Title
Characterization of the insulin reservoir in rat islets of Langerhans

Author
Gishizky, Mikhail L.

Publication Date
1988

Peer reviewed|Thesis/dissertation

CHARACTERIZATION OF THE INSULIN RESERVOIR IN RAT ISLETS OF LANGERHANS: EVALUATION OF HORMONE SYNTHESIS, PROCESSING, STORAGE AND SECRETION

by

MIKHAIL L. GISHIZKY

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

ENDOCRINOLOGY

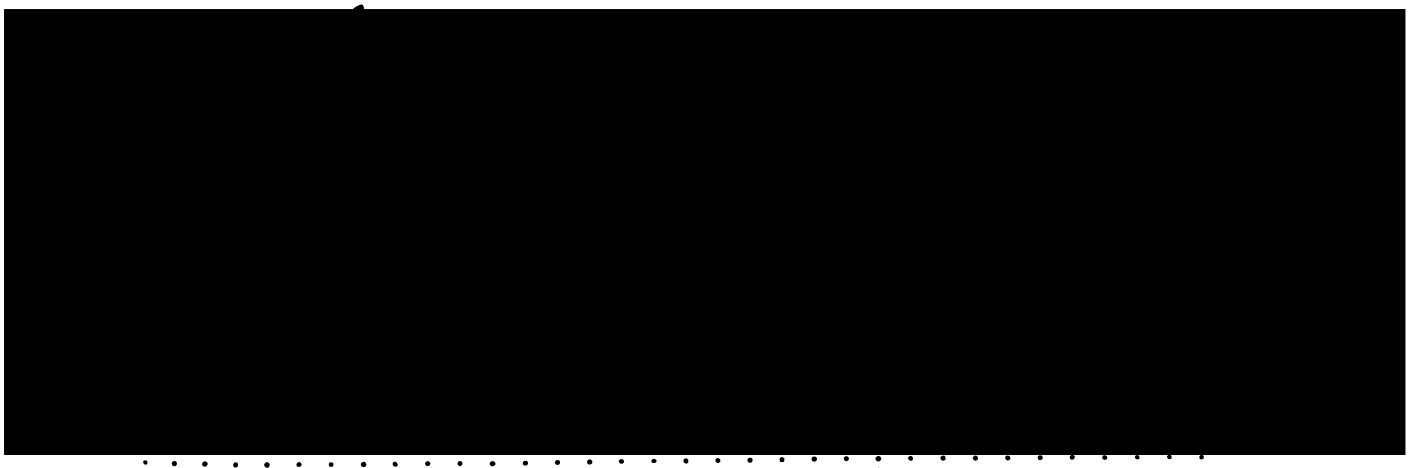
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



. . . . . .

Date **University Librarian**

Degree Conferred: JAN 3 1988 . . . . . .

CHARACTERIZATION OF THE INSULIN RESERVOIR IN RAT ISLETS OF LANGERHANS: EVALUATION OF HORMONE SYNTHESIS, PROCESSING STORAGE AND SECRETION.

PREFACE

"It seems to me we're all in the same boat with Columbus. He didn't know where he was going when he started. When he got there he didn't know where he was, and when he returned he didn't know where he'd been."

Mark Twain (paraphrased)

Many times through the course of this work I experienced frustration in realizing that with each discovery there is a greater awareness of ones ignorance. Indeed the nature of scientific investigation may be the quest to supplant that frustration with the discoveries of physical truth. This quest is associated with a great degree of anxiety and for myself, cannot be made alone.

I therefore dedicate this work to my parents, family and friends, for their support (emotional, spiritual, physical and intellectual) and bearing during the challenges of this experience.

ACKNOWLEDGMENTS

I would like to thank:

Dr. Gerold M. Grodsky, in whose laboratory this work was conducted, not only for his guidance in matters of science but also the direction and nurturing only a true mentor/friend can give; for his insight, humor and enthusiasm in all aspects of life.

Dr. Gerald Gold for his patients, insight, understanding and friendship through our many years of close association.

