cytometry
CVA	Coulter Volume Adapter
FALS	Forward Angle Light Scatter
90LS	Ninety-degree Light Scatter
PAP	Peroxidase-antiperoxidase
DAB	3, 3'-diaminobenzidine
FCS	Fetal Calf Serum
BSA	Bovine Serum Albumin
DNase	Deoxyribonuclease
FDA	Fluorescein Diacetate
EB	Ethidium Bromide
PLL	Poly-L-lysine
Hepes	4-(2 hydroxyethyl)-1 piperazineethane-sulfonic acid, sodium salt
Ab	Antibody
Ag	Antigen

CHAPTER ONE

INTRODUCTION

Glucose homeostasis is controlled by the endocrine pancreas which is spread throughout the entire gland as clusters of cells, the islets of Langerhans consisting of approximately 1000-3000 endocrine cells. Mammalian pancreatic islets represent a heterogeneous cell population composed of at least four different cell types. These cells have been identified as the alpha (A), beta (B), delta (D) and pancreatic polypeptide (PP) as described on the basis of histological, immunocytochemical and ultrastructural characteristics (1). A precise topography of islet cells has been recognized by immunofluorescent studies (2,3) with the A, D, and PP cells mostly located at the islet periphery and surrounding the B cells.

Through their secretory products and via membrane specializations, it has been proposed that these islet cells interact functionally as an integrated unit regulating pancreatic hormone release (2,4,5). Factors regulating islet hormone release may vary for each islet cell type due to the presence of specialized cell junctions amongst certain cells, and the distribution of cells within the islet (6,7). Therefore purified islet cell populations will best define the mechanisms regulating hormone release for each cell type.

Due to difficulties obtaining these cell populations, initially most in vitro studies on the endocrine pancreas have been carried out in heterogeneous cell populations. These studies include the perfused pancreas (8), pancreatic fragments (9), isolated islets (10,11) and dissociated islet cells (12-14). However, the desire to analyze

homogeneous islet cell preparations led to the use of B cell rich islets from obese hyperglycemic (ob/ob) mice (15) and of A cell enriched islets from streptozotocin or alloxan diabetic rats (16). Islets from the ob/ob mice represent a good model to study B cells and insulin release mechanisms. They are less appropriate to examine other cell types, especially for intercellular communication and, in addition, are less representative for normoglycemic conditions. Due to the effects that B cell destroying agents (streptozotocin and alloxan) have on A cells and glucagon release, the use of A cell enriched islets is questionable (17).

Physiological and pathological considerations have both led to the need for separating the various islet cell types. Purified preparations of each islet cell type is expected to define its specific regulation by intra- and extracellular factors (18-21), while studies on reassociated cells will define the suspected presence of functional cooperation between islet cells (19,20). The availability of purified islet cells also facilitates the search for agents or mechanisms involved in B cell destruction or dysfunction, as observed in streptozotocin-induced diabetes or diabetes mellitus (17). The availability of purified islet cells will greatly enhance our knowledge of the endocrine pancreas, ultimately providing a better understanding of diabetes mellitus, and possibly leading to a cure or alternate form of therapy.

To achieve islet cell purification a dissociation procedure for islet tissue must initially be developed to yield viable single cells. Secondly, cell parameters which differ significantly among the several cell types and which can form the basis for a separation technique must be identified. Finally, the selected purification procedure must be

adapted for separation of the various islet cell types without affecting their viability, structural cell coupling and respective cell numbers. These criteria must be reached to obtain purified islet cell populations, allowing for further in vitro analysis.

The objective of this thesis is to meet these criteria in order to successfully purify islet cell subpopulations. To obtain this:

(1) dissociation protocols were designed to disaggregate rat, canine and human pancreatic islets.

(2) flow cytometry of islet cells was investigated to determine differences in chemical, biological and physical parameters for the basis of identifying the various islet cell types.

(3) flow cytometry was used to obtain purified populations of A and B cells.

(4) both dissociation and separation protocols were submitted to quantitative and qualitative assessment to determine the efficacy of the procedures and the vital characteristics of the dissociated cells.

(5) immunocytochemistry (ICC) was used to characterize the separated cell populations to determine their purity.

The final goal is to determine which islet cells react with diphenylthiocarbazone (dithizone), as this compound has been recently used to identify pancreatic islets (22) for quantification of intact islet yield. It should also be noted that a major purpose of this study was to investigate the use of flow cytometry on characterizing and isolating islet cells. This stems from the fact that literature in this area is somewhat conflicting and only one investigator has reported success in isolating A and B cells (23).

CHAPTER TWO

LITERATURE REVIEW

2.1 PANCREATIC ISLET ISOLATION

The total mass of endocrine tissue comprises approximately 1-2% of the wet weight of the adult human and rat pancreas (24). Detailed studies of the function of the endocrine pancreas, therefore, depends on methods for preparation of sufficient quantities of tissue enriched in endocrine cells. The isolation process, although similar, has to be adapted to each species, whereas a major obstacle is to collect pure islets without contamination by aggregates of acinar cells. Historically, two approaches have been undertaken in order to isolate the islets of Langerhans from the surrounding acinar tissue: (1) microdissection, and (2) collagenase digestion.

Microdissection began with Bensley (25) who stained islets with a variety of dyes within the guinea pig pancreas, thus identifying them so that they could be picked out by hand for study. Pure islet tissue was first obtained by the freehand microdissection technique of Hellerstrom (11), which he applied to normal and obese hyperglycemic mice. These techniques used to isolate islets were extremely traumatic, producing only small numbers of islets. Thus, the need for less traumatic isolation procedures prompted the use of collagenase for enzymatic activity to obtain intact islets.

The major breakthrough for islet isolation began when Moskalewski (26) utilized the enzyme complex collagenase to separate intact islets from chopped guinea pig pancreas. Intraductal distension of the pancreas with a salt solution gave mechanical release which then

permitted increased enzymatic contact, improving the yield of isolated islets, as shown by Lacy and Kostianovsky (10).

Initially handpicking of islets from the digestate was the only method to purify the islets from the acinar tissue. Due to handpicking's impracticality for large-scale purification, Lacy (10) utilized sucrose gradients to separate islets from digestate, but in vitro studies revealed a non-uniform response to glucose. Lindall et al. (27) demonstrated an increased yield of islets as compared with sucrose, using Ficoll, a high molecular weight polymer of sucrose. However, this report did not include studies on the islets' ability to release insulin. It was not shown until 1973 (28) that insulin release from islets isolated with Ficoll purification was normal only if the Ficoll was dialyzed before its use. Collagenase digestion and Ficoll purification have now become the standard islet isolation technique, especially for rodent studies.

Rodent islet isolation procedures had to be modified to accommodate the more compact fibrous canine and human pancreases (29,30). Methods were developed to improve the distension of the gland, as the canine and human pancreases did not give the same result as ductal distension of the rat pancreas. Recently, major advances in obtaining high yields of purified canine and human islets have resulted from perfusing the gland throughout the digestion followed by teasing and subsequent trituration of the digested gland (31,32). Overall, these advances have increased islet yield and purity; however, more investigation is required for further improvements.

2.2 PREPARATION OF ISLET CELLS

The average sized islet is composed of 1000-3000 endocrine cells along with structural elements such as connective tissue and a rich capillary network. These cells are held together via the formation of specialized junctions and adhesive properties of surface molecules. A principle goal of any tissue dissociation method is to disrupt these intercellular bonds to obtain a good yield of single viable cells without provoking deleterious effects upon membrane components. However, the nature of these intercellular matrices is poorly understood, making it impossible to devise dissociation methods optimally suited for the tissue under study. For this reason, many techniques for obtaining islet cells in suspension are adaptations of procedures used for tissue dissociation of other sources of animal tissue (42). Usually, in most instances, one or a combination of the approaches are utilized.

2.2 (a) Mechanical Force

Application of mechanical force for breaking intercellular connections has been widely employed for various tissues, including such methods as loose homogenization vigorous, shaking, sieving, and trituration. Although simple and without enzyme suppression, these methods are often rejected for their traumatic effect and low yield of single cells.

The first report on islet dissociation described a mechanical disruption of individual guinea pig islets by placing them under a glass microscope slide and exerting pressure with a glass rod until the islet was dispersed into the surrounding medium (43). Its application was,

however, limited by the lengthiness of the procedure and low yield of single cells.

2.2 (b) Calcium Removal

The divalent cation calcium is involved in cell adhesiveness (44), and its omission from dissociating media, or its active removal, can be used to loosen cell-to-cell attachments. A specific ability to bind calcium selectively with the complexons (ethylene-dinitrilo) tetraacetic acid (EDTA) and ethylene glycol bis (beta-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) (45) has made techniques possible for separating cells by this means. In the liver and whole pancreas this treatment disrupted desmosomes but not gap or tight junctions (46,47). Mechanical force was necessary to complete dispersion of the remaining cell clusters, resulting in considerable cell damage.

Rat and mouse islets have been dissociated by a combination of calcium removal and mechanical force (48). This report describes a method involving vigorous shaking for a short time period with the islets suspended in a calcium free medium containing EGTA. A slightly longer shaking period produced smaller numbers of intact islet cells, prompting Lernmark to triturating the islets instead of the vigorous shaking (49). However, as reported by Pipeleers (50), rat islets may be dissociated mechanically in the absence of calcium, but the yield barely exceeds 35%. This suggests that mechanical force and calcium removal alone are not sufficient to obtain high numbers of single viable cells.

2.2(c) Enzymatic Digestion

Enzymes attack macromolecules such as proteins and glycoproteins, and have been used to digest intercellular bonds for more than thirty years (51). Presently, a variety of crude and purified proteolytic enzymes are employed for tissue disaggregation (42). However, the selection of an enzyme is rarely determined by a precise knowledge of the proteins to be digested. Simply, it derives, in most cases, from the empirical experience of other laboratories.

Proteolytic enzymes used for dissociation of intact islets include trypsin, dispase and collagenase, with some reports including the addition of a subsidiary enzyme, hyaluronidase. Many protocols have a combination of mechanical, chemical and enzymatic treatment to be employed simultaneously or successively (Table I). It is impossible to compare the yield in viable single islet cells for each method, as this information is either not reported or expressed under completely different parameters. Table I, therefore, illustrates the various approaches for dissociation of pancreatic intact islets, comparing the degrees and type of mechanical force, chemical manipulations and the type and concentration of enzymes used, as these are the crucial steps to be considered.

2.3 ISLET CELL PURIFICATION

To purify a heterogeneous cell suspension into individual cell populations, one must identify cell parameters which differ significantly among the cell types and which form the basis for a separation technique. Several biological, chemical and physical

TABLE 1
DISSOCIATION METHODS FOR INTACT ISLETS

Species	Enzyme	Calcium Removal	Mechanical Force	Reference
	Trypsin (%)			
Rat	0.5 at 37°C for 15 min	only no calcium	trituration	52
	0.25 at 37°C for 2-5 min	only no calcium	pipetting	14
	0.25 at 37°C for 15 min (3 times)	1 mM EDTA	stirring	53
	0.10 at 37°C for 6 min	3 mM EGTA	trituration	54
	0.02 at 37°C	1 mM EDTA	trituration	55
	0.01 at 30°C for 10-12 min	1 mM EGTA	pipetting	56
	0.0025 at 30°C for 10 min	1 mM EGTA	pipetting	22
Mouse	0.05 at 20°C for 10 min Dispase	2 mM EDTA	stirring	50
Mouse	0.5% at 4°C overnight	1 mM EGTA	shaking	57
Rat	37°C for 15 min (3 times)	1 mM EDTA	stirrir	53
	Collagenase (mg/mL)			
Rat	2.0 at 37°C for 30 min	1 mM EGTA	shaking	58
	2.0 + hyaluronidase at 37°C for 30 min	1 mM EGTA	shaking	58
	None	-	pressure	42
Guinea Pig	-	1 mM EGTA	shaking	48,58
Rat	-			

parameters have been used alone and in combination for the separation of a wide variety of cell types (59). Until recently, it was unknown whether one of these parameters could be applied to the purification of islet cells. Over the past seven years a few methods (1) elutriation, (2) gradient centrifugation, and (3) cell sorting have been utilized to separate islet cells into subpopulations with varying success.

2.3 (a) Elutriation and Gradient Centrifugation

The principles of cell separation by counter-streaming centrifugation (now known as centrifugal elutriation) were first described by Lindahl (60) and twenty years later adapted for biological applications (61). Since this early work, several techniques and instruments have been developed for separating particles on this basis, and have become widely employed in cell purification (62). This principle separates particles according to differences in sedimentation velocity, which is mainly determined by the radius of spherical particles, as described by Stokes' law.

Cell size was the first detected parameter for the separation of islet cells (56). Initial volumetric and surface analysis of islet cells revealed that B cells were two-to-three fold larger than the other islet cells types, thus having a higher sedimentation velocity. Elutriation distributed the islet cells over a first single cell fraction enriched in islet non-B cells, a second single cell fraction composed of more than 90% B cells and a third fraction of mostly structurally coupled cells (56). Using about 5×10^5 cells as a starting material, 15×10^4 cells could be recovered in the second fraction, which is sufficient for further functional studies on the B

cell. Elutriation of islet cells was shown to be attractive due to its short duration (35 minutes), its high cell recovery (70%), its minimal cell clumping and ability to separate single cells from coupled cells. This method demonstrated a high viability index (> 95%) as measured by trypan blue exclusion. It is, however, only limited to the purification of B cells from the non-B cells.

Isopycnic density gradient centrifugation was applied to the eluted cells in an attempt to further purify the non-B cell fraction into enriched A and D cell populations (56). According to Stokes' law, the sedimentation velocity of a spherical particle becomes zero when its density equals that of the surrounding medium. Isopycnic density gradient centrifugation uses this principle to separate cells with different densities, since the cells will bond at different regions as they sediment through a density gradient of appropriate range.

The usefulness of this technique for the separation of islet cells in the non-B cell eluted fraction was limited in view of the small differences in density (d) of the A cells (d=1.068), B cells (d=1.065) and D cells (d=1.070) (56). This gradient centrifugation was capable of further enriching A cell and D cell subpopulations. However, even though the elutriated and gradient centrifuged islet cells were viable, the extremely low cell yield (< 20%) of this procedure makes further separations of A and D cells dependent on other techniques.

2.3 (b) Cell Sorting

Differences in surface membrane components of closely related cells had led to procedures which coat the cells with a specific cell membrane marker. For example, cell-specific surface antibodies (Abs) which bind

to membrane antigens (Ags) of a particular cell type may be employed for cell separation. Isolation of particular cell types may be based upon Ab binding capacity according to the fluorescent intensity of the cells after fluorescent labelling of surface-bound Abs. Such separation may be accomplished by fluorescent activated cell sorting (FACS), where cells with a specific fluorescently- labelled surface Ab may be isolated from a heterogeneous cell population.

The availability of islet cell specific Abs (63,64) has created the possibility of isolating the various islet cells. These immunoglobulin (Ig)G Abs were primarily detected in recently diagnosed Type I (insulin-dependent) diabetic patients younger than thirty years of age. They have been predominantly used to fluorescently label the islet B cells (63,64) and somewhat the A cells (67) so that they become separable by FACS. Monoclonal Abs binding selectively to B cells have also been used for this purpose (65,66). These reports have been used exclusively on mouse and rat islet cells, producing B cell populations with up to 90% purity. Sorting of B cells labelled with selective Abs is, however, hindered by disadvantages: (1) high degree of cell reaggregation during pre-incubation with Ab; (2) inability to eliminate non-B cells attached to fluorescent B cells and which, therefore, contaminate the isolated preparation; and (3) the persistence of surface Abs on the isolated cells limiting further biological experimentation.

The knowledge that islet B cells were separable on the basis of their larger size (56) led to attempts to purify islet cells by flow cytometry, using cellular light scatter as the separation parameter (54,67,68). Rat islet cells in single cell suspensions were found to induce two peaks in forward low-angle light scattering intensity

(54,68). The A and D cells were more numerous the left peak (lower light scatter intensity) while the B cells w concentrated in the right peak (greater scatter intensity). This observation is not consistent with the data from Neilson et al. (67), who reports a single broad peak of light scattering intensity with A cells relatively concentrated to the left, B cells in the center and D cells to the right. Sorting of islet cell subpopulations using only light scatter intensity as the separation parameter produces two fractions: (1) a B cell enriched population, and (2) A and D cell mixed population (54,67,68). However, there exists a lower degree of purification and cell yield as found with other islet cell purification techniques (66).

FACS may also be employed to separate cells with sufficient differences in autofluorescence intensity (23,50). It has been shown that cellular autofluorescence in mammalian cells is due to their intracellular flavins and pyridine nucleotides, flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NAD(P)H, respectively (69,70). Islets represent a rich source of both constituents and, more importantly, undergo rapid glucose induced variations in the nucleotides fluorescence intensities (71-73). These observations led investigators to examine whether FACS could discriminate islet cell types based upon their endogenous fluorescence. Experiments with islets have clearly demonstrated this concept (22,74,75), providing purified A and B cell populations.

The method is based upon cell-specific variations in redox state and the subsequent changes in autofluorescence in conjunction with light scattering properties. AT 2.8 mM glucose the B cells have a higher FAD autofluorescence intensity than that of the non-B cells, allowing for

separation of the B cells. Whereas at 20 mM glucose the isolated non-B cells are reanalyzed while most cells maintain low NAD(P)H autofluorescence, some increase their autofluorescence. Sorting of the population with unchanged NAD(P)H fluorescence yields virtually pure A cells (23). However, Pipeleers' et al. (23) report is concise in regards to their methodology, little information is given detailing the flow cytometry technique.

2.4 IMMUNOCYTOCHEMISTRY

Histochemistry bridges the disciplines of biochemistry and histology to analyze the chemical composition of cells by developing methods to localize cellular constituents in situ without destroying structure. Immunocytochemistry (ICC) combines the basic principles of histochemistry with the high degree of molecular specificity inherent in the Ag-Ab reaction. Thus, ICC is the identification of a tissue constituent in situ be means of a specific Ag-Ab reaction tagged by a visible label (76).

It is important to realize that the Ab molecules must be either directly or indirectly labelled with some compound we can see. Therefore, a number of methods have been employed to localize Ags within cells by means of Ag-specific Abs. In the early 1940s, Coons et al. (77,78) laid the foundation of current immunoperoxidase methods by the use of fluorescein-labelled Abs to locate Ags in tissues. Coons illustrated that an Ab could be coupled (conjugated) with a fluorescent marker with full retention of the affinity for the Ag.

Immunofluorescent techniques are generally not difficult to perform and a wide range of conjugated products are commercially available.

However, there also exists a number of disadvantages, including the following: (1) masking of specific fluorescence by natural fluorescence of some tissues, (2) a fluorescent microscope is required, (3) rapid fading of fluorescence necessitating a photographic record, and (4) limited sensitivity and resolution plus the inability to visualize non-fluorescing tissue.

Enzymes were then introduced to replace fluorescein for labelling Abs (79). Since enzymes recognize substrates not only by the chemical groups attached to the substrate but also by the three-dimensional arrangement of these groups, specific enzymes act on specific substrates. Horseradish peroxidase forms a complex with hydrogen peroxide and upon the addition of 3,3'-diaminobenzidine (DAB), an electron donor, a second complex forms and eventually dissociates leaving the original intact enzyme and a brown precipitate, oxidized DAB (80).

Immunoperoxidase methods fall into two general classes: (1) conjugate, or enzyme labelled Ab procedures, and (2) nonconjugate, or unlabeled Ab procedures (81). The conjugate procedures include direct or indirect methods, both involving the covalent linkage of the peroxidase enzyme to the appropriate Ab. However, some Abs remain unlabeled following enzyme-Ab conjugation, competing with the enzyme conjugated-Abs for antigenic sites, thus limiting the sensitivity of the conjugate methods (82).

Peroxidase nonconjugate methods avoid the chemical procedures needed to link an enzyme to an Ab, thus producing a more sensitive technique. Introduced by Sternberger (83), the peroxidase-antiperoxidase (PAP) procedure has gained the widest acceptance of all

immunoperoxidase techniques. It takes advantage of the fact that if whole serum containing antiperoxidase Abs is mixed with horseradish peroxidase Ags, a complex is formed that precipitates. This PAP molecule can be dissolved to form a soluble reagent for immunoperoxidase techniques (82).

Immunoperoxidase histochemistry thus offers the following advantages for localizing tissue Ags: (1) no special equipment is needed to visualize the end product; (2) histological detail is preserved; (3) sections are permanently stained for years, hence allowing for retrospective studies, and (4) sensitivity is excellent.

2.5 DITHIZONE STAINING OF ISLET TISSUE

Dithizone, an organic dye, has been used in histochemical techniques for the detection of zinc in animal tissue (84). Zinc has been demonstrated in small quantities in most plant and animal tissues. Except for being a prosthetic group for the enzyme carbonic anhydrase, zinc's physiological or structural role is uncertain. First utilized in the early 1940s, the technique was applied to sections of pancreas in which it was shown that dithizone stained islets of Langerhans while not the acinar tissue.

Dithizone is a violet-black solid which is water insoluble, but readily dissolves in alcohol. Seventeen different metals have been shown to form complexes with dithizone, resulting in a red color being formed (84). To produce a dithizone solution which is specific for zinc, it has been reported that a complex-forming buffer must be used in order to form non-reactive complexes with other metals (84,85). That is, a dithizone complex-forming solution containing thiosulfate and

potassium cyanide will react with zinc without interference from other dithizone reactive metals. If a particular solution is zinc specific or not, still is unclear basically due to the lack of investigation.

Regardless of whether one uses a dithizone solution non-reactive to other metals or not, both approaches have stained islet tissue reddish while leaving the acinar tissue unstained (85). Dithizone is capable of staining islet tissue of rabbits, dogs, rats, mice and cats, using tissue sections (85,86) and isolated islets (87). The only reported exception has been islet tissue of the guinea pig (86) which may be in lieu of the fact that guinea pig pancreas contains less insulin than that of other species studied (88).

Of great interest is the distribution of zinc between the different cell types within the islets (or which cells are dithizone positive). Administration of diabetogenic agents into rabbits such as alloxan produced a rapid disappearance of the stainable zinc in the islets with dithizone (86). Also the injection of dithizone itself into rabbits produced a prolonged period of hyperglycemia (89). These reports suggest that in the rabbit zinc is predominantly or exclusively in the B cells.

Further evidence linking zinc to the B cells comes from Scott's (90) observation that crystalline insulin contains zinc. Under conditions where insulin secretion is stimulated only small amounts of zinc can be demonstrated with the use of dithizone (86). On the other hand, more zinc is found when insulin is being stored (86). However, this data was obtained from fish islet tissue, making it difficult to allow comparisons to the mammalian model.

Maske (86) also states that zinc is found in the B cells of dogs and mice, whereas A cells of the rat contain more zinc than B cells. This report does not provide any data or references to support this information. With such little investigation into this area, it still remains unclear to dithizone (or zinc's) specificity to islet cells. However, dithizone is a powerful tool to recognize islet tissue and differentiate it from the surrounding acinar tissue (87).

CHAPTER THREE

INTRODUCTION TO FLOW CYTOMETRY

3.1 THE FLOW CYTOMETER

The fluorescence activated cell sorter (FACS) is used both for analysis and separation of particles in suspension. Flow cytometry involves sensing particles as they move single file in a fluid stream past a laser illumination system coupled with a set of highly sensitive detectors. The particles may be cells, large molecules, latex beads, or other particles.

When the cells pass through the laser beam, light is scattered and fluoresced in all directions, much like a dust particle scatters sunlight in a room. Light scattered at low angles is detected by a photodiode, while photomultiplier tubes (PMT's) detect right-angle light scatter and emitted fluorescence. This detected light is then transduced to voltage pulses by the PMT's, then integrated and converted to a digital signal by a 256-channel analog-to-digital converter (ADC). If the cytometer is equipped with four PMT's, three are usually used to detect fluorescence at different regions of the color spectrum, with right-angle light scatter collected in the fourth. Therefore, if low angle light scatter and cell volume are also being detected, the FACS may simultaneously measure six different cell parameters. Data is then accumulated in computer memory as 256-channel histograms or as list mode for subsequent analysis and/or comparison.

FACS analysis data collected can be displayed either as a one parameter histogram as seen in Figure 1a, or as a two parameter histogram, as shown in Figure 1b. One parameter histograms are

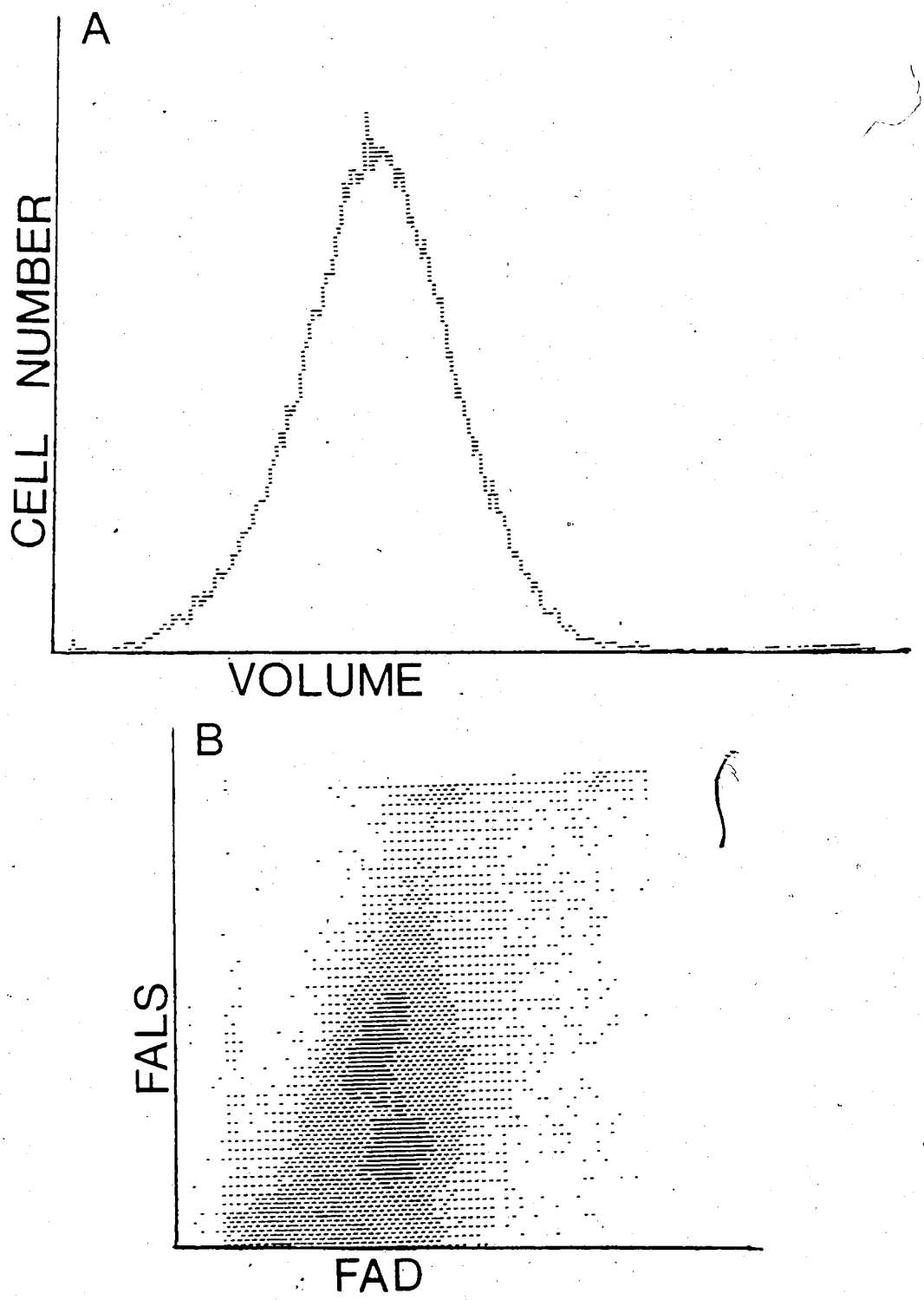

FIGURE 1 One parameter histogram (A) of volume (horizontal axis) vs number of cells (vertical axis), and a two parameter histogram (B) of FAD (horizontal axis) and FALS (vertical axis). The third dimension is the number of cells.

presented in the form of two dimensional histograms in which the horizantal axis displays intensity of fluorescence or light scatter (or the parameter being detected), and the vertical axis shows frequency of cells. Two parameter histograms illustrate three variables, with two FACS parameters (ie:volume and FACS) used for the X and Y axes, and the third dimension represented by contour lines, is the number of cells. Both terms, "one-parameter" and "two-parameter", are accepted in the field of flow cytometry (91).

FACS histograms serve two key functions: (1) as primary analytic data, they provide a quantitative basis for the definition of sub-populations, by displaying the frequency of cells in the population as a function of parameter expression, the histograms allow visualization of clustering of cells within a restricted parameter range, and (2) provide an index for establishing thresholds for analysis of cells expressing one parameter in a sub-population defined on the basis of one (or two) other parameters. This latter function, usually called "gating", is best defined using one parameter histograms, allowing one to investigate the relationship between two parameters. The capability of two parameter histograms to graphically show the relationship of two parameters, provides an easy method to define or locate cell sub-populations.

The capacity to simultaneously measure and compare several variables for each individual cell in a heterogeneous cell sample makes the flow cytometer a very powerful and unique instrument, for the characterization of cell populations.

3.2 MULTIPARAMETER CELL ANALYSIS

Flow cytometry has found a wide range of applications in cell biology, expanding into such disciplines as: immunology, physiology, genetics, micro-biology and pathology. By far the largest number of flow cytometry studies to date, have involved the identification of sub-populations of lymphocytes carrying surface antigens detectable with fluorescein conjugated antibodies (92). However, this trend is beginning to change, with more investigation directed to other sources of cell populations (93).

The number of measurable cellular parameters detectable via flow cytometry is very extensive, therefore, the following discussion describes only the parameters used in this study. Based upon Shapiro's (93) classification, parameters are classified as extrinsic and intrinsic or whether or not they can be measured with or without the use of reagents. Extrinsic parameters are those which depend upon the application of dyes or labels to identify cell populations. Intrinsic parameters include light scatter and volume (structural characteristics) and auto fluorescence (functional characteristic). Extrinsic parameters were not used for this study.

Light Scatter

Forward or low angle light scatter measurements have been widely used for estimation of cell size since 1970 (94). A near linear theoretical relationship between size and forward light scatter is true only when light is scattered at small angles (0.5 - 2.0^{o}) from the incident laser beam, and only within a certain range of diameters of smooth spherical objects of the same refractive index. Forward angle light scatter (FALS) is however a complex function of several variables,

influenced by cellular properties other than size. These include the differences in refractive and reflective properties of the cell and suspending medium (95), wavelength of light (96), properties of the plasma membrane and internal structur of the cell (97).

When analysing FALS data, one must therefore be careful drawing conclusions based upon the relationship between low angle light scatter and cell size. McGann et al (98) demonstrated using human lymphocytes and hamster fibroblasts, that the intensity of FALS varied inversely with cell volumes in hypertonic and hypotonic solutions. Cell damage induced by freezing and thawing was also found to result in low viability with little change in cell volume, but a significant reduction in FALS intensity was observed. This data clearly illustrates that FALS and cell volume vary independently, and FALS is a complex sensitive index of the state of the cell.

FALS provides a convenient means of distinguishing live from dead cells, and therefore may be used to exclude dead cells from analysis (99). Dead cells have characteristically low intensity FALS, and gated analysis of scatter histograms show that the low intensity peak is entirely composed of dead cells (95). However, small cells and debris may also exhibit low intensity of FALS, and can be eliminated by the "gating" procedure. This reduction in light scatter of dead cells is not due to size, but may involve a change in the cell's refractility, possibly related to the differences in appearances of live and dead cells with phase contrast microscopy (91). Whatever the physical explanation, this observation is extremely useful for FACS analysis.

Ninety degree light scatter (90LS) is believed to be closely related to internal structure of cells. It has been suggested that 90LS

could be used to detect lipid storage vesicles in the cytoplasm, as 90LS is sensitive to refractive index changes of cells (95).

Volume

Electronic volume measurement by the Coulter principle (100,101) is the most common flow cytometric technique for determining cell size. This is basically due to the large number of electronic cell counters in use, but very few optical flow cytometers are equipped with this capability. Recently Coulter Electronics (Hialeah, Fla.) has made available the Coulter Volume Adapter (CVA) as an accessory to the Coulter EPICS flow cytometer. As opposed to cell counters, the CVA allows for simultaneous measurement of volume and photometric parameters.

The CVA is based upon a modification of the Coulter transducer, which measures changes in electrical resistance caused by a particle suspended in an electrolytic medium traversing a narrow aperture. The passage of a cell through the aperture produces a voltage pulse which, according to theory, should have an amplitude proportional to the volume of the cell. Variables influencing the change in resistance are either cell-specific (volume, orientation, shape, and electrical resistivity of the internal milieu of the cell compared to the surrounding medium) or aperture-specific (geometric dimensions, and current passing through). The addition of the CVA is a powerful tool for displaying the relationship of cell volume to other cellular parameters.

Autofluorescence

The autofluorescence of most mammalian cells is due primarily to the presence of pyridine and flavin nucleotides (69,70). Many investigators have been concerned with autofluorescence solely as a source of

interference with measurement of weak extrinsic fluorescence signals. However, pyridine and flavin nucleotide fluorescence is a function of the oxidation-reduction, or redox state of cells. Fluorescence measurements of NAD(P)H have been used to monitor redox state of cells, tissues, and organs since the technique was described in 1959 (102). Therefore, by detecting and measuring the intrinsic parameter autofluorescence, one can gain understanding in the functional characteristics of a cell population.

3.3 CELL SORTING

The FACS also has the capability of sorting out of the sample stream those particles of special interest. Any parameter(s) measurable in a flow cytometer can provide a basis for selection of cells. The degree of purity which can be achieved in the selected population is set primarily by the precision with which the selection parameter(s) can be measured. To the novice, flow sorting can be extremely overwhelming at times. However, the procedure involves only three major steps to follow: (1) droplet generation, (2) droplet charging and deflection, and (3) decision logic. Figure 2 illustrates these three steps, used almost universally for flow sorting (103).

Cells leave the reservoir and are injected under sligh ressure into the center of a stream of cell free sheath fluid, then pass through the detection zone where they are illuminated by the laser. An ultrasonic transducer vibrates the sample/sheath stream, causing it to break into droplets containing the individual cells. The average number of droplets formed per second is approximately 32,000, depending upon the size of the flow chamber tip, and the frequency of the ultrasonic

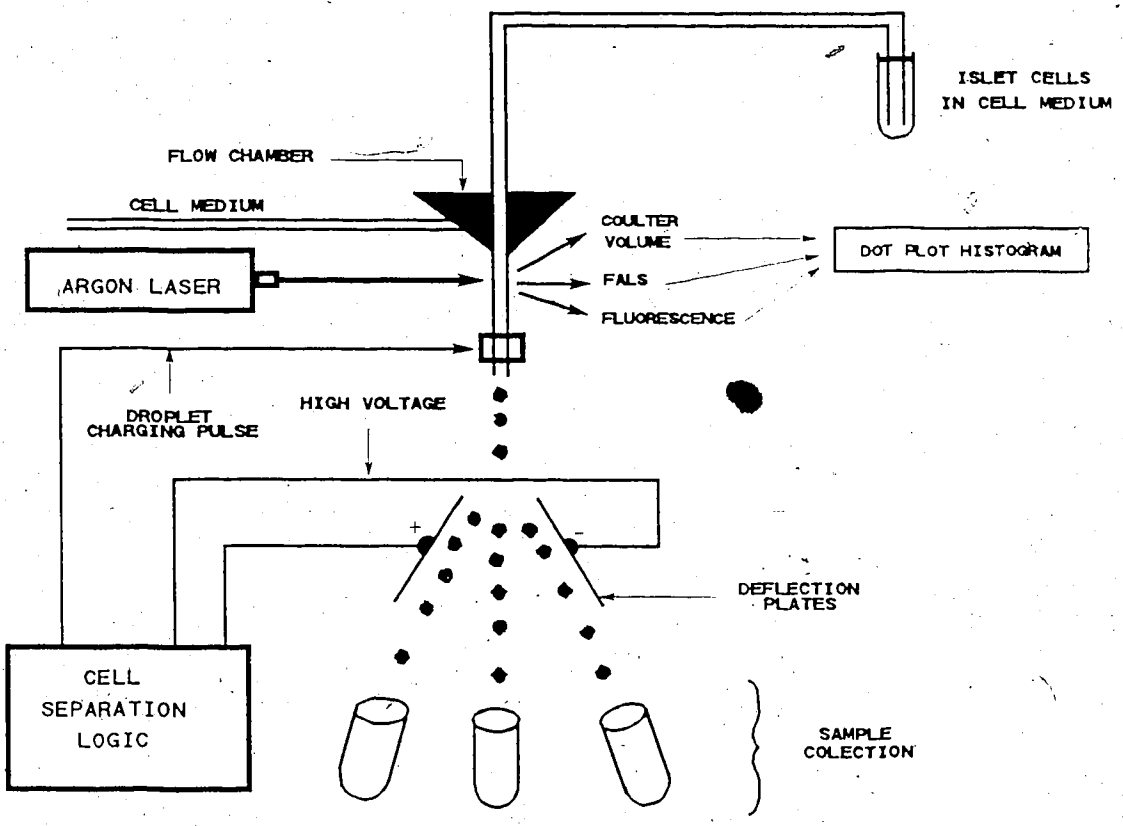

FIGURE 2 PROCEDURE FOR SORTING CELLS

waves. Under these conditions, cells which have passed the detectors continue down the stream, reach its tip and become encased in droplets, where usually only 1/6th of the droplets contain a cell.

When a droplet contains a particle we wish to sort, a voltage potential is placed on the stream and changes the droplet as it breaks off. The droplet is charged negative or positive, depending on whether the deflection is to be left or right. The charged droplet is then passed between two high voltage deflection plates and is pulled out of the stream toward the oppositely charged plate. The charged droplets can be collected by a container or slide placed an appropriate distance out of the stream. All uncharged droplets continue directly downwards and fall into a collection container or the discard vessel.

Before a droplet (or cell) can be sorted a decision must be made as to what particles are to be sorted and in which direction, left or right. Based upon one or two parameter histograms, the criteria for sorting is set into the instrument as decision logic. Simply, a lower and an upper channel are selected on one parameter histograms to select the desired cell population to be sorted. If two different populations need to be sorted, a lower and an upper channel are selected for sorting left and another pair can be selected to sort right. When using two parameter histograms, lower and upper limits are selected for each parameter which blocking off the clustering of a cell population. Therefore, for a droplet to be sorted it must satisfy both parameters for selection in the desired direction. This selection of sort criteria, generates a signal which will cause the droplet containing the selected cell to be charged. However, a sort-delay must also be set to determine the time between which cells are detected and selected for

sort windows and the point at which the droplets break off and become charged.

CHAPTER FOUR

MATERIALS AND METHODS

4.1 ISOLATION OF ISLETS OF LANGERHANS

4.1 (a) Rat Islets

The method of islet isolation was based upon that of Lacy and Kostianovsky (10) using modifications based on other techniques (27-29). Six to ten Sprague-Dawley rats were anesthetized with sodium pentobarbital (40 mg/kg) (Somnotol, MTC Pharmaceuticals, Hamilton, Canada). The pancreases were cannulated via the common bile duct with Intramedic non-radiopaque PE-50 (internal diameter, 0.023 inches) polyethylene tubing and distended with 10 mL of chilled Hanks Balanced Salt Solution (HBSS, Gibco, Grand Island, NY) containing 1 mg/mL D-glucose. Once excised and placed in chilled HBSS, the pancreases were removed, picked free of contaminating lymph nodes and fatty tissue and then chopped vigorously with scissors in HBSS.

The minced tissue was divided into two or three 50 mL Falcon conical centrifuge tubes, usually containing 3-4 g of tissue in each tube. HBSS (2.0 mL/g tissue) and 15 mg/g tissue of Type V collagenase (Sigma, St. Louis, MO) were added to the tissue and gently shaken by hand in a 37°C water bath until an individually assessed digestion end point was reached (10-12 min). Digestion was stopped by the addition of chilled HBSS to a volume of 50 mL, and the tubes were spun at 450 g and the supernate removed.