And all my collaborators within the metabolic unit whose ideas and friendship provided the basis for this endeavor.

I would also like to express my gratitude to the members of Achievement Rewards for College Scientists foundation for their continued support throughout my graduate career. Being an ARC'S scholar has not only provided the financial security and honor of their recognition, but even more importantly has afforded me the opportunity to share my work with a group of interested and wonderfully spirited individuals.

ABSTRACT

It has been reported that acute glucose stimulation of islets results in the preferential release of newly synthesized insulin. This suggests that the large islet hormone reservoir may represent a heterogeneous pool. In these investigation we characterized the nature of the islet hormone reservoir and evaluated possible mechanisms responsible for its regulation.

Our studies demonstrated that under stimulated secretory conditions normal pancreatic islets secreted newly synthesized insulin in preference to their large stored hormone content. The preferential release pattern was observed with all secretogogues tested and was not restricted to a specific subset of islets. Aided by computer model analysis, we proposed that the islet insulin reservoir represented a heterogeneous pool composed of at least two hypothetical compartments - labile and stable. Evaluation of the islet hormone reservoir under different *in vivo* and *in vitro* conditions demonstrated that in response to prolonged stimulation, the hypothetical labile compartment apparently decreased in size. This augmentation in the compartmental character was associated with 1) decreased amount of insulin secreted, 2) increased proportion of newly synthesized insulin secreted and 3) an increased rate of prohormone conversion with no alteration in the rate of hormone synthesis. Thus parameters which defined the islet hormone reservoir represented a dynamic system that responded to the islets milieu.

Preferential release of newly synthesized insulin was

not an intrinsic property of insulin secreting cells. Furthermore, the mechanism responsible for the compartmentalization of the insulin reservoir did not discriminate between the two non-allelic murine insulins. Our studies indicated that differences in the amino acid structure of the two prohormones apparently resulted in proinsulin I being transported to the conversion compartment faster than proinsulin II. However, glucose regulation of the synthesis and secretion of insulins I,II (new and stored) was the same.

Although we cannot exclude the possible contribution of intra-cellular secretory granule segregation, our investigations support the hypothesis that the basis for islet insulin reservoir compartmentalization and the dynamic kinetics of the secretory response are a consequence of complex intercellular interactions involving recruitment of heterogeneous B-cell populations within the islet.

PUBLICATIONS RESULTING FROM THIS WORK

Gold, G, Landahl, H.D., Gishizky, M.L., Grodsky, G.M.: Heterogeneity and Compartmental Properties of Insulin Storage and Secretion in Rat Islets. Journal of Clinical Investigation 69:554-563, 1982.

Gold, G., Gishizky, M.L., Grodsky, G.M.: Evidence that Glucose "Marks" Beta Cells, Resulting in Preferential Release of Newly synthesized Insulin. Science 218:56-58, 1982.

Grodsky, G.M., Gold, G., Landahl, H.D., Gishizky, M.L., Nowlain, R.E.: Evidence of Glucose-Sensitive Process in the Beta Cell that Regulates Preferential Secretion of Newly Synthesized Insulin. In The Importance of Islets of Langerhans for Modern Endocrinology (K. Federlin and J. Scholtholt eds.), Raven Press New York, 1984.

Gold,G., Gishizky, M.L., Chick, W.L., Grodsky, G.M.: Contrasting Patterns of Biosynthesis, Compartmental Storage and Secretion of Insulin in Rat Tumor and Islet Cells. Diabetes 33:556-561, 1984.

Gold,G., Pou, J., Gishizky, M.L., Landahl, H.D., Grodsky, G.M.: Effects of Tolbutamide Pretreatment on the Rate of Conversion of Newly Synthesized Proinsulin to Insulin and the Compartmental Characteristics of Insulin Storage in Rat Islets. Diabetes 35:6-12, 1986.