Prior to Ficoll (Type 400-DL, Sigma, St. Louis, MO) purification, any remaining collagenase and exocrine debris were removed by a series of washes with 20 mL HBSS and centrifugations at 450, 200, 120, 50 and

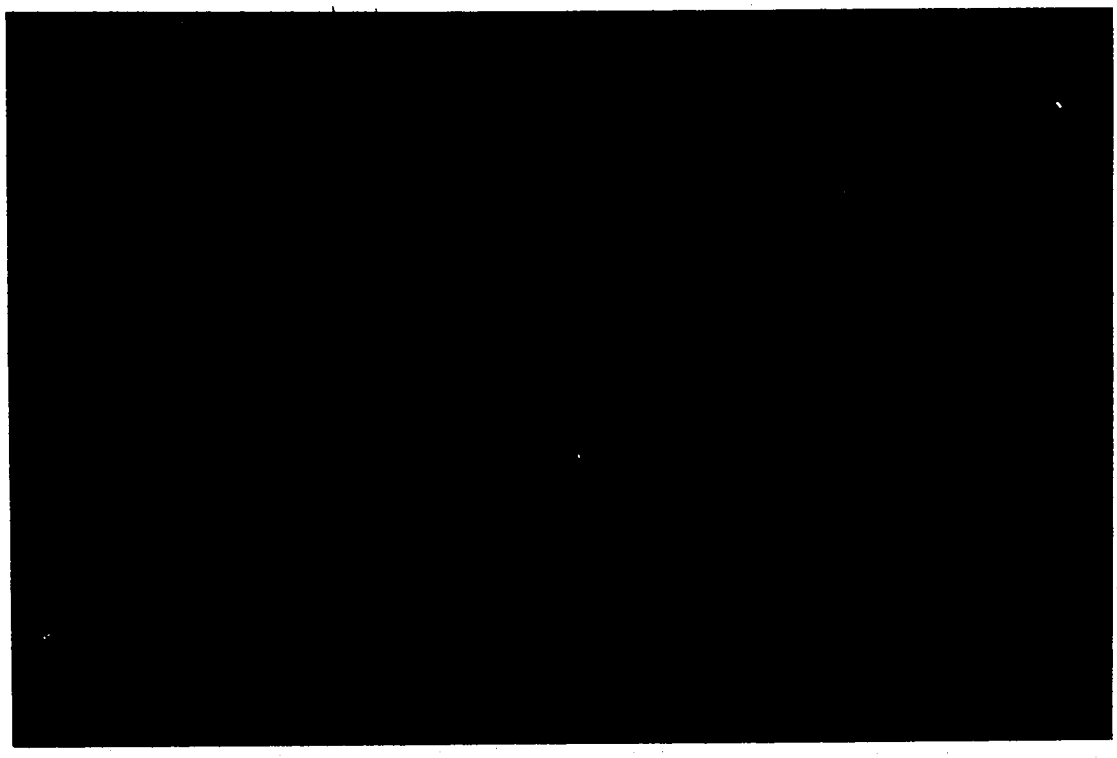

PLATE 1 Freshly isolated rat islets of Langerhans (25X).

450 g. Following the final wash, 5 mL of 25% Ficoll was added to the tissue and the tubes were vortexed to disperse the tissue. Ficoll solutions of 23%, 20.5% and 11% (5 mL each) were gently layered on top, followed by centrifugation at 850 g for 20 min.

Isolated islets were found at the interface of the 23% and 20% Ficoll layers, removed with a siliconized Pasteur pipette and placed in Medium 199 containing 25 mM Hepes buffer, Earle's Salts, L-Glutame and 1 mg/mL D-glucose, supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin (PS). The islets were washed 3-4 times using the Medium 199 to remove any contaminating Ficoll. Once transferred to a petri dish containing supplemented Medium 199, islets were handpicked with a finely drawn siliconized Pasteur pipette and counted. Plate 1 shows freshly isolated rat islets as viewed under a Wild Leitz dissecting microscope using a modification of the reflected green light technique of Finke et al. (104).

4.1 (b) Canine and Human Islets

After an overnight fast the adult mongrel dogs of both sexes, were given 32 mg/kg sodium pentobarbital, endotracheally intubated, an intravenous line established, and the abdomen prepped with a povidone-iodine solution. A midline abdominal incision was made and the pancreas was mobilized with all major vascular connections maintained. Cannulas (PE90) were inserted into the right and left branches of the main duct and into the left duct via a cutdown approximately 6 cm from the distal end. Blood vessels were then clamped and divided, and the pancreas was removed, weighed and placed in chilled HBSS. The ducts were immediately filled by injection slowly through each of the cannulas

with a total of 150 mL of chilled HBSS, containing 0.5 mg/mL collagenase (Sigma type XI, 2200 U/mg, St. Louis, MO), and brought to the islet laboratory for the isolation of intact islets.

Human pancreases were obtained from heart beating cadavers in Edmonton and the Southwest Organ Bank (Dallas, Texas). During harvest, the gland was cooled and flushed by an infusion of chilled Collins solution, packaged in a sterile manner and transported on ice to the islet laboratory.

Islets were isolated with a modification of the technique devised by Warnock et al. (105) and is outlined in Figure 3. Human pancreatic ducts were exposed by a vertical incision in the middle of the gland and the divided ends of the duct were cannulated (16 gauge, Medicut). The cannulas of the dog and human pancreases were connected to a recirculating perfusion apparatus and perfused with chilled HBSS containing 0.5 mg/mL collagenase (Sigma type XI, 2200 U/mg, St. Louis, MO) (dogs) or 1.0 mg/mL collagenase (Sigma type XI, 2200 U/mg, St. Louis, MO) in HBSS containing 5 mM calcium chloride (humans) at a pressure of 250-300 mm Hg (dogs) and 80-100 mm Hg (humans).. After 10 min, the temperature of the perfusate was increased slowly to 37°C and the perfusion was continued until the gland became soft and mucoid (10-12 min for dogs, 14-16 for humans). The digested tissue was transferred to a beaker containing HBSS, teased apart with forceps, washed (3 X's), resuspended in HBSS and mechanically dispersed by trituration through graded needles (14-, 16-gauge). The tissue was combined, centrifuged and resuspended in chilled Medium 199 (4 mL/mL of digested tissue) with 25 mM Hepes, 10% FCS 100 U/L penicillin and 100 μg/mL streptomycin (PS).

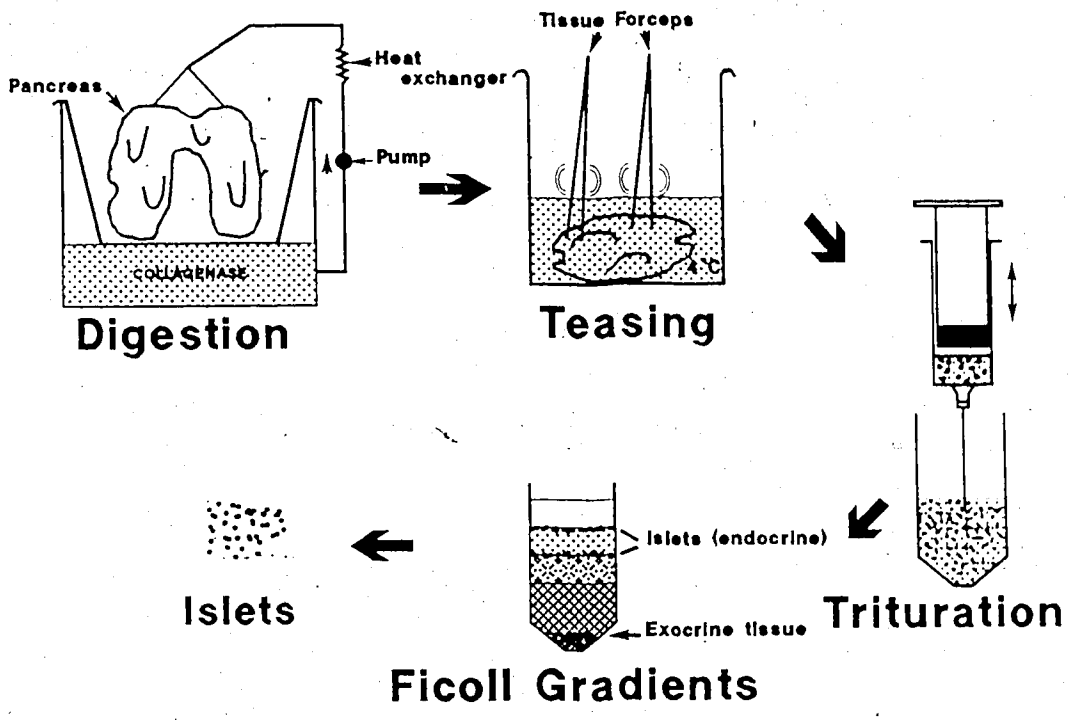

FIGURE 3 ISOLATION OF CANINE AND HUMAN ISLET OF LANGERHANS

Ficoll purification of islets was achieved by placing 4 mL aliquots of tissue suspended in culture medium into 50 mL conical tubes, adding 4.3 mL of Ficoll (density 1.125, Sigma, St. Louis, MO), gently vortexing and overlaid with 4 mL aliquots of Ficoll with densities 1.085, 1.075 and 1.045. The tubes were centrifuged at 550 g for 25 min, tissue removed from the 1.045/1.075 and 1.075/1.085 interfaces, washed, combined and suspended to a final volume of 30 mL in Medium 199 with 10% FCS and PS.

A 0.5 mL sample of the islet suspension was combined with 1.5 mL of 1 mg% dithizone (Sigma, St. Louis, MO) and allowed to react for 10 min. When the dithizone stained the islets pink, ten 20 μL aliquots were placed on slides for counting. The number of islets was calculated from the mean number of islets per sample. Plate 2 shows freshly isolated human islets stained with dithizone as viewed under a dissecting microscope.

4.2 DISSOCIATION OF ISLET CELLS

4.2 (a) Rat Islets

The method for dissociating freshly isolated rat islets into single cells employed calcium removal, mechanical force and enzymatic disaggregation. All solutions contained 2.8 mM glucose, 10 mM Hepes, and 0.2% bovine serum albumin (BSA, Sigma, St. Louis, MO), and are filtered through a 0.22 μm filter for sterilization.

Isolated islets were washed with three sedimentations in calcium-free HBSS (Gibco, Grand Island, NY) containing 1 mM EDTA (Sigma, St. Louis, MO), with 1000 islets/5 mL. The islets were then gently aspirated in this suspension using a siliconized Pasteur pipette (size 9

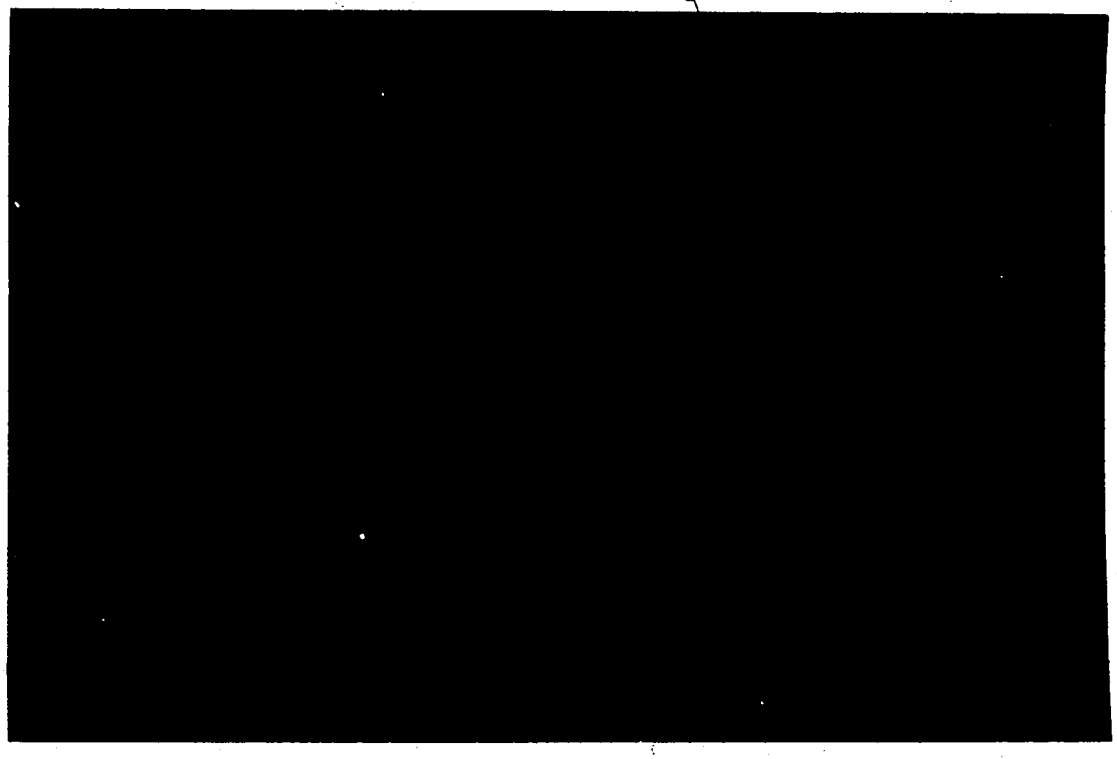

PLATE 2 Freshly isolated human islets of Langerhans stained with Dithizone (25X).

in) for 8 min at room temperature. This suspension was then supplemented with trypsin (Gibco, 1:250) to a final concentration of 25 μg/mL and 2 μg/mL bovine pancreatic deoxyribonuclease (DNase, Sigma, St. Louis, MO). The aspiration of the suspension was then continued at 30°C until approximately 50-60% of the cells appeared single (10-14 min), as observed under an inverted light microscope. The cells were then diluted with cold HBSS (no calcium), filtered through a 60 μm nylon screen, centrifuged at 300 g for 6 min and resuspended in Medium 199 (10% FCS, 0.2% BSA, PS). Islet cells were then placed in an incubator (37°C under 5% CO_2) until further studies were carried out.

4.2 (b) Canine and Human Islets

The dissociation of freshly isolated or cultured canine and human islets was also based upon calcium removal, mechanical force in conjunction with enzymatic dispersion. All solutions were prepared and sterilized as with those of dissociating freshly isolated rat islets. Approximately 5000 islets were incubated at room temperature for 60 min in 10 mL of HBSS (no calcium), supplemented with 3 mM EGTA (Sigma, St. Louis, MO). The incubating media was then removed and a freshly prepared solution of 1 mg/mL trypsin (Gibco, 1:250) plus 5 μg/mL DNase was added. The islets were aspirated several times through a Pasteur pipette, incubated at 37°C for 6 min, then disrupted by gentle aspirations through 16-, 18- and 20-gauge needles successively until a single cell preparation was obtained, as determined by microscope inspection. Subsequent steps in the dissociation of these islets were identical to those following the trypsin digestion of the fresh rat islets.

This islet cell preparation protocol was also utilized for obtaining single cell suspensions of cultured rat islets. Figure 4 illustrates the two protocols used for islet dissociation, both employing calcium removal, mechanical force and enzymatic activity.

4.3 FLOW CYTOMETRIC (FC) ANALYSIS

4.3 (a) Equipment and Media

FC analysis of the freshly dissociated islet cells was performed using an EPICS V Cytometer (Coulter Electronics, Hialeah, Florida), equipped with a Coulter Volume Apparatus (CVA). An argon laser (Innova 90 Series, Coherent Laser Products Division, Palo Alto, CA) with the excitation wavelength set at 488 nm, was used throughout the FC analysis. Laser power was controlled automatically at a constant output of 300 mW. The details of PMT high voltage and settings, optical filter setups and gating parameters are included in Appendix A.

The islet cells suspended in RPMI (Gibco, Grand Island, NY) medium, supplemented with 2 μg/mL DNase, 0.2% BSA, 2.8 mM glucose and 25 mM Hepes, were stored on ice until analysis. Once transferred to the sample vessel, the islet cells were gently but continuously stirred. A 70 μm nozzle was selected for the dispersion of the aspirated cells in a narrow fluid stream, utilizing normal sterile saline as the sheath fluid. Reaggregation of the 75% single cells was minimized by keeping cell concentrations under 5 x 10^5/mL, and occasionally submitting them to gentle pipetting. The cells could now be individually analyzed for several parameters.

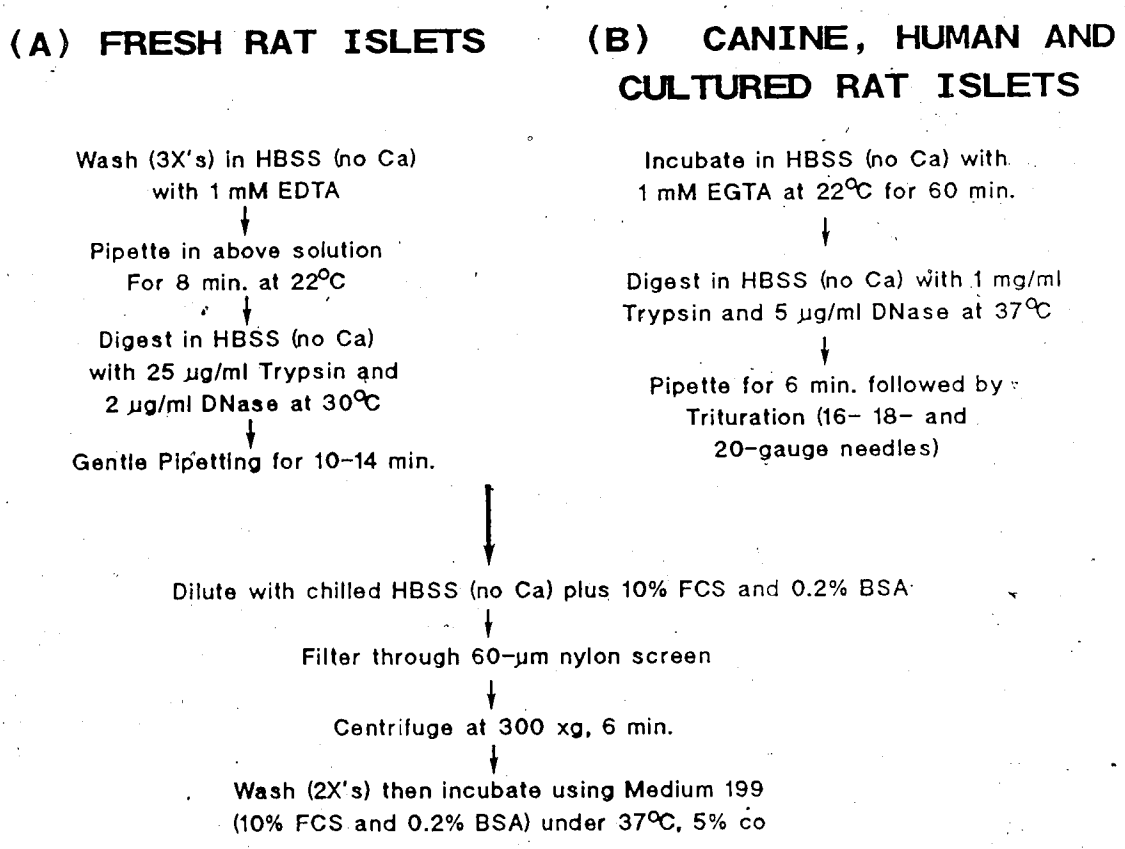

FIGURE 4 PROTOCOLS FOR DISSOCIATING ISLETS

4.3 (b) Data Analysis

Experiments to determine the feasibility of purifying A and B cells, or to identify these cells, were initially conducted before attempts were made at sorting these cells. The intrinsic parameters: volume, FALS, 90 LS, and endogenous auto-fluorescence were measured to identify various differences among the heterogeneous islet cell suspension.

Cellular autofluorescence (or FAD) was activated at 488 nm then detected by measuring the emitted fluorescence between 510 and 550 nm. This was accomplished by using a suitable band pass interference filter, and collecting the log-integrated signal. Light scatter was measured as near forward scattered light intensity (3°-13°) in order to eliminate other scattered light, and at 90 LS. Cell volume was detected with the CVA, with all the current and gain settings listed in Appendix A.

The converted and amplified signals resulting from measuring the intrinsic parameters were plotted on a visual display. A dot plot histogram was constructed by choosing gain and high voltage settings to place the peaks of the histograms in the middle of the 256 channel range. To eliminate contaminating debris from the analysis of the the other parameters, data accumulation gates were set to exclude the lower channels in both FALS and volume histograms.

4.4 PURIFICATION OF ISLET CELL FRACTIONS

4.4 (a) Setting up the FACS

The CVA was dismantled the flow cytometer and in its place a flow chamber was inserted, ch the capability of sorting cells. Deflection plates were inserted, their charging wires connected and

allowed to warm up. A two- droplet sort pulse in conjunction with a 15 msec delay were employed. Once deflecting streams were established via the deflection plates, the droplet breakoff point was established to coincide with the preset delay. This enabled all cells being sorted to deflect properly, once they had been charged. Hence, this initial procedure fine tuned the FACS.

4.4 (b) Cell Sorting

The sorting procedure is based upon that of Pipeleers (23), using FALS in conjunction with FAD and NAD(P)H auto- fluorescence. Equipment, media and cell sorter specifications are the same as those reported in FC analysis.

Freshly dissociated islet cells were submitted to FACS analysis, illuminating the cells at 488 nm band collecting FALS and FAD autofluorescence (emission at 510-550 nm). Islet cells exhibiting high FAD autofluorescence and high FALS were sorted in one direction, while the cells with lower FAD and FALS intensities in the other direction. This procedure was carried out at 17°C (sample and sheath fluid) and 2.8 mM glucose. Cells were collected in RPMI media containing 10% FCS.

A second purification step was conducted on the islet cell population which had been isolated on the basis of their lower light scatter activity and FAD autofluorescence. At 20 mM glucose (10 min pre-exposure) and 37°C the cells were sorted using an ultra violet source (351-363 nm) for illumination then measuring emission between 400 and 470 nm. At these wavelengths NAD(P)H autofluorescence is being detected. Under these conditions, the cell population with low NAD(P)H fluorescence was sorted, while the remaining cells were discarded.

Therefore, three different cell populations were obtained from FACS: (1) high FAD fluorescence - high FALS intensity (I-B), (2) low FAD fluorescence - low FALS intensity (I-A), and (3) low NAD(P)H fluorescence (II-A). All three populations were subjected to qualitative and quantitative analysis.

4.5 QUALITY CONTROL

4.5 (a) Quantitative Analysis

To assess the efficacy of both the dissociation and purification procedures, cell recovery was determined by the total cell number and the degree of cell coupling, in a hemocytometer (Bright Line, American Optics, Buffalo, NY). For the purified A and B cells, counting of these purified populations was also integrated by the cell sorter.

4.5 (b) Qualitative Analysis

Fluorescein diacetate (FDA, Sigma, St. Louis, MO) and ethidium bromide (EB, Sigma) were used as indicators of membrane integrity. FDA is a nonpolar compound and readily passes into the cell where it is hydrolized by esterases to form a green fluorescein. This product is polar and cannot cross the intact plasma membrane and therefore accumulates intracellularly. EB, in contrast, does not penetrate intact membranes, but once inside a cell, it complexes with double stranded nucleic acids to form a red fluorescein (106,107).

FDA was prepared as a 1×10^{-4} M stock solution in acetone and stored at -20°C. The working solution was prepared by a 1/100 v/v dilution of the stock preparation with HBSS. The final concentration of FDA in the cell suspension was 10 μM. EB was dissolved in ethanol to a

concentration of 2.5 x 10^{-4} M and stored in the dark at 4°C. A working solution of EB was prepared as FDA, to a final concentration of 25 μM in cell suspension. Samples of both freshly dissociated and purified islet cells were then examined under a Leitz fluorescent microscope to determine the number of FDA and EB positive cells. The viability of the cells was also tested by assessing their survival after a 24 h culture period (37°C under 5% CO_2) in Medium 199 (10% FCS, 100 mg% glucose).

4.6 COMPOSITION OF PURIFIED ISLET CELLS

Pancreatic islet cells were tested for their cell composition using an immunoperoxidase technique based on the methods of Sternberger (108) with modifications. Approximately 50,000 unpurified cells were allowed to settle on to poly-L-lysine (PLL, Sigma, St. Louis, MO) coated slides, and were used for negative and positive controls. The purified cell fractions obtained via the FACS were directly sorted on the PLL slides, at 10-15,000 cells/slide. Once the cells were settled onto the slides, they were fixed in Bouins for two hours, washed in absolute alcohol and stored in 70% alcohol at 4°C until immunocytochemistry was performed.

The samples were stained for the presence of insulin, glucagon, somatostatin, pancreatic polypeptide and amylase. Included in each series was a positive control for each hormone being detected, plus a negative control for the series. Slides were incubated with guinea pig antiserum to human pancreatic insulin (Dako, Cedarlane Laboratories), rabbit antiserum to synthetic glucagon (Immunonuclear Corp., Stillwater, Minn.) and somatostatin (Dako, Cedarlane Laboratories), rabbit antiserum to bovine pancreatic polypeptide (Milab, Malmo, Sweden), and rabbit antiserum to human amylase (Sigma, St. Louis,

MO). These primary reagents (PRA, Figure 5) were incubated on the slides for 48 hours at 4°C.

Linking reagents (LRA) were protein A (E-Y Laboratories, San Mateo, CA) for insulin and goat antirabbit IgG (Zymed, Cedarlane Laboratories) for all other hormones. After a 60 min incubation at room temperature, excess LRA was washed off with saline, and the developing reagent (DRA) rabbit peroxidase-antiperoxidase (Dako, Cedarlane Laboratories) was added for 30 min at 4°C. Following this incubation, slides were washed, DAB with peroxidase was added to slides, washed off after 8 min and slides were mounted.

Slides were later analyzed to determine the cell composition of the purified fractions obtained from FACS. Routinely, 5-700 cells were counted per slide to determine the hormone content or cell composition in each sorted fraction.

The hormone content of the purified cell fractions was measured in acid extracts of the cells. Samples of 5-10,000 cells were placed in acid-alcohol (sulfuric acid-ethanol) for 24 h at 4°C. Samples were then centrifuged (1000 g, 15 min), supernatants fractions neutralized to a pH of 7.3 with saturated sodium bicarbonate. The samples were collected and insulin-glucagon concentrations were measured by double-Ab radioimmunoassay.

1. Immobilized Ag

wash

2. Addition of PRA

wash

3. Addition of LRA

wash

4. Addition of DRA

wash

5. Addition of DAB

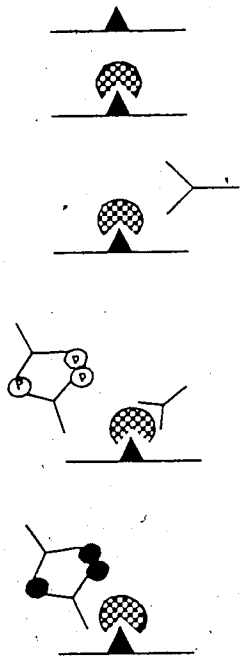

FIGURE 5 ICC TECHNIQUE FOR PURIFIED CELL FRACTIONS

PRA = PRIMARY REAGENT, LRA = LINKING REAGENT

DRA = DEVELOPING REAGENT

4.7 DITHIZONE STAINING OF ISLET CELLS

A non-complexing forming dithizone solution was used to stain the single pancreatic islet cells. Dithizone (Sigma, St. Louis, MO) was dissolved in ethanol and stored at $4^{o}C$ as a stock solution. A final working solution was prepared by diluting the stock solution with Krebs Ringer Solution to a final working concentration of .0003%. Purified non-B cells and B cells from both rat and canine pancreatic islets were sorted into the depression of a hanging drop slide. This depression was then filled with the dithizone solution and allowed to incubate for approximately 10 minutes. After the incubation period the number of red cells or cells staining with dithizone were calculated for each cell fraction under a light microscope. Approximately 300 islet cells were assessed per sample and a total of five samples were collected for each cell fraction being tested.

Due to difficulties of visualizing the red coloration within the individual islet cell the same procedure was performed on pellets of approximately 50,000 cells to determine if dithizone stained the cells. This was used only as a second measure of the specificity of dithizone for the islet cells.

CHAPTER FIVE

RESULTS

5.1 PREPARATION OF ISLET CELLS

Fresh rat islets dissociated by method A (Figure 4) routinely provided a predominantly (80 - 95%) single cell preparation of 1250-1500 cells per islet. After a short incubation, dissociated cells assume a spherical form as illustrated in Plate 3. Cell viability was 90-95%, determined by incubating the cells in FDA-EB and counting viable cells (green fluorescein) in a hemocytometer chamber using fluorescence microscopy (Plate 4). Cultured rat islets (24-48 hours) provided a 75-80% single cell population with 1000-1200 cells per islet. Cell viability was also 90-95%, with assessment using FDA-EB.

Cultured (1-7 days) or freshly isolated canine and human islets prepared by method B, produced a 80-95% single cell preparation of 950-1100 cells per islet. These dissociated islet cells also possessed a spherical form and a viability of 90-95%. Table 2 summarizes the results obtained from the above sources of intact islets.

5.2 FLOW CYTOMETRIC ANALYSIS OF ISLET CELLS

Data is presented as one and two parameter histograms, with data aquisition gates set using only the FALS distribution for each experimental condition, to eliminate debris from the analysis of the other parameters.

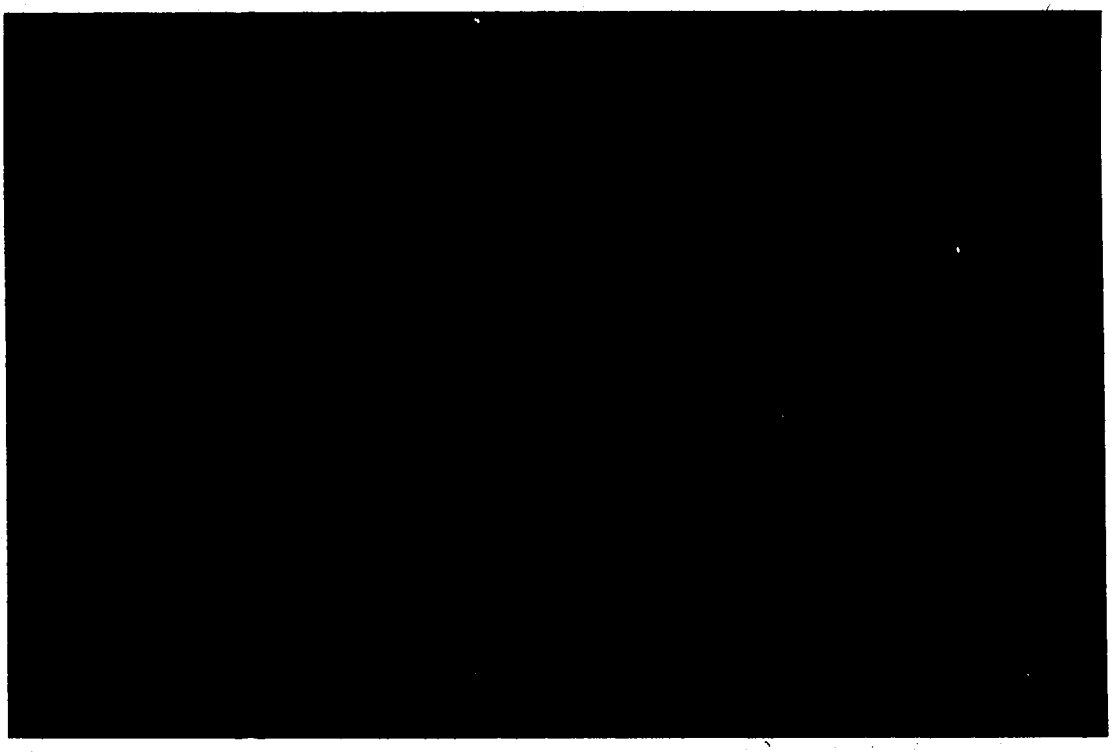

PLATE 3 Dissociated rat islet cells after a 20 min. incubation (100X).

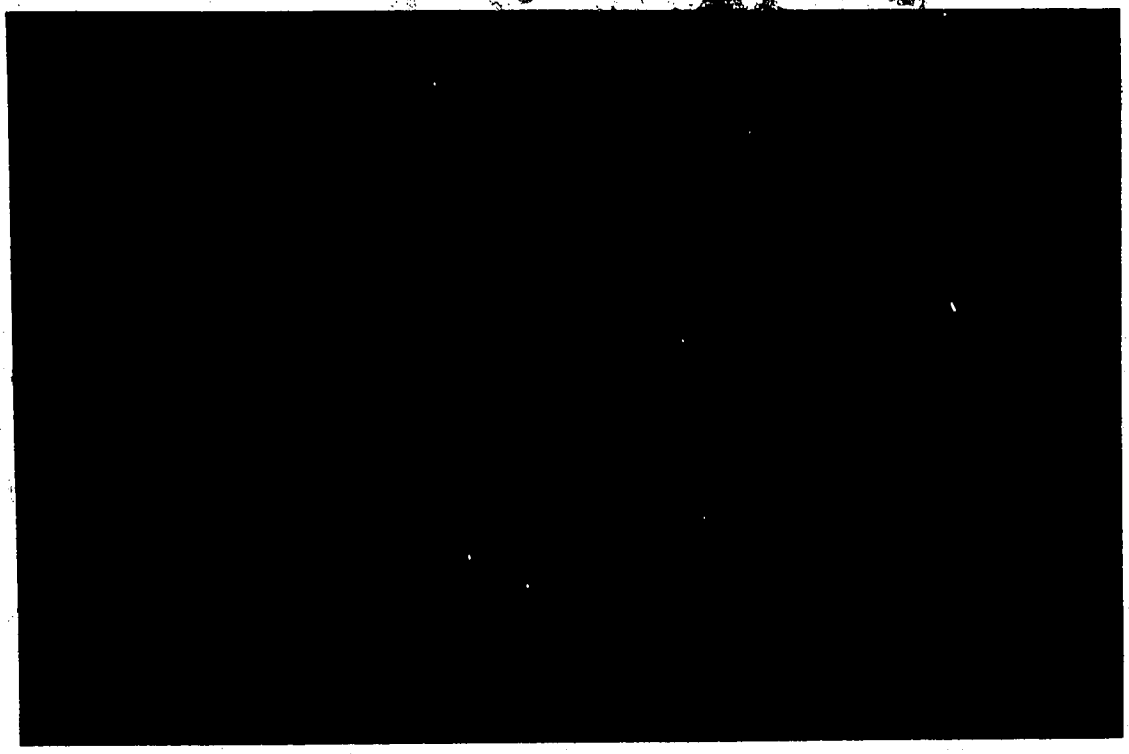

PLATE 4 Fluorescein diacetate assay of rat islet cells (100X).

TABLE 2
DISSOCIATION OF INTACT ISLETS

Intact Islets	% single[a]	cells/islet[a]	viability%[b]
Fresh Rat	80-95	1250-1500	90-95
Cultured Rat	75-80	1000-1200	90-95
Canine-Human	80-95	950-1100	90-95

a = Determined in hemocytometer

b = FDA-EB Assay

5.2 a) Rat Islet Cells

When dissociated rat islet cells (obtained from fresh and cultured intact islets) were analyzed in a RPMI-Hepes buffer at room temperature and 2.8 mM glucose the intensity distribution of light scattered at a small forward angle (FALS) generated a reproducible histogram of four peaks (Figure 6-a). The peak farthest to the left was produced mostly by cellular debris, whereas the peak farthest to the right from coupled cells. Therefore, FALS yields two distinct populations of single islet cells, one narrow peak with low light scatter intensity and a broader peak expressing higher FALS intensity.

Volume measurements of the rat islet cells revealed the presence of two well defined groups of cells with varying degrees of volume distributions. The cellular debris does not appear in the volume histogram (Figure 6-b) as it was eliminated by "gating" on FALS. These two populations found in both FALS and volume one parameter histograms are further defined and correlated using two parameter histograms (Figure 7-c). Analysis of 90LS (Figure 8-b) generates only one broad peak with no definition of individual cell populations.

When endogenous autofluorescence or FAD content was detected at 2.8 mM glucose two discrete populations were observed. Figure 8-a displays the FAD content, with the initial peak corresponding to low FAD, whereas the second peak represents high FAD levels. When two parameter histograms of FAD versus FALS and Volume were constructed (Figures 7-a and 7-b, respectively) two distinct populations were easily discerned. However, when the islet cells were analyzed at 20 mM glucose (Figure 9-a), endogenous autofluorescence of FAD content produced only one peak.

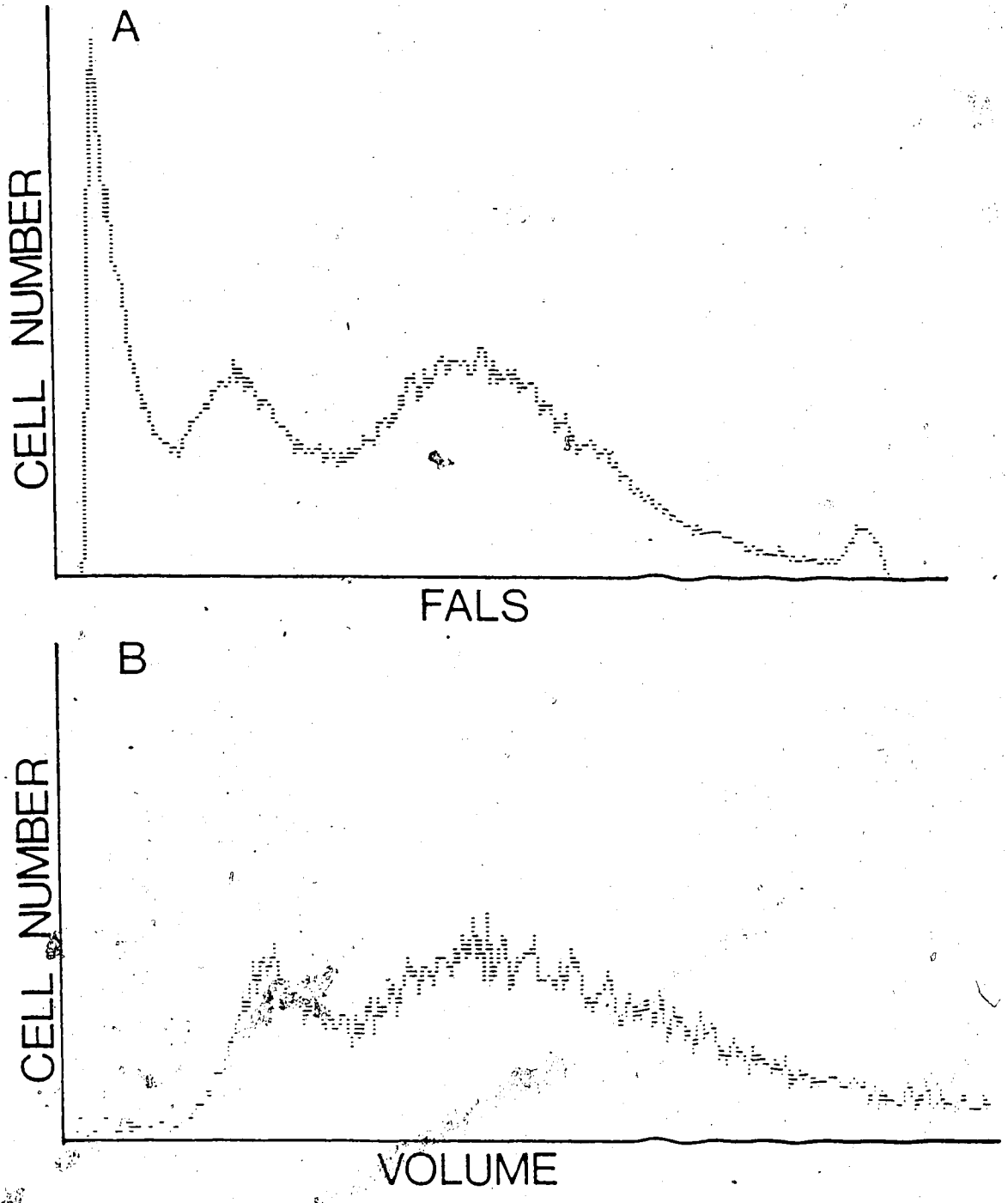

FIGURE 6 One parameter histograms of FALS (total cell number 75,000) (A) and Cell Volume (total cell number 75,000) (B) of rat islet cells at 2.8 mM glucose.

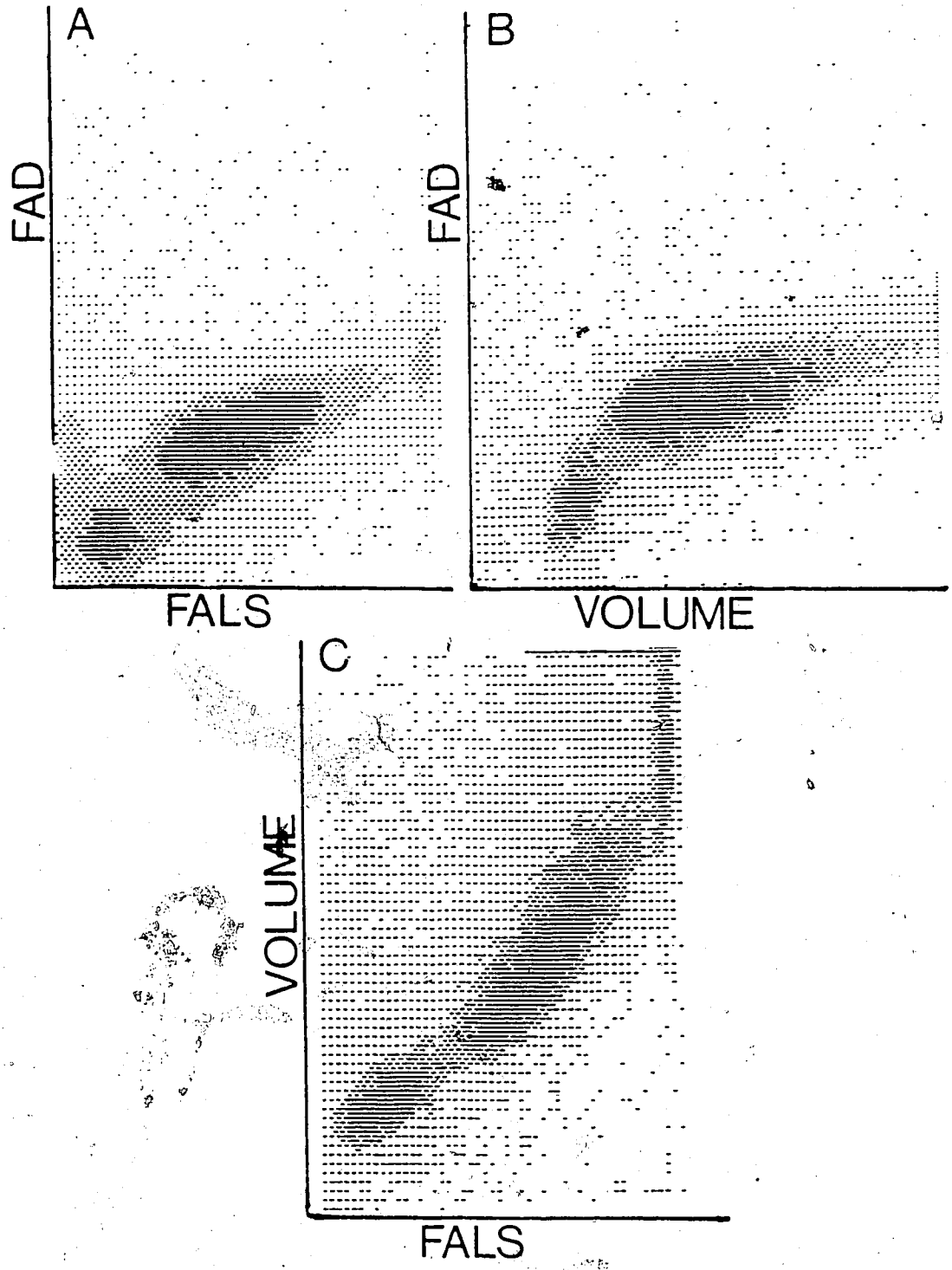

FIGURE 7 Two parameter histograms of FAD vs FALS (A), FAD vs Volume (B), and Volume vs FALS (C) of rat islet cells at 2.8 mM glucose. Total cell number for each histogram is 75,000.