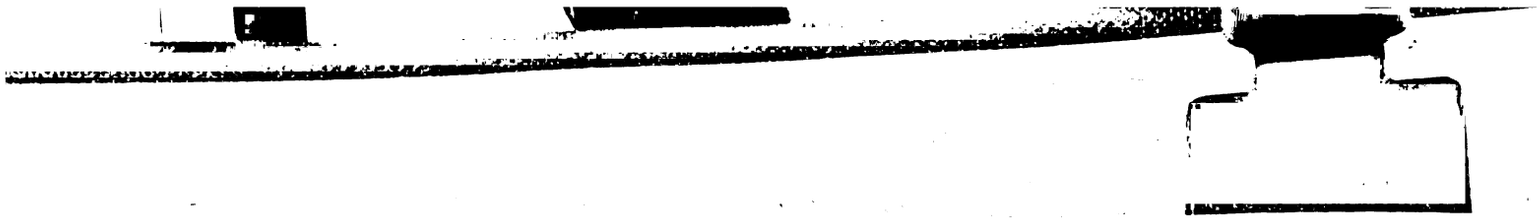
Gishizky, M.L., Grodsky, G.M.: Differential Processing of Rat Insulin I and II in Rat Islets of Langerhans. (in press 1988).

Gishizky, M.L., Gold, G., Nagamatsu, S., Grodsky, G.M.: Differences in Rat Proinsulin I and II Amino Acid Structure Result in their Differential Rates of Intracellular Transport and Conversion. (manuscript in preparation).

Gishizky, M.L., Gold, G., Grodsky, G.M.: Altered Compartmental Configuration of the Islet Insulin Reservoir in Response to Prolonged Glucose Exposure. (manuscript in preparation).

ADDITIONAL PUBLICATIONS

Gishizky, M.L., Frankel, B.J., Grodsky, M.L.: Cyclophosphamide Treatment of Prediabetic Chinese Hamsters. Acta Phisiologica Scandanavia 121:81-84, 1984.



SUMMARY

Rat islets of Langerhans, like other regulated secretory tissues, contain a large hormone reservoir. In the absence of additional hormone synthesis this reservoir is theoretically capable of sustaining maximal glucose stimulated insulin secretion for >10 hrs. Interestingly, however, islets cultured overnight exhibit a diminished glucose stimulated insulin secretory response, with relatively little change in their insulin content. This suggests that a portion of the islets insulin content is not available for secretion. Pulse labeling experiments, demonstrating an apparent preferential release of newly synthesized insulin in response to glucose stimulation, further support the hypothesis that the islet insulin reservoir (content) may represent a heterogeneous pool. In these studies we investigated the nature of the insulin reservoir in islets and insulin secreting tumors, characterized the parameters which define it (hormone synthesis, processing, storage and secretion) and evaluated possible mechanisms responsible for its regulation.

Pulse-labeling experiments demonstrated that, in freshly isolated islets, hormone synthesis reached a maximum rate by 45 min of glucose exposure and was sustained at that level for over 3 hrs. This allowed us to characterize the insulin reservoir in freshly isolated islets and evaluate hormone processing, storage and secretion under conditions of steady state hormone synthesis.

Following a 45 min preincubation with 20 mM glucose, proinsulin to insulin conversion proceeded with a $t_{1/2}$= 50 -

60 min and was not effected by alterations in the islets secretory state. Furthermore, under all stimulated secretory conditions (irrespective of secretogouge) islets secreted radioactive insulin at a greater fractional rate than immunoreactive insulin, evidenced by secreted insulin having a higher specific activity (cpm/ng) than cellular insulin resulting in a secreted/cellular specific activity ratio of greater than unity. This confirmed that the islet insulin reservoir represented a heterogeneous pool which we proposed to be comprised of at least two compartments - labile and stable. With the aid of a computer model, we estimated that the size of the hypothetical labile compartment in freshly isolated islets represented approximately 33% of the islet insulin content. However, this configuration did not remain constant.