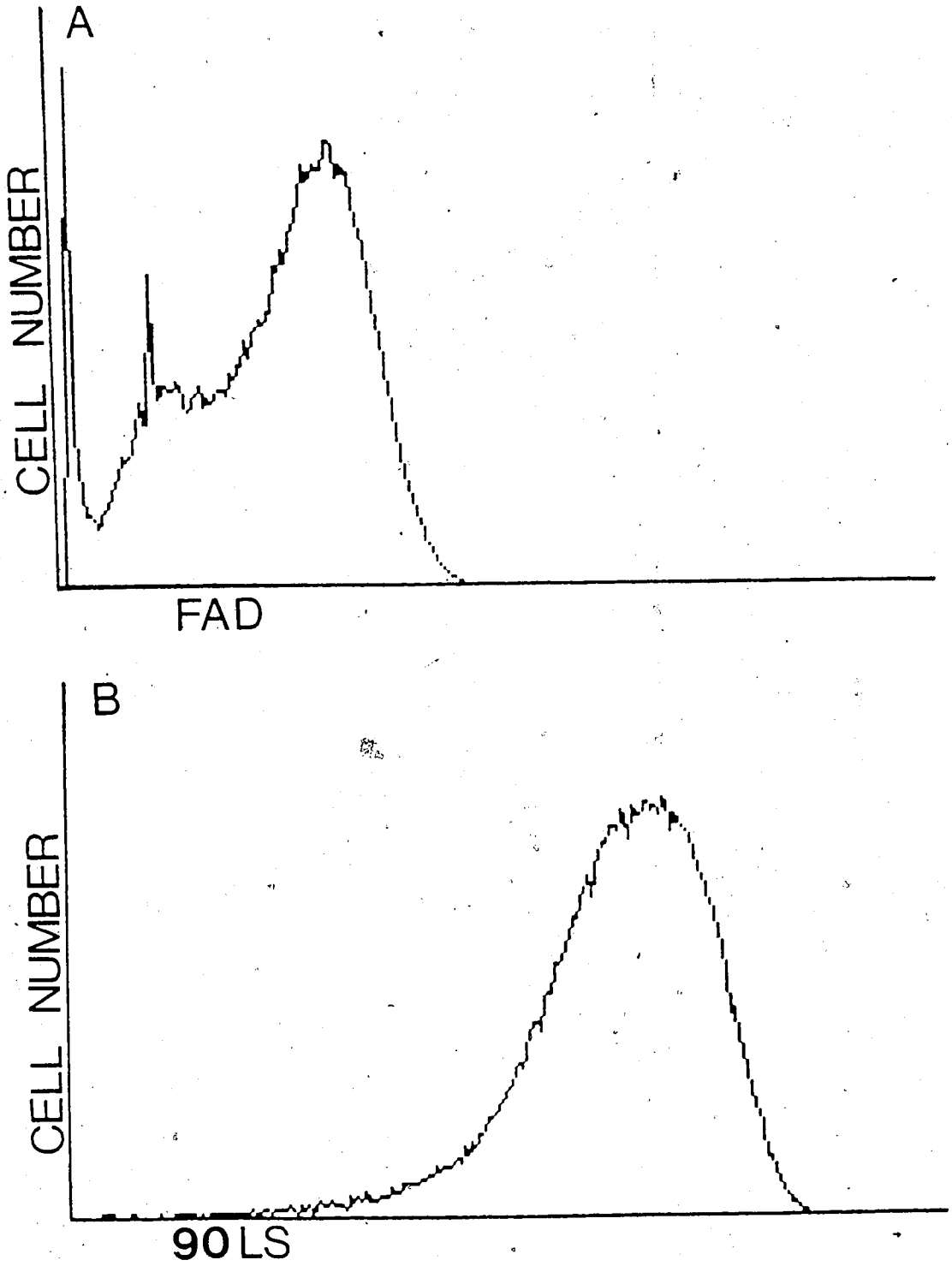

FIGURE 8 One parameter histograms of FAD autofluorescence (total cell number 75,000) (A) and 90LS (total cell number 50,000) (B) of rat islet cells at 2.8 mM glucose.

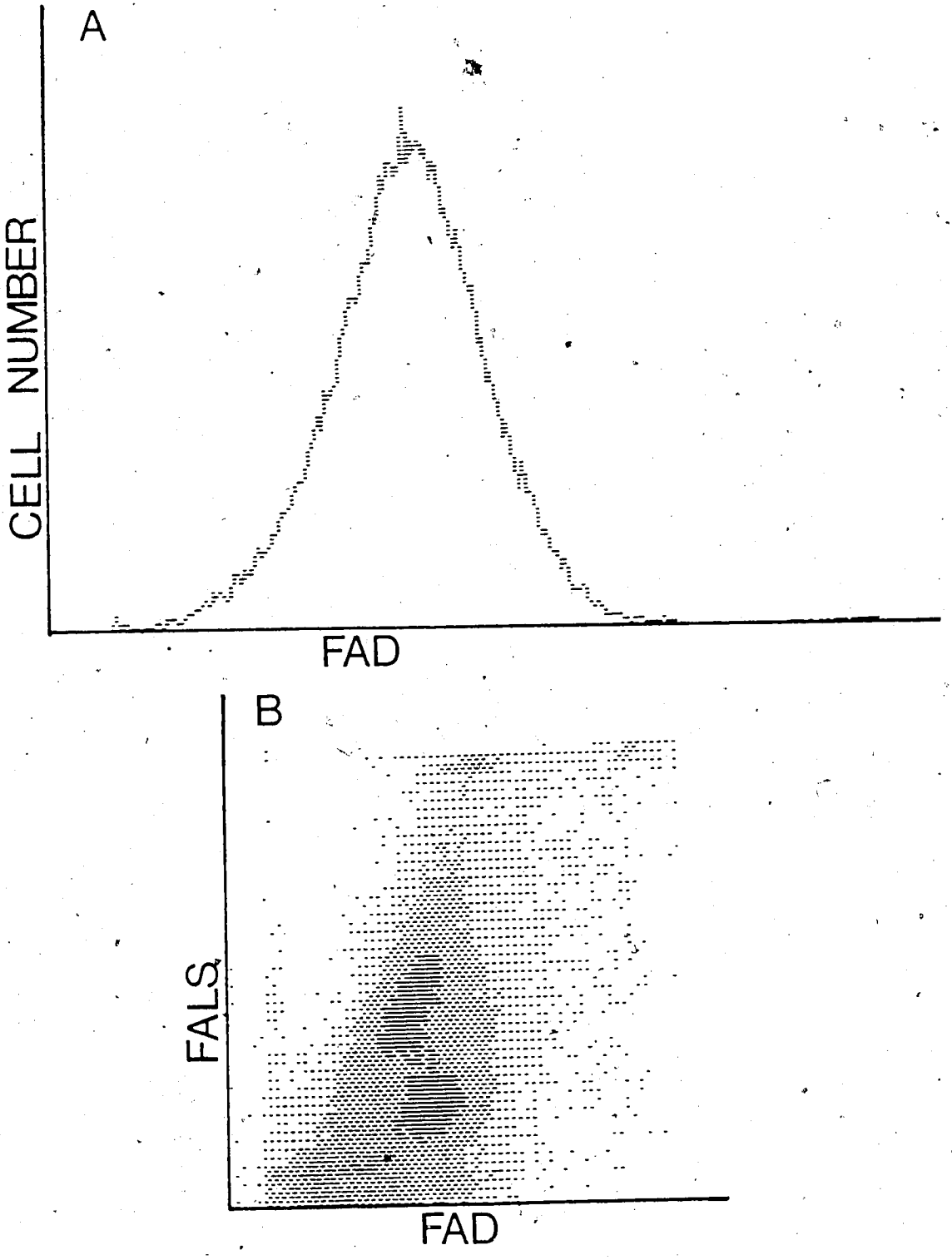

FIGURE 9 One parameter histogram of FAD autofluorescence (A) and a two parameter histogram of FALS vs FAD (B) of rat islet cells at 20 mM glucose and 37°C. Total cell number for each histogram is 75,000.

Figure 9-b illustrates this affect of high glucose, causing only one peak in FAD fluorescence but maintaining the two normally found in FALS.

In summary, flow cytometric analysis of rat islet cells at 2.8 mM glucose displays two peaks or cell populations in FALS, volume and FAD autofluorescence measurements, but only one in 90LS. These were believed to be the non-B cells (lower FALS intensity, volume and FAD content) and the B cells (higher FALS intensity, volume and FAD content).

5.2 b) Canine Islet Cells

When unpurified canine islet cells were analyzed under the same conditions as rat islet cells, the same two populations were immediately detected. FALS intensity of canine islet cells, however, did not express the two distinct populations as well in rat islet cells. (Figure 10-a). The initial peak corresponding to cellular debris was present but further definition of the two single cell populations seen in rat islet cells was not evident, as only one narrow peak was seen with a small shoulder.

Autofluorescence of canine islet cells exhibited two well defined cell populations, with an initial small peak followed by a larger peak with two distinct shoulders. Figure 10-b represents the FAD content with the low FAD population appearing as a small peak or shoulder next to the larger peak of high FAD content. The first peak in Figure 10-b was not a measurement of FAD autofluorescence, as at 20 mM glucose (Figure 11) this peak is still present and unaltered. However, at 20 mM glucose the larger peak has shifted to lower fluorescence intensity or FAD content, thus producing only one peak with no shoulders. Figure

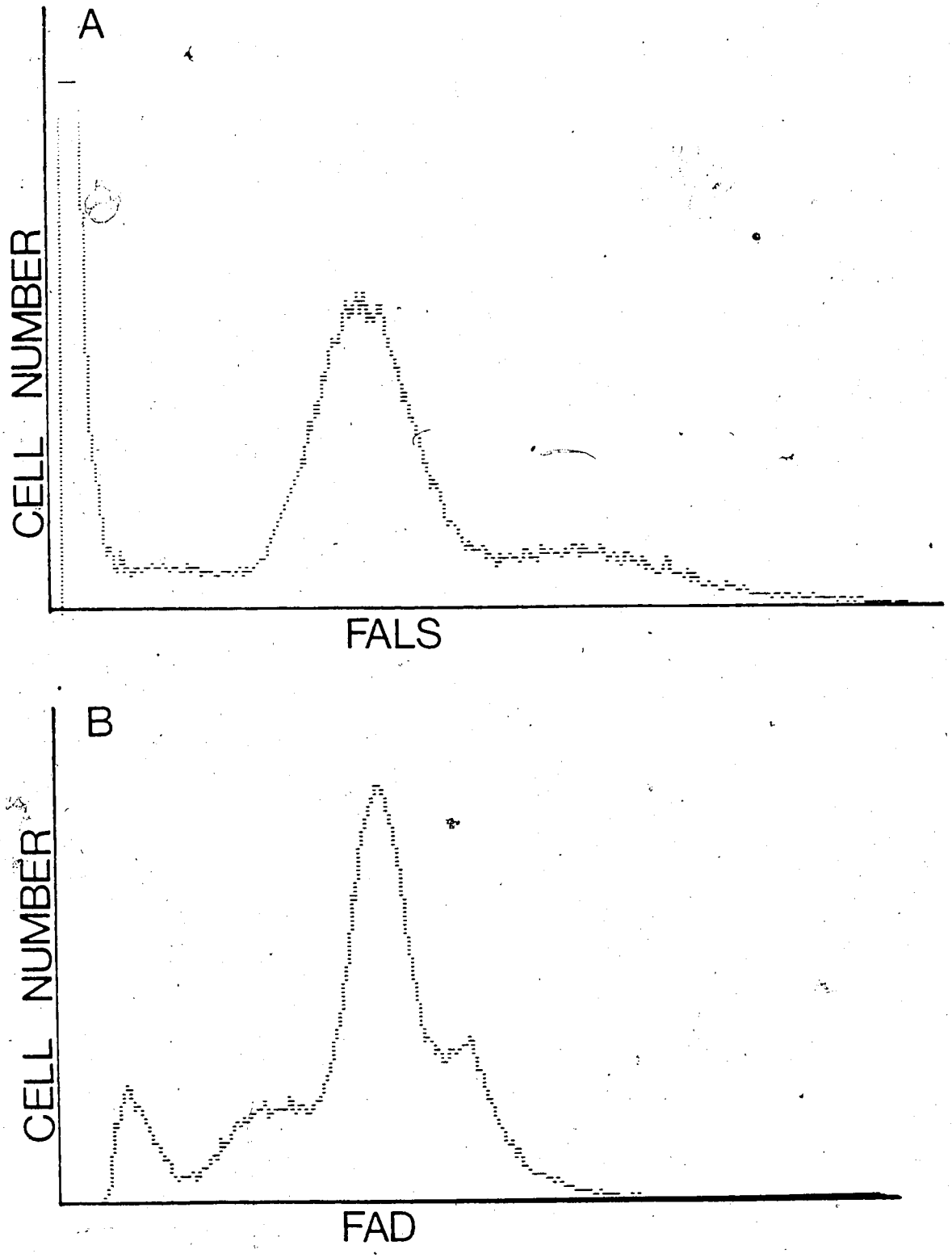

FIGURE 10 One parameter histograms of FALS (A) and FAD (B) of canine islet cells at 2.8 mM glucose. Total cell number for each histogram is 75,000.

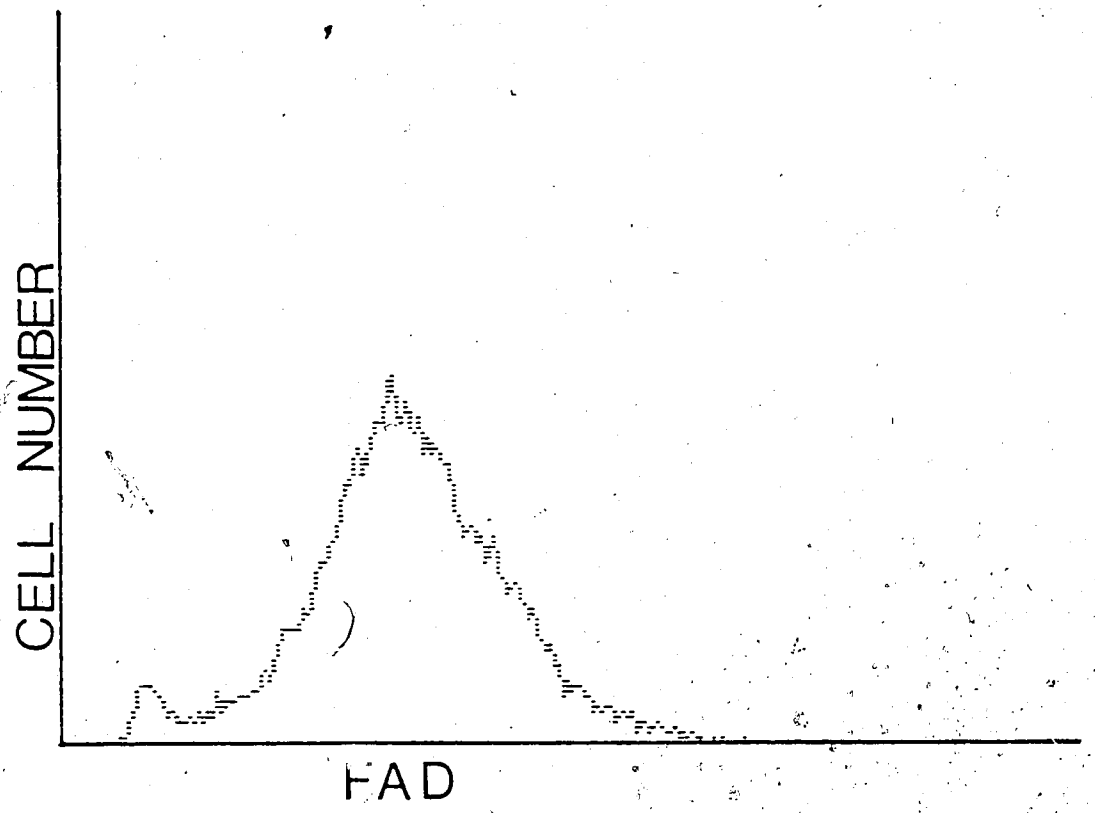

FIGURE 11 One parameter histogram of FAD autofluorescence of canine islet cells at 20 mM glucose and 37°C. Total cell number is 75,000.

12-a illustrates the two cell populations differing in FAD content at 2.8 mM glucose, but after exposure to 20 mM glucose at 37°C the two populations are no longer distinguishable (Figure 12-b). Therefore, as with rat islet cells, canine islet cells express two peaks or cell populations with different degrees of FAD autofluorescence, but also possess a cell population with a low intensity autofluorescence of unknown origin.

5.2 c) Human Islet Cells

Flow cytometric analysis of dissociated human islet cells was not as successful at distinguishing individual cell populations, as that of rat and canine islet cells. FALS intensity had only one narrow peak of single cells along with the initial peak of cell debris. Volume studies were slightly more defined than FALS, producing two distinct peaks. Figure 13 represents the data obtained from FALS and volume distribution from six separate groups of human islet cells.

Autofluorescence of human islet cells at 2.8 mM shows the presence of two distinct cell populations (Figure 14). The first peak does not appear to represent autofluorescence due to intracellular FAD, as only the second peak is altered by the presence of high glucose with a slight shift to the right (data not shown). Therefore, human islet cells also possess a cell population with low autofluorescent intensity, but exhibit no differences in the cells FAD content. Figure 15 represents a series of two parameter dot plot histograms comparing the various parameters measured.

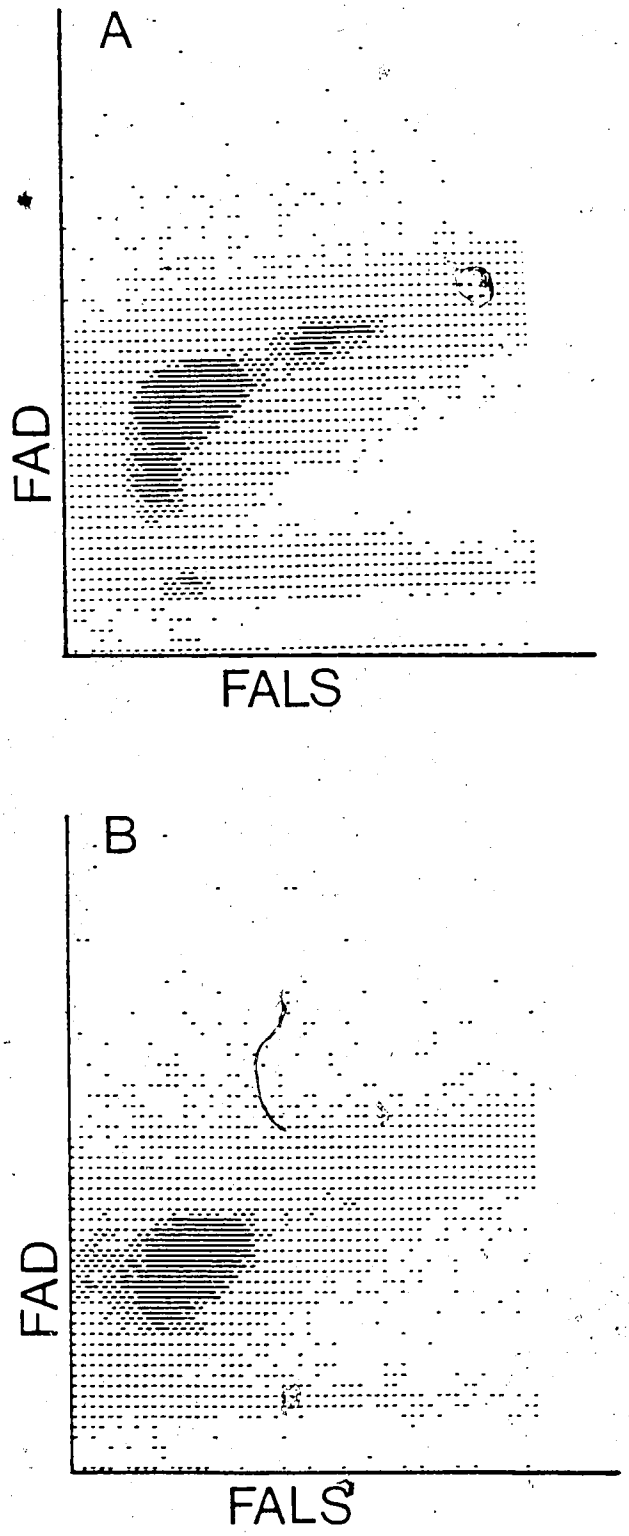

FIGURE 12 Two parameter histograms of FAD vs FALS of canine islet cells at 2.8 mM glucose (A) and 20 mM glucose (B). Total cell number for each histogram is 75,000.

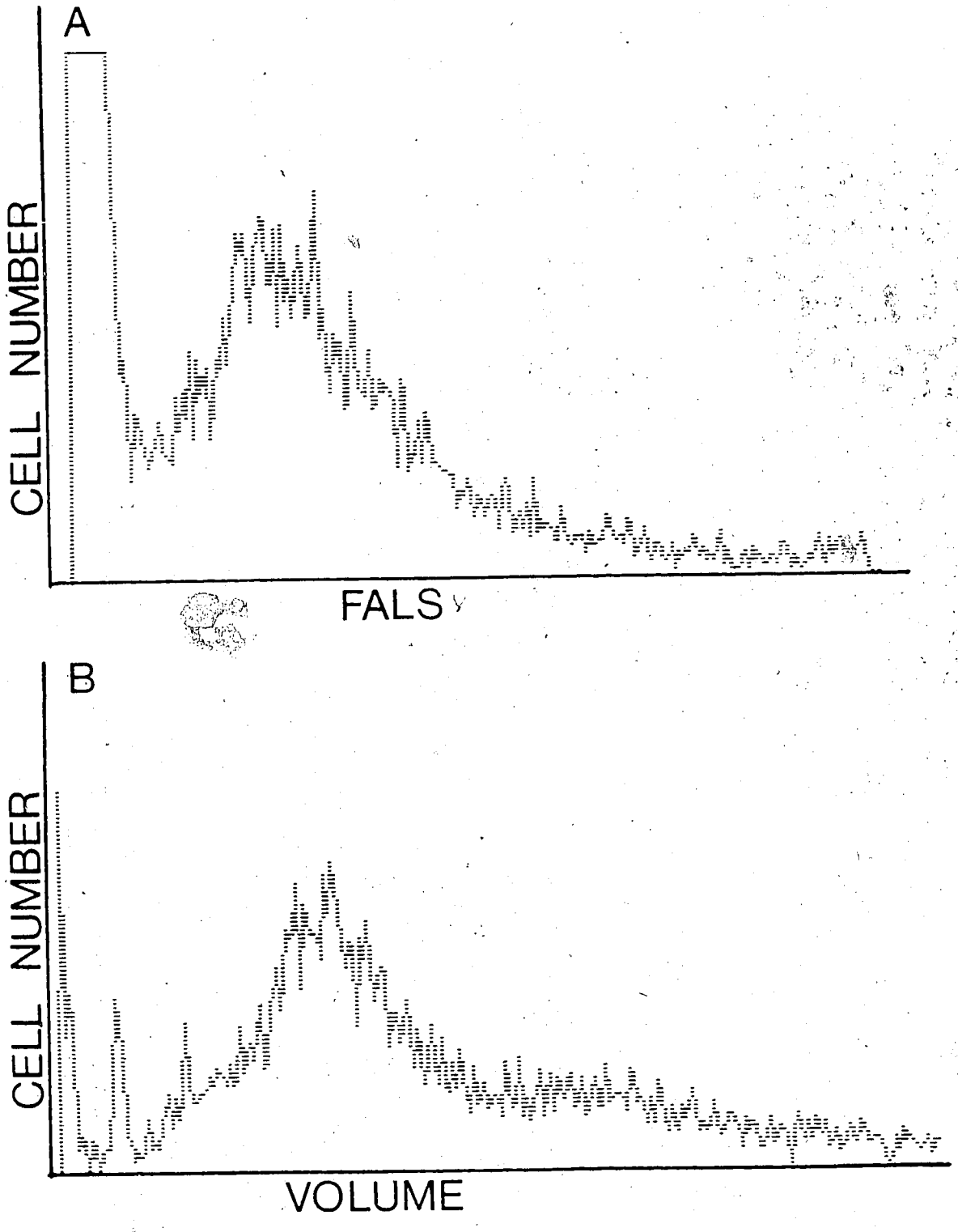

FIGURE 13 One parameter histograms of FALS (A) and Cell Volume (B) of human islet cells at 2.8 mM glucose. Total cell number for each histogram is 30,000.

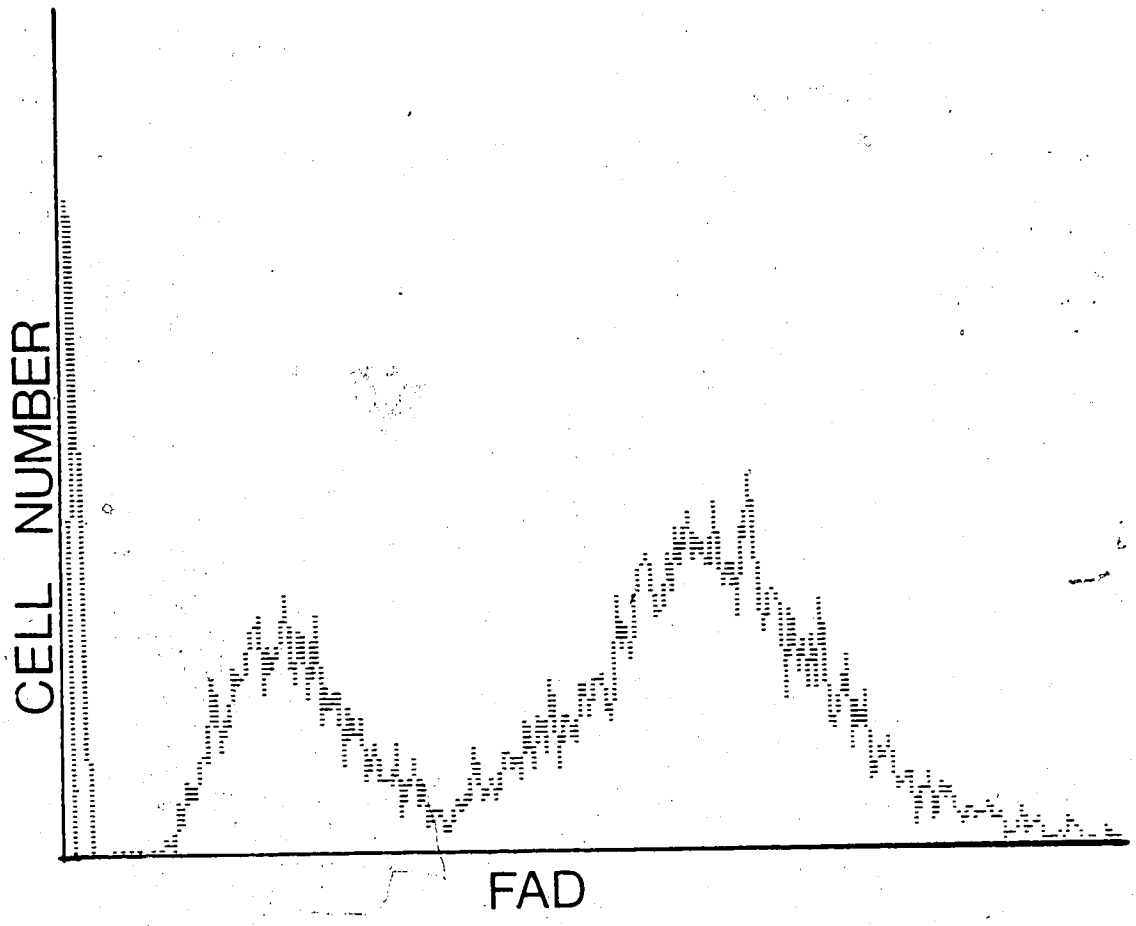

FIGURE 14 One parameter histogram of FAD autofluorescence of human islet cells at 2.8 mM glucose. Total cell number is 30,000.

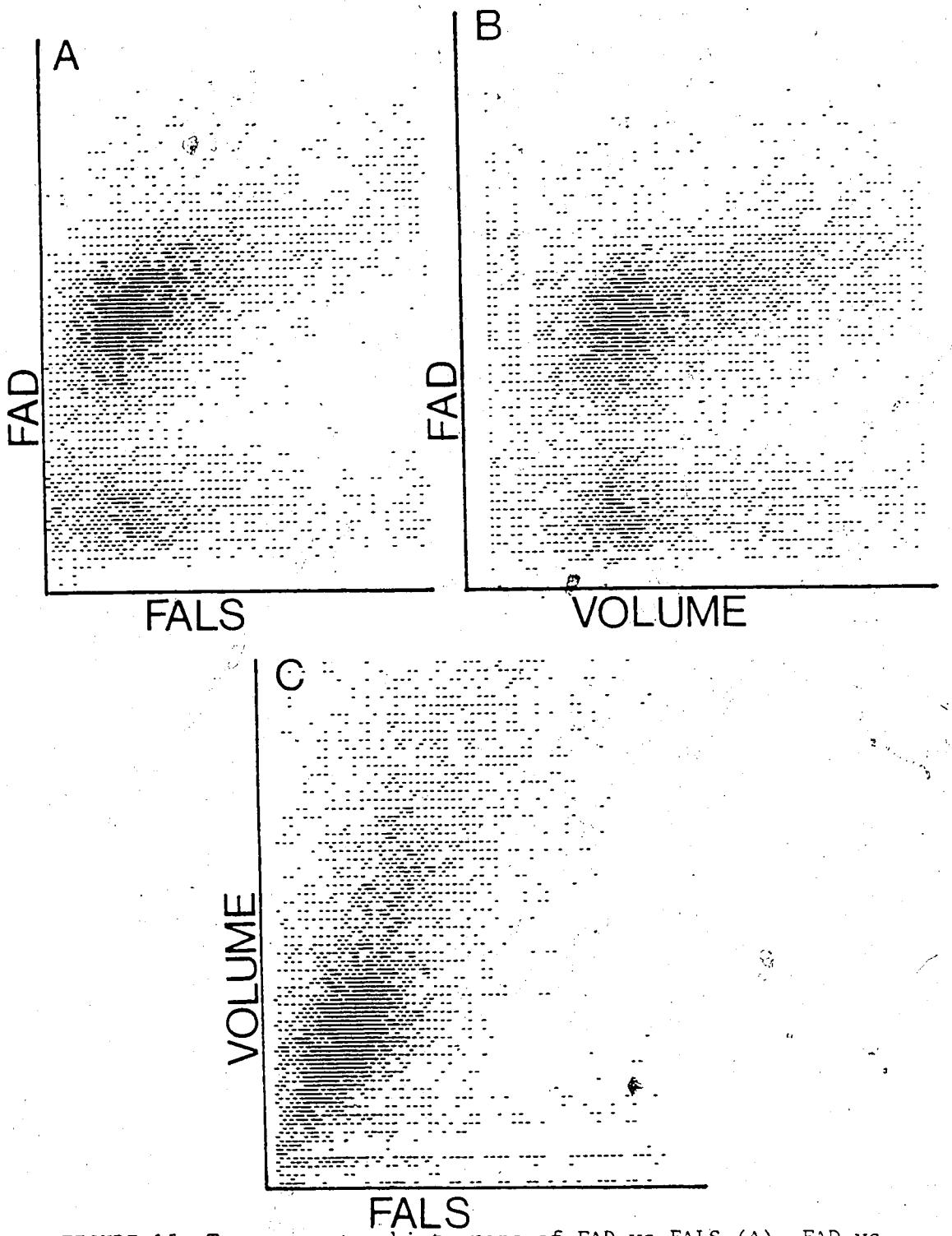

FIGURE 15 Two parameter histograms of FAD vs FALS (A), FAD vs Cell Volume (B), and Cell Volume vs FALS (C) of human islet cells at 2.8 mM glucose.
Total cell number for each histogram is 30,000.

5.3 PURIFICATION OF RAT AND CANINE ISLET CELLS

5.3 a) Sorting of Islet Cells According to FAD-FALS Analysis

At 2.8 mM glucose and 17°C (sample and sheath fluids) two islet cell populations were distinguished according to differences in FAD content and FALS activity. Figures 16 and 17 represent the selected sort windows for sorting rat islet cells, while Figure 18 illustrates those for dog islet cells. These specifications were maintained throughout all experiments.

Two cell fractions were sorted and collected for rat and canine islet cells, while a third fraction was directed to the discard vessel. Fraction I-A with low FAD-FALS intensity, was composed of more than 90% single cells and contained on average 30% of the sorted cells (Table 3 and Figure 16). On the other hand, Fraction I-B, also more than 90% single cells, representing the high FAD-FALS cells, made up 45% of the sorted cells (Table 3, Figure 16). These two fractions together contained approximately 75-80% of the cells in the initial preparation. The remaining 20% that were discarded, appeared as aggregates of five to eight cells. Similar results were obtained for sorting canine islet cells (Table 3).

The cellular composition of each fraction was determined by ICC and acid extractions for hormonal content. When the rat islet cells Fraction I-A was stained for the four pancreatic hormones plus amylase, 80-85% of the cells were found to contain glucagon and only 1-3% were positive for insulin (Table 3, Plate 5). In Fraction I-B, more than 95% of the rat islet cells were found to be insulin-containing B cells

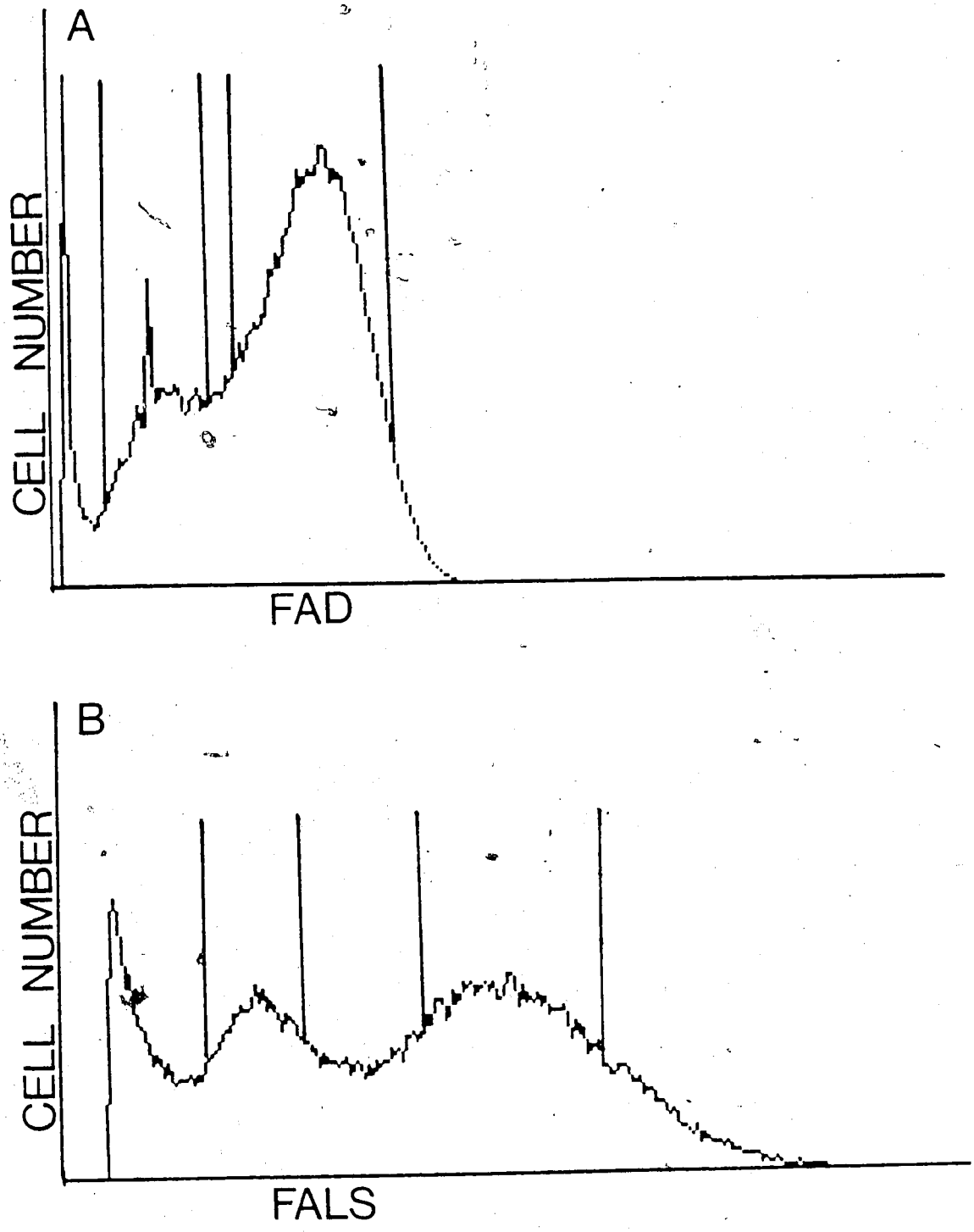

FIGURE 16 One parameter histograms of FAD (A) and FALS (B) of rat islet cells at 2.8 mM glucose and 17°C illustrating selected sort windows.

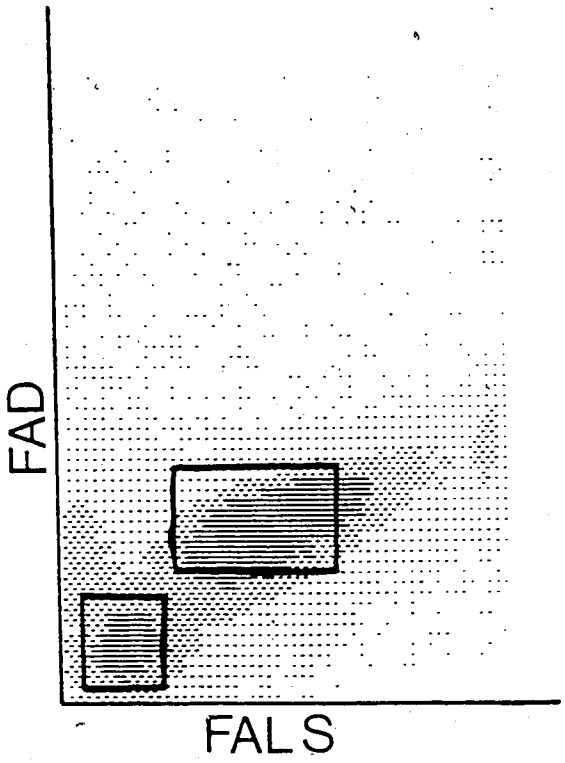

FIGURE 17 Two parameter histogram of FAD vs FALS of rat islet cells at 2.8 mM glucose and 17°C illustrating selected sort wimdows.

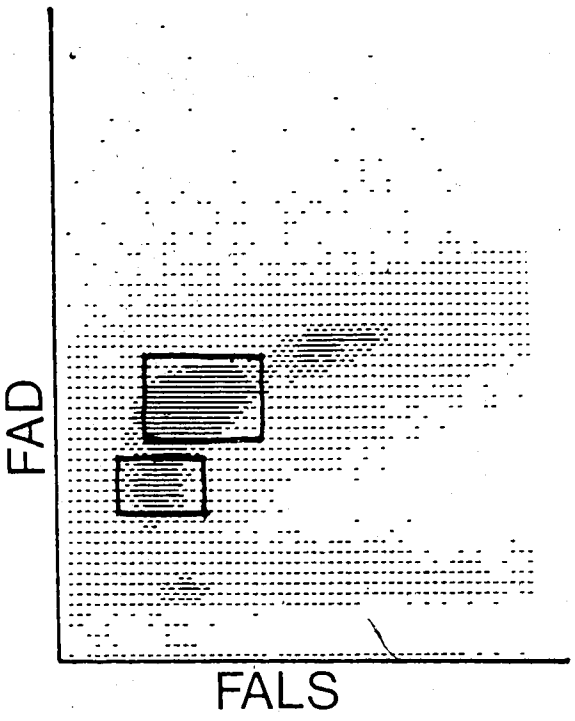

FIGURE 18 Two parameter histogram of FAD vs FALS of canine islet cells at 2.8 mM glucose and 17°C illustrating selected sort windows.