Prolonged hyperstimulation of islets, *in vivo* (3 days of tolbutamide treatment, orally) and *in vitro* (225 min preexposure to 20 mM glucose, in culture), resulted in a dramatic increase in the fractional secretory rate of newly synthesized insulin which was interpreted as a decrease in the relative size of the hypothetical labile compartment. This augmentation in the hormone storage properties of islets was associated with: 1) decreased amount of immunoreactive insulin secreted and 2) an increased rate of prohormone conversion. Thus under conditions of steady state hormone synthesis the parameters which define the compartmental nature of the islet insulin reservoir (hormone processing, storage and secretion) represented a dynamic system that responded to the

islets milieu.

Although heterogeneity in the hormone reservoir was not restricted to a select subset of islets, preferential release of newly synthesized insulin was not observed in the absence of extra-cellular calcium or following tolbutamide treatment in vivo. Furthermore, insulin secreting tumors, which have a high rate of constitutive insulin release, secreted newly synthesized and stored insulin at the same fractional rates. This demonstrated that preferential release of newly synthesized insulin was not an intrinsic property of insulin secreting cells. Rather, it was restricted to those cells which utilized a "regulated" exocytotic mechanism and secretory tissue which contained a large amount of stored hormone.

We further demonstrated that the mechanism responsible for the compartmentalization of the insulin reservoir (content) was not dependent on maintaining the integrity of the microtubule network and did not discriminate between the two murine insulins. Unlike most mammals rats and mice synthesize two distinct insulins (I,II) that are products of non-allelic genes. Our studies indicated that differences in the amino acid structure of the prohormones apparently resulted in proinsulin I being transported to the conversion compartment faster than proinsulin II with a concomitant faster rate of proinsulin I to insulin I conversion. However, glucose regulation of the synthesis and secretion of insulins I,II (newly synthesized and stored) was the same.

Although we cannot exclude the possible contribution of intra-cellular secretory granule segregation, our investiga-

tions, and those of other laboratories, support the hypothesis that the dynamic hormone storage and secretory properties observed in islets are a consequence of complex intercellular interactions involving recruitment of heterogeneous B-cell populations within the islet.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Insulin synthesis, in pancreatic B-cells, follows the classic intra-cellular pathway originally described by Jameson and Palade for secretory proteins in pancreatic acini cells [reviewed in Palade 1975]. Temporal assessment of the morphologic distribution of radioactive (pro)insulin has demonstrated an analogous vectorial compartmental transfer in B-cells during this process [Orci 1974]. Translation of insulin mRNA results in the synthesis and translocation of preproinsulin across the RER membrane. Following removal of the signal sequence proinsulin is transported from the ER through the Golgi apparatus and sequestered into secretory granules. In secretory granules and possibly the trans Golgi compartment proinsulin is converted to insulin. Once processed, the mature insulin remains in the secretory granules and undergoes a characteristic hexameric condensation while awaiting its ultimate fate (secretion or degradation). Each of these events consists of multiple steps that are potential regulatory sites for production and release of this hormone.

TRANSLATION OF INSULIN mRNA

Synthesis of insulin, as with other proteins begins with mRNA translation. In the cytoplasm, insulin mRNA translation appears to conform to the mechanism outlined in the "signal hypothesis" of cotranslational translocation of secretory proteins across the RER membrane [Blobel 1975, 1980]. This process which involes a translational arrest mechanism, has been reviewed in great detail [Sabatini 1982, Walter 1984]. The

elongation arrest is due to a complex interaction between the ribosome, the translated amino terminus portion of the hormone precursor and the signal recognition particle (SRP;[Walter 1981]) which is believed to specifically recognize the translationally active complex and prevent further synthesis by binding to the active ribosome [Walter 1984]. Binding of the SRP arrested complex to the docking protein (SRP receptor) on RER derived membranes [Meyer 1982, Gilmore 1982], dissociates the SRP from the complex and allows translation to proceed [Walter 1979, Myer 1980, Gilmore 1982,].