TABLE 3
CELL COMPOSITION OF ISLET CELL FRACTIONS

	Sorting Criteria			Cell Composition (%)[b]				
Cell Fraction	FALS	AutoFluorescence	Cell Number[a]	A	B	D	PP	Amylase
Rat								
I-A(n=12)	Low	Low FAD	401(±13.2)	80-85	1-3	2-6	5-10	<1
I-B(n=12)	High	High FAD	570(±22.7)	<2	95-100	<2	<2	<1
II-A(n=5)	Low	Low NAD(P)H	301(±27.[illegible]	[illegible]5-100	<1	<2	<2	nil
Dog								
I-A(n=5)	Low	Low FAD	298(±[illegible]	[illegible]-85	2-4	5-10	5-10	<1
I-B(n=5)	High	High FAD	439(±19.[illegible]	<2	95-100	<2	<2	<1

Fractions I represent cells purified at 2.8 mM glucose and 17°C.

Fraction II represents cells purified at 20 mM glucose and 37°C.

a= Mean cell number ±SEM, calculated per intact islet, as observed when 3000-5000 intact islets were dissociated and purifed per experiment (n).

b= Determined after immunostaining for the various hormones.

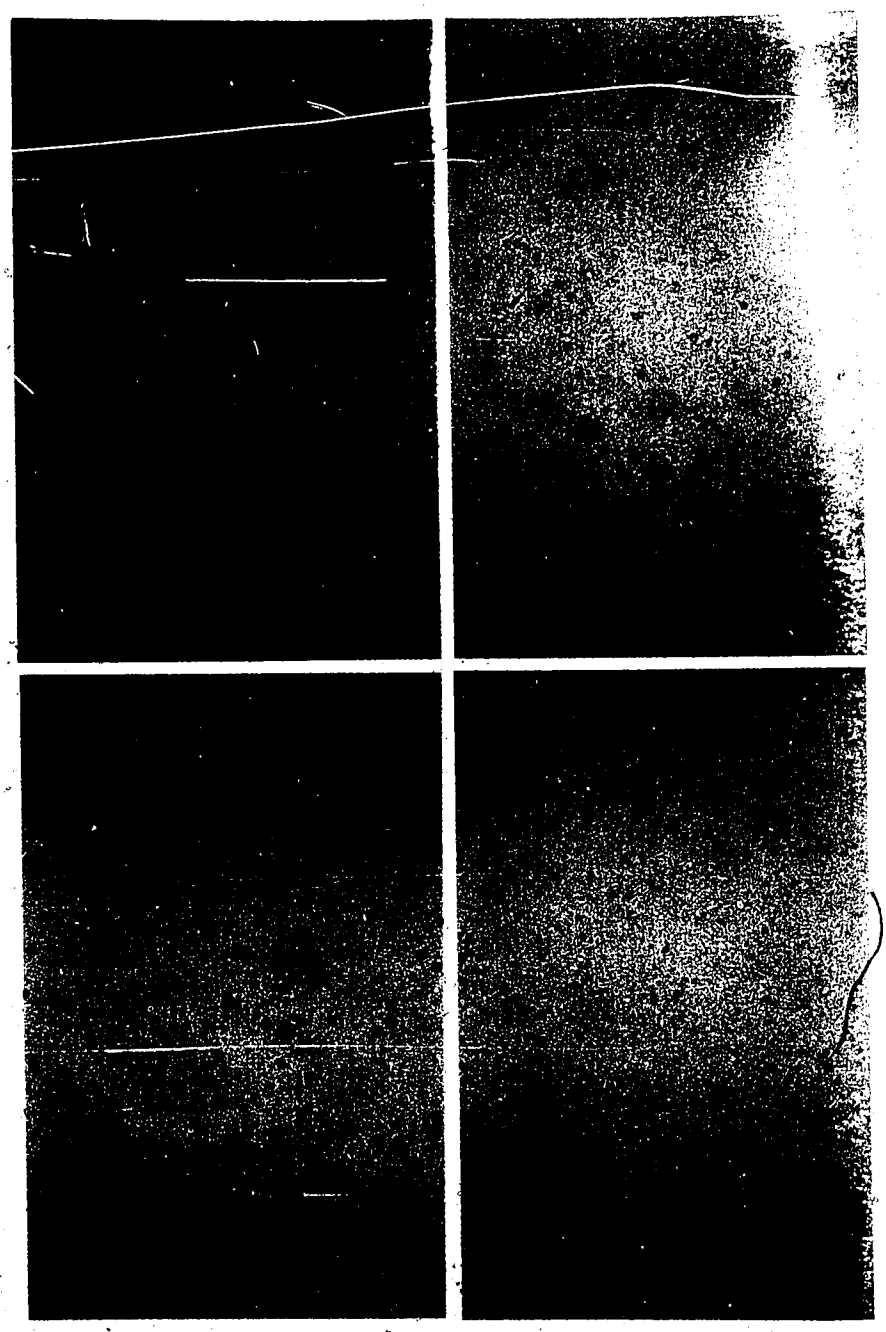

PLATE 5 Immunocytochemistry of Fraction I-A.
(A) Rat islet cells stained for Glucagon (100X).
(B) Rat islet cells stained for Insulin (100X).
(C) Canine islet cells stained for Glucagon (125X).
(D) Canine islet cells stained for Insulin (100X).

(Table 3, Plates 6) and less than 2% stained positive for glucagon. Similar results were also found for the purification of canine islet cells (Table 3, Plates 5,6). Acid extractions of these sorted fractions are expressed in insulin/glucagon ratios, further demonstrating the degree of purity of the islet cell subpopulations (Table 4).

5.3 b) Sorting of Islet Cells According to NAD(P)H Levels

Fraction I-A (non-B cells) of the rat islet preparation was further analyzed for its NAD(P)H content at 20 mM glucose and 37°C. At these conditions the majority (>90%) of the cells exhibited no increase in NAD(P)H levels, thus allowing for selection of appropriate sort windows (Figure 19). This purified population (fraction II-A) was composed of 95% glucagon-containing cells, with minor contamination from the other endocrine cells (Table 3, Plate 7). It also expressed a lower insulin/glucagon ratio as compared with fraction I-A.

All cell fractions obtained from the FACS were immediately tested for structural integrity using the FDA-EB assay. At this time they possessed more than 95% viability, and 90% after a 24 hour culture period.

5.4 DITHIZONE STAINING OF ISLET CELLS

Purified non-B cells and B-cells (fractions I-A and I-B, respectively) from both rat and canine islets were tested for their staining characteristics to Dithizone. In the rats, only the non-B cells composed of 80-85% A cells stained positive or appeared red when incubated with Dithizone. The B cells had no reaction to the stain, where > 75% of the non-B cells did react. On the other hand, more than

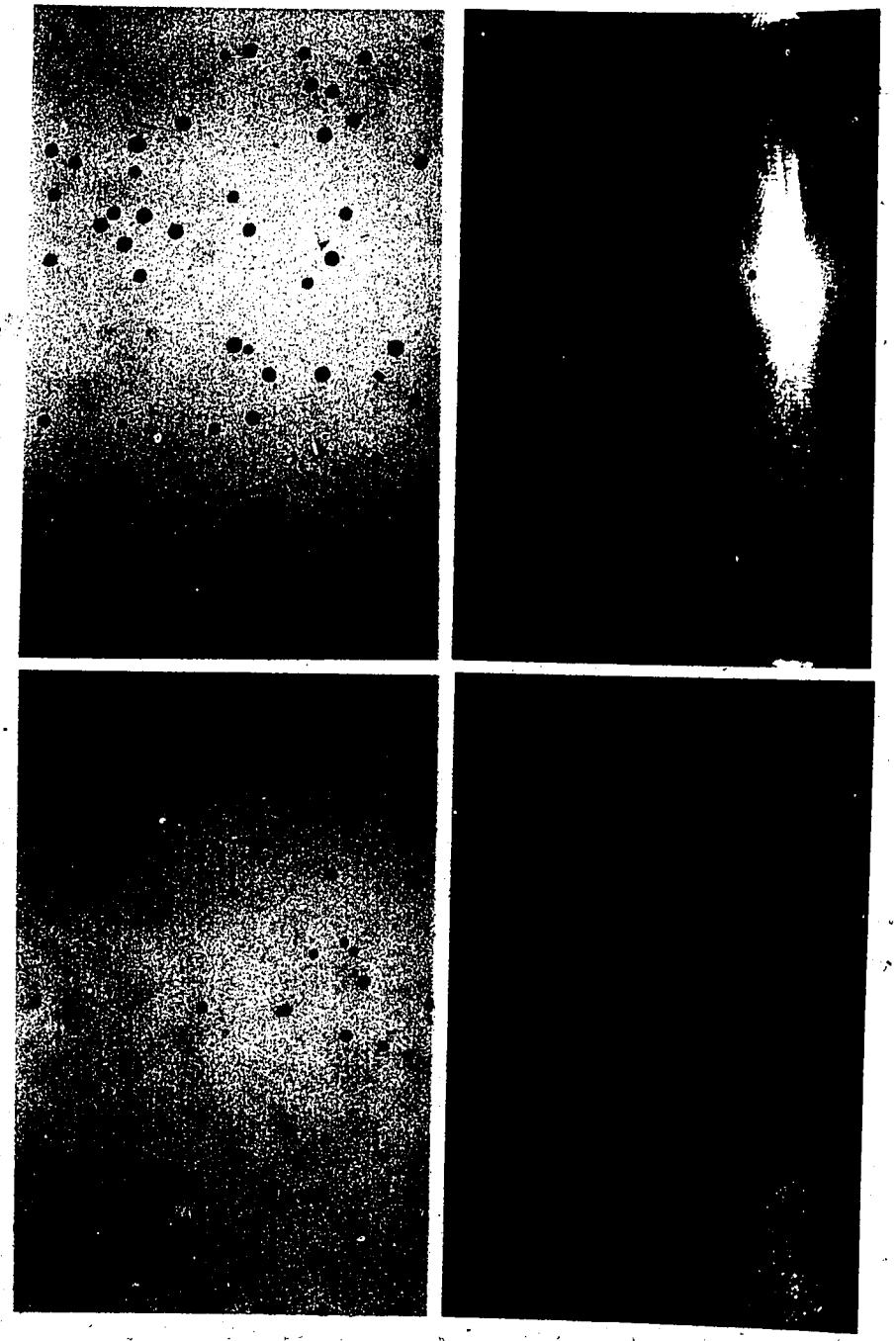

PLATE 6 Immunocytochemistry of Fraction I-B.
(A) Rat islet cells stained for Insulin (125X).
(B) Rat islet cells stained for Glucagon (125X).
(C) Canine islet cells stained for Insulin (125X).
(D) Canine islet cells stained for Glucagon (125X).

85% of the B cells from canine islets stained with Dithizone, with no reaction in the non-B cell population. Therefore, Dithizone is specific for the non-B cells in rat islets and canine B cells.

TABLE 4

HORMONE CONTENT OF ISLET CELL FRACTIONS

Cell Fraction	INS[a]	GLU[a]	INS/GLU
Rat			
unpurified	314.4±21.8	15.8±1.4	19.9
I-A	6.3±0.8	66.7±1.7	0.09
I-B	498.2±24.3	1.8±0.7	276.70
II-A	5.1±0.2	76.7±3.8	0.07
Dog			
unpurified	298.2±19.9	14.2±4.1	21.0
I-A	5.9±0.6	62.1±4.8	0.10
I-B	472.6±32.1	2.2±0.9	214.80

INS, Insulin; GLU, Glucagon

a- Data illustrates mean ± SEM of 3 different cell preparations, and expressed as nanograms per 10^4 cells.

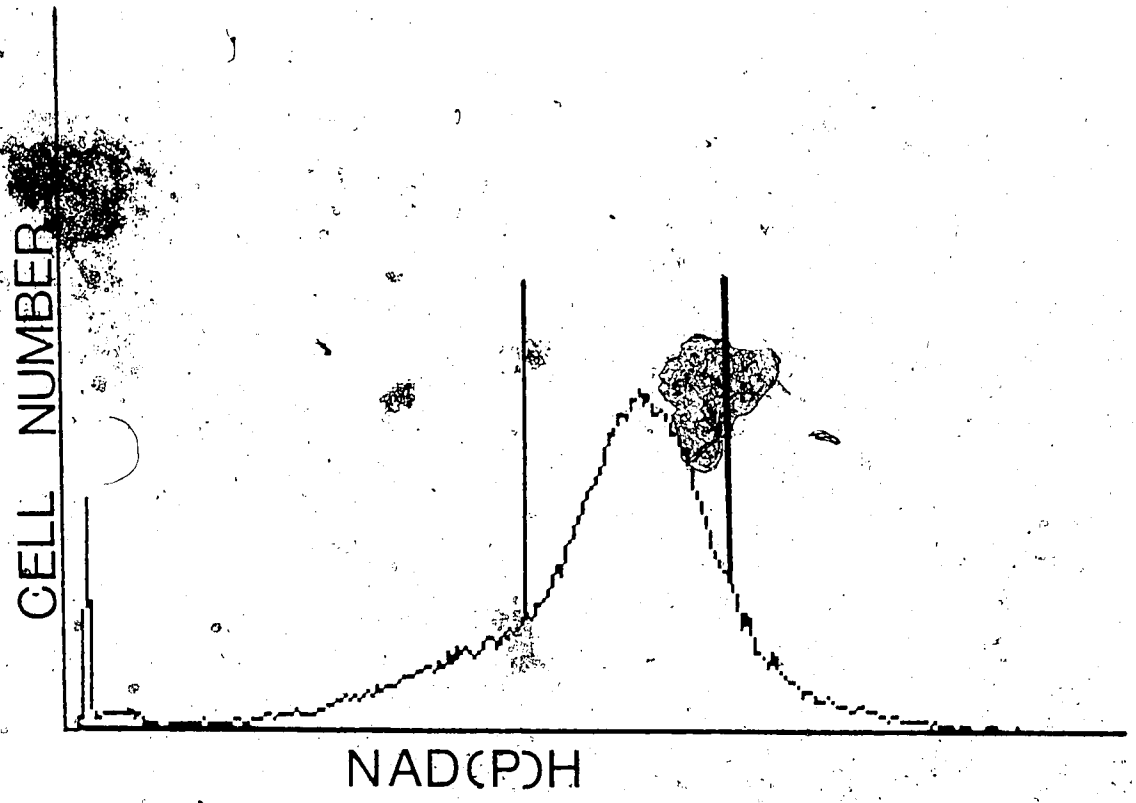

FIGURE 19 One parameter histogram of NAD(P)H autofluorescence of rat islet cells (fraction I-A) at 20 mM glucose and 37°C, illustrating selected sort windows.

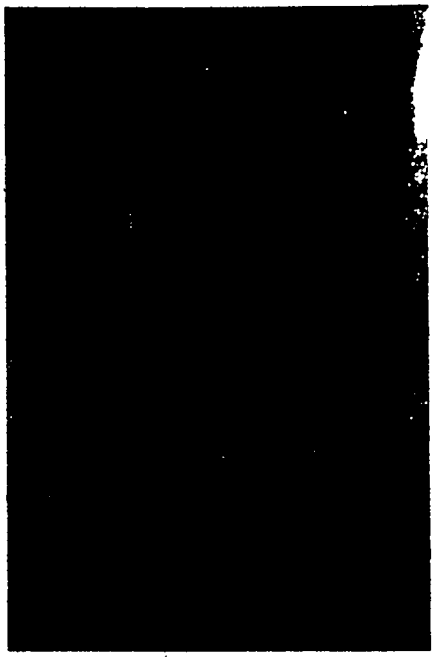

PLATE 7 Immunocytochemistry of rat islet cells Fraction II-A stained for Glucagon (100X).

CHAPTER SIX

DISCUSSION

6.1 OBTAINING SUFFICIENT NUMBERS OF ISLET CELLS

Before undertaking any attempts toward the purification of islet cells, a dissociation protocol(s) must be developed to yield sufficient numbers of viable cells. Initially many methods were investigated, but the most successful techniques employed a two-step disaggregation of islet tissue. The preparation of single rat, canine and human islet cells required the combination of calcium removal and enzymatic digestion, in conjunction with mechanical force. However similar in the basic approach, one method was required for fresh rat islets and another for canine, human and cultured rat islets (Figure 4).

Calcium removal in both techniques was achieved by using the chelators EDTA (fresh rat) and EGTA (others). Both of the chemicals are capable of removing calcium from extracellular medium, where no differences were observed between their respective affects on dissociating islet tissue. The two chelators were used, as the techniques were based upon their employment in two separate dissociation protocols (23,54).

The major difference between the two methods is that fresh rat islets required a 40-fold lower concentration of trypsin for dispersion into single cells. Three possible factors may contribute to this difference: (1) rat islets were handpicked and therefore exhibit a higher degree of purity as opposed to canine and human islet preparations, hence they could be digested more readily without interference from contaminating exocrine tissue, (2) canine and human

islets are isolated via different protocols from the rat islets, thus may require greater enzymatic activity for dissociation, (3) culturing of rat islets may allow for possible repair of delitrious affects occurring during the collagenase digestion, making them more difficult to disperse than fresh rat islets. Therefore the degree of purity and condition of the initial intact islet preparation possibly determines the degree of enzymatic digestion required. This also explains why fresh rat islets are digested at 30°C as temperatures under 37°C for trypsin are associated with reduced cell damage (109).

The addition of DNase reduced cell clumping, as has been observed in other reports (56). BSA was added to all dissociation solutions as a protective agent to coat the cells and perhaps prevent them from adhering to each other or to the vessel in which they were contained. No protease inhibitors were employed to terminate trypsin activity, but cold media was added to accomplish this. This media was also supplemented with FCS, as serum is believed to contain multiple protease inhibitors (42).

With the present methods, sufficient numbers of single islet cells were obtained (Table 2) for subsequent analysis in the flow cytometer. However, the wide differences in experimental conditions exclude any conclusion based upon other reported dissoeiation techniques. For example, many techniques are employed for obtaining islet cell monolayers, where the degree of single cells is not of major importance. Thus, this information is usually not reported, making comparisons impossible.

6.2 FLOW CYTOMETRIC ANALYSIS OF ISLET CELLS

Flow cytometric analysis of dispersed rat islet cells identified differences within this heterogeneous cell suspension for all the intrinsic parameters measured except for 90LS. The intensity distribution of FALS (Figure 6-a) is consistent with the results of Fletcher (68) and Rabinovitch (54), all consisting of two distinct populations of single islet cells. Pipeleers (23,74,75) reports that FALS histograms of unpurified rat islet cells correspond only to one broad peak with A cells scattering less light than B cells. These differences are likely due to variations in the settings selected to detect FALS (laser power, gain, optical properties of the detection system) producing inconsistent data from report to report. Therefore, depending upon how the flow cytometer is set up, some will detect one broad or two distinct peaks when analysing rat islet cells for FALS intensity.

A general consensus does exist when comparing the respective light scattering intensities of the various islet cell types. This report plus others (54,68,75) indicates that A, D and PP islet cells display a lower FALS intensity than B cells. Neilson (67) however, states that D cells are found in the peak farthest to the right or display the greatest FALS. Pancreatic D cells possess multiple long microvilli rendering them coupled to B cells or to other D cells (56). It is most likely that Neilson observed most of the D cells in this region of high FALS intensity as they were structurally coupled to other cells producing a higher FALS intensity as opposed to being analysed as single cells.

In summary, this study confirms that FALS histograms of single rat islet cells corresponds to distinct cell populations of low FALS intensity (non-B cells) and high FALS intensity (B cells). Any inconsistencies are likely due to misinterpretations of data or differences in the various flow cytometers in use.

Most flow cytometric studies on dispersed rat islet cells assume that FALS properties is largely dependent upon cell size (23,54,68). Furthermore, Pipeleers and Pipeleers - Marichal (56) demonstrated that single cells dissociated from rat islets are distributed into two populations, small-sized A and D cells (cell volume 200-600 μm^3) and large-sized B cells (cell volume 600-1500 μm^3). These workers used counterflow elutriation to separate B cells from A and D cells on the basis of size differences. However, no reports have definitely confirmed that low angle FALS intensity is capable of detecting size differences of rat islet cells. As described previously (3.2), a near linear relationship between size and FALS is true only when light is scattered at small angles (0.5-2.0°) and only within a certain range of diameters of smooth spherical objects with the same refractive index.

This report described a method to measure cell volume and to compare its relationship to FALS intensity using the flow cytometer. Figure 6-b represents a volume distribution of single rat islet cells illustrating two well defined cell populations. When a two parameter histogram is constructed of volume versus FALS intensity, a "near" linear relationship is clearly visible between these two parameters (Figure 7-c). This histogram shows that the low cell volume of non-B cells correspomds to a low FALS intensity, and the B cells with a larger volume exhibit greater FALS intensity. It is also evident that the B

cells represent a more heterogeneous cell population as their FALS and volume distributions are much more diverse as compared to the non-B cells. Therefore, this study confirms that flow cytometric analysis of FALS intensity is closely related to the cell volume of single rat islet cells. However, caution must be stressed as FALS is also a complex function of several other variables, and conclusions should be based only within the study of rat islet cells.