Through a yet undetermined mechanism [Wickner 1985, Sabatini 1982, Engelman 1981, Blobel 1980], interaction of the ER membrane with the emerging signal sequence facilitates the transfer of the nascent polypeptide across the membrane. Removal of the signal sequence is believed to be a cotranslational event mediated by a peptidase located on the luminal surface of the ER membrane [Jackson 1977, Walter 1979, Sabatini 1982]. Physical restriction in this process indicate that a polypeptide must attain a minimal length of 70-90 amino acids [30-40 within the ribosome + 20 to cross the membrane + 18-30 residues of the signal] to become accessible to the peptidase [Blobel 1975, Palmiter 1977]. Amino acid sequence analysis of many secretory and membrane protein signal regions has not demonstrated any obvious primary structural homologies. Sequence orientation or specific position within the amino terminus do not appear critical for function and recent reports suggest that non signal related translocation mechanisms do exist [Kaiser 1987]. However, a general trend in

accumulation of hydrophobic amino acids in the signal region of many translocated proteins has been reported [von Heijne 1985] and in the case of insulin, signal sequence mediated translational arrest appears to be a requisite for directing translocation of preproinsulin across the ER membrane [Shields 1977].

In Isolated rat islets preproinsulin has been identified and its processing demonstrated to be very rapid [Patzelt 1978]. Half life for cleavage of the pre sequence is approximately five minutes and results in the presence of soluble proinsulin within the ER lumen. It is believed that the disulfide bonds within the proinsulin are formed coincident with preproinsulin translocation and signal cleavage. Evidence implicating the SRP-arrest mechanism in regulating insulin mRNA stability [Welsh 1985], translational active - inactive pool distribution [Welsh 1986] and translational efficiency [Cordell 1982] have been reported for insulin and other "signal" containing proteins. Thus this mechanism may not only serve to segregate cytosolic proteins from those requiring membrane translocation but also provides a multitude of potential regulatory sites.

PROINSULIN TO INSULIN PROCESSING

Following removal of the signal peptide and release of the proinsulin within the ER lumen, further proinsulin to insulin processing can be thought of occurring in two general steps: 1) transport of proinsulin from the ER to the conversion compartment and 2) enzymatic modification. Both these steps

imply a degree of selectivity which in turn is a potential regulatory site.

Intra-cellular Transport

Protein sorting and targeting: Three molecular mechanisms have been proposed for sorting and selectively targeting secretory proteins to their specific destinations [Kelly 1985]. The first suggests that every secretory protein contains a "sorting domain" which is recognized by a "carrier" that transports (or retains) the protein to its correct destination [Blobel, 1975, 1980]. Another proposal is that soluble proteins in the lumen of the ER or Golgi are not sorted but their probability of going by one pathway or the other is proportional to the volume of the transport vesicles and the number of vesicles that leave the compartment per unit time. According to this model if the vesicles in different pathways have different surface to volume ratios, the ratio of membrane to secretory proteins in the two pathways can differ. A third possibility is a combination of "carrier-mediated" and bulk-flow process (analogous to fluid-phase endocytosis which accompanies receptor-mediated endocytosis; [Steinman 1983]). By this model, in addition to carrier specific exocytosis the bulk phase flow would externalize any protein that was present within the transport vesicle, unless there was an exclusion mechanism. At present none of the models can be conclusively established or disproved, however, the mechanism predicted by the third model appears more plausible.

Most investigations have supported the contention that

externalization of all proteins, membrane and secretory proteins follows the same intra-cellular pathway (RER to Golgi to surface; [Strous 1980, Novick 1980, 1981, Bergmann 1983, Green 1981]) with no apparent regional specialization [Roth 1980, Slot 1983, Bergmann 1984]. Although similar intra-cellular compartments appear to be involved, the actual mechanism for transport may differ for different classes of proteins and cell types. At least two classes of protein externalization mechanisms have so far been demonstrated: constitutive and regulated [Gumbiner 1982].

The main features of the constitutive mechanism include: 1) lack of an intra-cellular storage pool - thus making secretory rate directly dependent on the rate of protein synthesis, 2) transport vesicles which have a "short half-life" and do not contain the characteristic "dense core" found in electron micrographs of classical secretory granules, 3) insensitivity to external stimuli; exocytosis does not require the presence of calcium in the external media. This mechanism is believed to regulate common homeostatic function and thus should be present in all cells, the most common include hepatocytes, fibroblasts, lymphocytes, muscle cells and yeast.