The FALS intensity of canine and human islet cells differs from that of rat islet cells, in that the two peaks were not well defined in canine islet cells (Figure 10-a) and only one broad peak was found with human islet cells (Figure 13-a). However, the volume distribution of human islet cells (Figure 13-b) displayed two distinct populations based on cell size. When compared with FALS intensity in a two parameter histogram (Figure 15-c), light scatter is not proportional to volume of the human islet cells. This data further reinforces the fact that FALS is a complex function of several variables influenced by cellular properties other than cell size. Possibly the refractive and reflective properties of the islet cells differs from rat to human, altering their respective FALS properties. Hence, for FALS to be used to estimate cell size, depends upon the type of cells being analysed and most likely varies amongst different flow cytometers.

Van De Winkel et al (74) described a flow cytometric measurement of FAD content based upon endogenous autofluorescence at 2.8 mM glucose to distinguish between non-B and B rat islet cells. Two well defined peaks were displayed in this study when rat and canine islet cells were analysed for their FAD content, corresponding to low (non-B cells) and high FAD content (B cells). To confirm that FAD autofluorescence was

actually being detected, rat and canine islet cells were analysed at 20 mM glucose and 37°C. This increase in glucose will cause the reduction of FAD to $FADH_2$ in functionally viable cells, thus causing a decrease in FAD autofluorescence. Therefore, when histograms were displayed there was no longer defined islet cell populations based upon FAD content. The B cells normally with higher FAD content at 2.8 mM glucose, had decreased in autofluorescence intensity, and were no longer distinguishable from the non-B cells.

The measurement of autofluorescent intensity of human islet cells displayed an initial peak of low intensity, which was also present in canine islet cells but not as predominant. Exocrine tissue may have been responsible for this low intensity peak, displaying a distinct peak with low autofluorescence. This peak was completely absent in rat islet cells and present in small quantities in canine islet cells, as these cell suspensions were routinely obtained from purer intact islet preparations. Thus, the increased exocrine contamination in human islet cell suspensions is possibly responsible for this low intensity peak. Attempts were unsuccessful at sorting out this population, possibly due to a malfunction in the FACS or the level autofluorescence intensity was too low. The exocrine contamination may have also interfered with the detection FAD autofluorescence, thus resulting in the loss of distinction between non-B cells and B cells as demonstrated in rat and canine islet cells.

6.3 PURIFICATION OF ISLET CELLS

While islet cell purification via elutriation (56) is a simple, rapid, and reproducible technique, it is limited by its inability to simultaneously purify islet cell types other than B cells. In an attempt to prepare highly purified A and B cells, this report tested whether the differences in islet cell FALS intensity and autofluorescence might thus lead to a fast, visually monitored islet cell purification by FACS. Although already successful in purifying rat A and B cells by Pieleers et al (29) no other investigators have reported success nor has it been attempted with canine islet cells.

Regardless of whether FALS intensity histograms of unpurified islet cells distinguish cleanly between both cell types, it is known from other cell systems that minor differences in light scatter become a useful parameter in cell sorting when they are associated with differences in cellular fluorescence (110). Single A cells exhibited more fluorescence than B cells, making both populations easily distinguishable in FACS histograms of unpurified islet cells. Visual selection of sorting criteria facilitated the elimination of contamination by debris and dead cells and minimized variations in cellular composition of the sorted fractions. However, by increasing the size of the sort window, a greater cell yield could be obtained but with a lower degree of purity. Therefore, trial runs had to be executed in order to obtain the maximum number of sorted islet cells per sort window to correlate with the highest degree of purity.

At 2.8 mM glucose and 17°C the sorting of rat and dog islet cells according to their light scatter activity and FAD autofluorescence

results in the purification of 570 and 439 pure single B cells per isolated islet, respectively. Canine islets yield fewer purified islet cells because on the average these islets are smaller than rat islets, and dissociation of canine islets yields fewer single cells. This procedure also foresees in the preparation of A cell populations with an 80-85% purity for rat islets and 75-85% for dog islets.

Due to the greater number of rat experiments, the enriched A cell fraction was further analysed at 20 mM glucose and 37°C to produce a cell population of 95-100% A cells. The few contaminating islet cells with increased NAD(P)H are removed by sorting the cells with no increase in NAD(P)H levels. This second step further increases the degree of purity but there is also a drop in cell yield. Subsequent experiments on the A cells should determine if this step is required for desired experimental design.

The data obtained from these purification experiments is impossible to compare with that of the previous report by Pipeleers et al (23), as this report expresses cell yield per 20 rat pancreases. Table 3 represents cell yield per islet since different amounts of islets were used for each experiment, allowing for comparison from one experiment to the next.

By comparing the values for the insulin content per 10^4 cells (Table 4) in the two I-B fractions and the percent insulin - containing cells in these fractions (Table 3) led to a mean insulin content of 52.42 and 49.74 pg per rat and canine pancreatic B cells, respectively. Using this same approach for the I-A fractions gave 8.34 and 7.76 pg of glucagon per rat and canine pancreatic A cells, respectively. The mean

glucagon content of 8.07 pg/rat pancreatic A cell was obtained in the same manner from fraction II-A.

From the insulin content per 10^4 cells in fraction I-A and the mean insulin content per B cell (52.42 pg) it can be calculated that only 1% of the cells of this sorted population correspond to insulin - containing B cells. Similar values are obtained in fraction II-A and I-A of purified canine islet cells. These values are indeed also obtained after ICC identification of the cells (Table 3). The same calculation led to a 2% contamination of both the rat and canine single B cell preparation by glucagon - containing cells. This value is also compatible with the ICC analysis of the I-B fractions. Therefore, both ICC studies and hormonal composition from acid extractions confirm the purity of the sorted islet cell populations.

The present procedure does not succeed in the purification of PP or D cells. Since PP cells are not present in large quantities within intact islets, while islets isolated from the PP rich lobe of the pancreas (107) may provide a conceivable means of purifying these islet cells. The purification of D cells might be more difficult due to the numerous microvilli rendering them attached to other islet cells (56).

In employing two separation parameters, namely FALS and endogenous fluorescence activity, this report succeeds in purifying single B cells from both canine and rat islet cells. It also achieves highly enriched populations of A cells in both the rat an canine models. This technique offers the considerable advantage of visually monitoring the separation procedure. The main disadvantage to this technique is the high cost of the purchase and maintenance of the instrument. Another well known disadvantage is the duration of the sorting procedure.

6.4 QUALITY CONTROL

6.4 a) Quantitative Analysis

Cell recovery of the purified single A and B cell fractions were determined within a hemocytometer, but were also counted during the sort procedure. The cells when sorted are numerically integrated by the cell sorter giving an estimate of each fraction. These values did not differ for more than 10% from the numbers obtained from calculations in the hemocytometer.

Numbers integrated by the cell sorter are accurate only when single cells are being sorted. When coupled cells are detected the sorter only indicated the number of particles and thus will markedly underestimate cell numbers when few cells occur as single cells. However, seeing as sorted cell fractions are > 90% single cells these integrated values are closely related to values determined within the hemocytometer. Cell numbers recorded in Table 3 were therefore obtained from the hemocytometer as this was the most accurate method.

6.4 b) Qualitative Analysis

The few reports on obtaining islet cell fractions from the FACS use either neutral red (23) or FDA (54) to determine the cells structural integrity. This report calculated structural integrity utilizing both fluorescent dyes, FDA and EB. Since FDA requires esters within the cytoplasm to be hydrolysed by esterases, fluorescein fluorescence is therefore an indicator of both membrane integrity and enzyme activity. As an added control cells from both the dissociation and sort procedure were incubated for 24 hours then assessed for their structural

integrity. Cells exhibited more than 95% viability immediately after being sorted and 90% after the 24 hour incubation period, implying that the sort procedure has little if any affect on the cells viability. This finding has also been reported by Pipeleers during his sort sequence (50).

It might appear at first that the functional integrity of islet cells purified via FACS is best determined by secretory response which is identical to that of intact islets. This approach however is not applicable, as the fucntional integrity not only depends upon the number of islet cells but also on the intercellular signals which amplify the individual cells response (18,19).

The data from such a study can be extremely misleading when interpreting the cell fractions functional integrity. Fletcher et al (68) reported that A/D cell enriched fractions had higher insulin secretion than that of a B cell enriched fraction when exposed to 16.7 mM glucose. The A/D cell fraction had a better response to glucose because the few B cells present in the population could respond more actively due to the cellular interaction of the A and D cells present. Therefore, a study of this nature was not included in this report, as this approach is misleading when interpreting functional viability of purified islet cell fractions.

A rapid and appropriate release of insulin requires a sensitive glucose recognition unit, where glucose - stimulated insulin release is mediated by the generation of reducing equivalents (72). This will therefore lead to an increase in NAD(P)H to NAD(P) ratio and a decreased FAD to $FADH_2$ ratio. These alterations in the B cell redox were observed in this report and occured within 10 minutes after the rise in glucose

and persisted during their exposure. A decrease in the B cells FAD autofluorescence was the visual monitor for this change in redox state, representing a valid parameter for the glucose sensitivity of the purified islet B cells. This change in fluorescence or redox state can be correlated with the cells increased secretory capability (112). Therefore giving an indication of the islet cells functional viability.

6.5 SPECIFICITY OF DITHIZONE TO ISLET CELLS

The availability of purified islet cells prompted the investigation into the distribution of zinc between the different types of cells within the islet. However, the specificity of a dithizone solution to zinc is still unclear, and since a non-complex forming dithizone solution (non-specific for zinc) was used in this study, data was interpreted as whether or not dithizone reacted with cells, staining them reddish. The most widely accepted interpretation is that dithizone is specific for the zinc within the pancreatic B cell. Therefore it was investigated if in fact this was the case for islet cells from rat and canine islet tissue.

The purified B cell preparation from canine islets was stained red by dithizone but not the non-B cells. Whereas, the opposite was observed in purified rat B and non-B cells. This data is consistent with the report from Maske (86) however he presents no data to confirm his statement. Therefore, this report is the only data representing whether dithizone stains pancreatic B cells or not.

When intact canine and rat islets are stained with dithizone, the canine islets appear to have a darker appearance than rat islets. Since canine B cells react with dithizone, which are more numerous than the

reactive non-B cells of the rat islet, more cells will stain with dithizone within canine islets. Therefore, it is expected that canine islets appear darker, as more individual cells are reacting with dithizone.

Therefore, this report revealed that dithizone is specific for the non-B cells in rat islet but only with B cells of canine islet. The fact that > 75% of rat non-B cells, consisting of 80-85% A cells, stained with dithizone suggests that the A cells were predominantly reacting to dithizone.

CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS

The dissociation of intact pancreatic islets requires a two step procedure of calcium removal and enzymatic digestion, associated with gentle mechanical force. Different concentrations of trypsin were required to obtain single cell suspensions, depending upon the purity and initial condition of the intact islet preparation. It was found that islet suspensions with large amounts of contaminating exocrine tissue required much more trypsin. Exocrine tissue interfered with trypsin activity, making it more difficult to disaggregate the islets. An increase in trypsin was also required when the rat islets were cultured, suggesting the incubation period altered the islets structural state.

Protocols required to dissociate islets must therefore be based upon the type and condition of intact islets. If fresh hand-picked islets are incubated for even a few hours, changes may have to be made to obtain single cell suspensions. The use of islet dissociation techniques devised in other laboratories should only be used as a guide to determine individual protocols. For example these methods may not be optimal as intact islets may be isolated by different techniques, affecting the degree of enzymatic activity required. Therefore, when determining techniques to dissociate islets, one must assess the optimal conditions for the source of islet tissue.

Effective quantitative and qualitative analysis should be applied to each dissociation technique to assess the efficiency of the

procedure. A 24 hour culture period followed by a vital stain is suggested. As islet cells with a high degree of viability immediately after dissociation may not survive an overnight incubation.

A "near" linear relationship between FALS and cell size may be present only within certain cell populations, as single rat islet cells were the only cell suspension to display this relationship. FALS intensity is commonly used to estimate cell volumes, particularly in flow cytometry of rat islet cells. This however is not true for human islet cells, as volume distributions display two populations, with only one broad peak in FALS. Therefore, caution must be stressed when interpreting FALS data, since FALS and cell volume can vary independently.

Canine and rat pancreatic B cells display higher FAD autofluorescence and FALS intensity as compared to non-B cells. These differences in FAD autofluorescence, in conjunction with FALS distribution formed the basis for islet cell purification. Purified B cells (>95%) and A cells (>80%) were obtained by FACS analysis at 2.8 mM glucose and 17°C. Sorting of the rat A cell fraction based upon NAD(P)H content, allowed for further purification of A cells (>95%).

The FACS is an effective means of purifying islet cell populations without affecting cell viability or structural coupling. However, to achieve highly purified cell fractions with sufficient numbers, FALS must be used in combination with FAD content, and not alone. The few attempts at sorting islet cells based on FALS alone were not successful in obtaining highly purified populations.

To measure FAD autofluorescence, emitted light must be detected between 510-560 nm with the proper filter. Therefore, before making any

attempts at purifying islet cells, absorbance spectra of filters should be determined to ensure accurate measurements. Glucose concentration should also be maintained at 2.8 mM to achieve a high FAD to $FADH_2$ ratio, and optimal results were found at 17°C.

It is conceivable that further investigation with human islet cells will yield purified A and B cell populations based upon FAD content and FALS intensity. If exocrine contamination in this report interfered with accurate measurement of FAD content, methods of obtaining highly purified islet populations should alleviate this problem. With pure human islet cell preparations, definitive results could be obtained to determine if human B cells possess higher levels of FAD as opposed to non-B cells.

A FACS is a complicated instrument to operate and requires a certain level of skill to use, especially when sorting cell populations. Lymphocytes are more economical and easier to obtain than are islet cells, and should be used to become familiar with the flow cytometer. Once this has been achieved, islet cells should be analysed initially to identify distinct subpopulations, before attempting cell sorting.

Dithizone was found to stain canine pancreatic B cells, and rat non-B cells. Most reports on the use of dithizone naturally assume that B cells react to dithizone, since zinc has been found in insulin. However, this is not true for rat islet cells, as only the non-B cells would react with dithizone.

The availability of purified islet cells will greatly enhance our knowledge of the physiology and pathology of intact islets of Langerhans. An independent in vitro analysis of the islets single endocrine cells is expected to define the specific regulation of

hormonal secretion for each type of islet cell. Therefore, subsequent studies on purified islet cells will increase our understanding of the mechanisms involved in islet physiology.

Cryobiological studies of the islets single cells will provide a better understanding of the cryobiology of intact islets, directed towards developing an optimal freezing protocol for the intact isolated islet. For example, it is unclear if cryopreservation protocols optimized for the survival of B cells are sufficient to maintain the stability of the frozen-thawed islets which also contain the A and D cells. Hence, protocols are required that optimize the cryopreservation of all the islet's endocrine cells since these cells function in vivo as an integrated unit. By measuring the osmotic permeability properties of each pancreatic endocrine cell individually, one could apply this information to predict low temperature responses and optimal conditions of addition and removal of cryoprotectants of the islet cells to select the optimal cooling conditions.

By defining the optimal conditions for the cryopreservation of the single endocrine cells, protocols may be selected to maximize damage to the donor's passenger lymphoid cells in the islet while preserving the endocrine cells, possibly reducing the islets immunogenicity. Therefore, an in vitro analysis of the islets single cells may increase the percent survival of cryopreserved pancreatic endocrine cells, which will greatly benefit the clinical use of islet cell transplantation.

In conclusion, this report succeeds in purifying islet cells based upon distinct differences in FAD content and FALS intensity. Previously, this has only been achieved by one investigative group using rat islet cells. As these results show, the same parameters used to

purify rat cells can be applied to sort canine islet cells. Flow cytometry provides a useful tool for obtaining highly enriched islet cell fractions without a loss in cell viability. Sufficient numbers are also achieved to permit further in vitro analysis of the pancreatic islet cells.

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APPENDIX A

FLOW CYTOMETER SPECIFICATIONS

The lazer power was maintained at 300 mW throughout this study. A laser wavelength of 488 nm was used for all flow cytometric analysis except when islet cells were sorted according to NAD(P)H levels. For this sort procedure an ultra violet light source (351-363 nm) was employed to excite the islet cells.

The filter set up used in this study was designed to eliminate unwanted stray light and to maximize detection of the desired scattered and fluoresced light. The following is the sequence used for the filters: 457-502 nm laser blocking filter at 90^{o} to the path of light, 488 nm dichroic filter at 45^{o} to the 90LS detector, and a 525 nm bandpass filter directly in front of the green detector. When NAD(P)H autofluorescene was detected a 418 nm long band pass filter was used in place of the 525 nm band pass filter. The absorbance spectrum of both these latter two filters is described in Figure 20, as detection of emitted light at narrow band passes was crucial for measuring FAD and NAD(P)H autofluorescence.

Gain and high voltage settings used for the photodiode and each PMT are presented in Table 5.

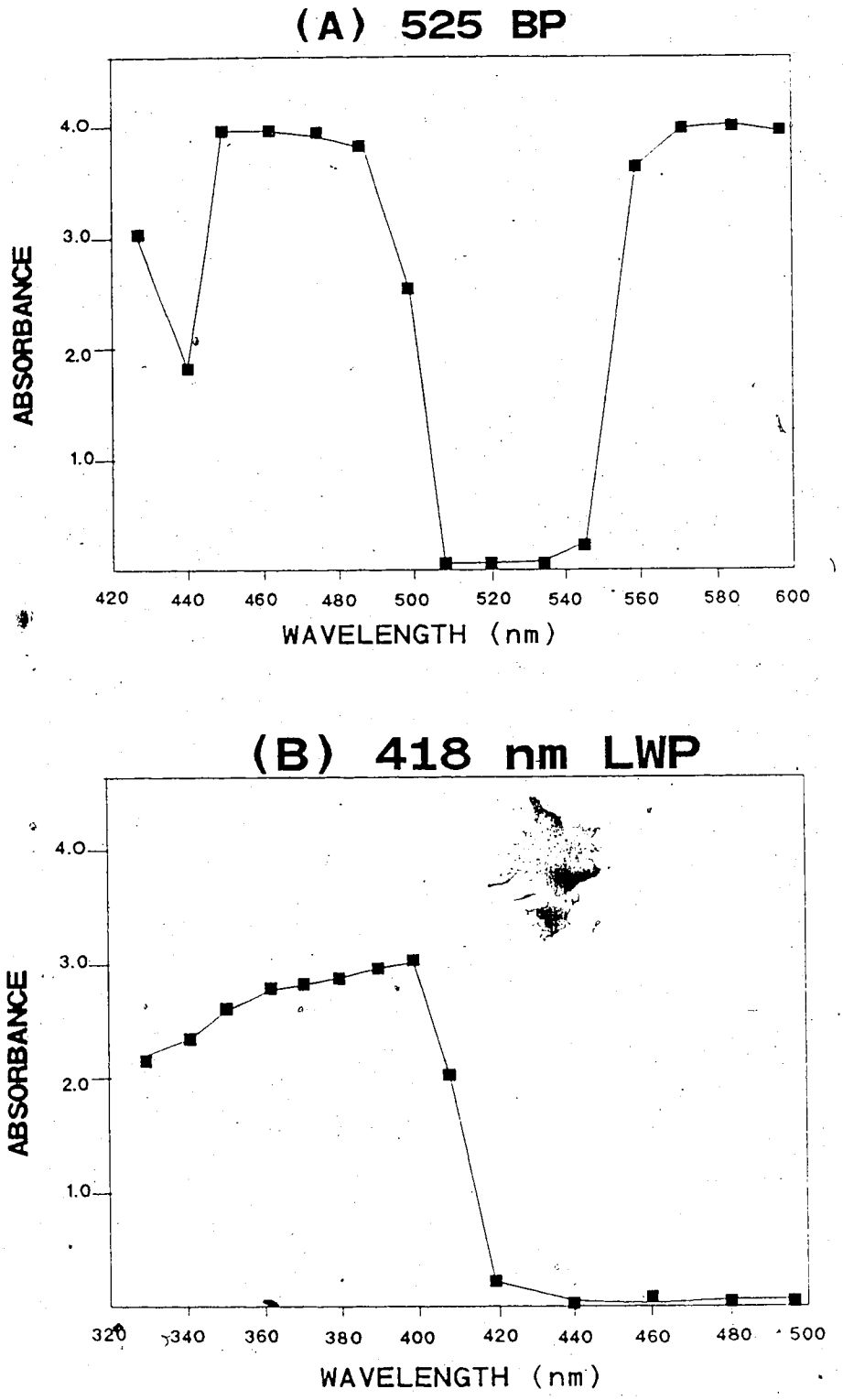

FIGURE 20 FILTERS USED TO DETECT FAD (525 BP) AND NAD(P)H (418 LWP).

TABLE 5

GAIN AND HIGH VOLTAGE SETTING USED FOR EACH DETECTOR

DETECTOR	PARAMETER	GAIN	HIGH VOLTAGE (mW)
LIGHT SCATTTER	FALS	001	--
PMT 1	FAD AUTOFLUORESCENCE	--	1200-1400
PMT 1[a]	NAD(P)H AUTOFLUORESCENCE	--	1200-1400
PMT 4	90LS	--	450
CVA	VOLUME	002	--

a- PMT 1 used to detect NAD(P)H autofluorescence at 20mM glucose and 37°C