The regulated mechanism is present in specialized secretory cells (including B-cells) which exocytose large amounts of protein in response to external stimuli. These cells have the ability to externalize proteins at rates greater than their synthesis. This is achieved by maintaining these proteins in recruitable intra-cellular storage reservoirs. The proteins are concentrated and stored in vesicles (secretory

granules) which provide one of the characteristic morphological features of secretory cells - the "dense core" granules observed in electron micrographs.

The demonstration of both these classes of exocytosis in the same cell type [Gumbiner 1982] has led to an intensive effort to identify the mechanisms by which determination to each of these pathways is made. With the cloning of many secretory protein genes and their transfection into different cell types an overall picture of the process is emerging.

Introducing DNA encoding rat or human proinsulin into COS cells (a fibroblast derived cell line), resulted in high levels of transient proinsulin expression and secretion [Laub 1983, Lomedico 1982]. In this cell system, proinsulin was not processed to insulin and prohormone externalization appeared only dependent on the rate of protein synthesis. Thus as predicted by the model, COS cells appear to lack a regulated pathway so that externalization must occur via the constitutive mechanism. Conversely, stable transfection of human proinsulin into AtT-20 cells (a cell line of endocrine origin capable of synthesizing and secreting ACTH) resulted in synthesis of human proinsulin and its copackaging with endogenous ACTH containing secretory vesicles [Moore 1983]. In addition, unlike COS cells, proinsulin was apparently proteolytically processed to insulin and released along with mature ACTH when the cells were stimulated to secrete. Similar studies using other secretory proteins [Moore 1986] and cell systems [Hellerman 1984] have reported in all cases that the foreign prohormone is packaged into secretory vesicles and released upon

stimulation. Furthermore, transgenic mouse experiments in which human growth hormone was being expressed in pancreatic islets demonstrated that, synthesis and secretion of exogenously introduced human growth hormone was under the same glucose regulated control mechanism as endogenous mouse insulin [Welsh 1986]. It appears then that all hormone precursors can be targeted to the regulated secretory pathway and packaged into secretory granules of any endocrine cell, irrespective of their tissue or species of origin, indicating the permissive nature of the targeting mechanism. Furthermore, evidence is accumulating which is incompatible with the existence of only a bulk flow model for protein externalization [Moore 1983b, 1986, Burgess 1984, 1985]. Some of the most compelling evidence is the ability of chloroquine to divert ACTH precursor in AtT-20 cells from the regulated to constitutive pathway [Moore 1983c]. This indicates that hormone precursors must contain "sorting domains" which are responsible for their selective targeting [Blobel 1980]. The nature of these domains, their mechanism of recognition and the level within the secretory pathway at which they function (ER, Golgi or other) remain to be determined. Discussion of the possible regulatory sites in these mechanisms and their relation to (pro)insulin transport, processing and secretion is addressed below.

Transport to the Golgi Apparatus: The first step in transport involves export of proinsulin from the RER to the cis Golgi face. This is an energy dependent process and is believed to be mediated by small smooth surfaced vesicles,

called transitional vesicles [Howell 1969, Palade 1975, Rothman 1980, Lodish 1987]. Depletion of cellular ATP blocks this transport and results in the accumulation of the newly synthesized protein within the "transitional elements" of the ER [Jamieson 1968]. Cell fractionation and electron microscopic autoradiography during pulse chase experiments have demonstrated that, in islets, the RER to Golgi transit time for proinsulin occurs with a half life of approximately 10-15 min [reviewed in Orci 1974, Steiner 1983]. Until recently, export of proteins from the ER was viewed as nonspecific and protein sorting usually considered the function of the Golgi apparatus [Farquhar 1981, Rothman 1981, Orci 1982]. However, recent studies indicate that protein sorting may already be affected at the level of the ER.

Studies with hepatocytes [Fries 1984], hepatoma cells [Lodish 1983, Ledford 1983] and exocrine pancreas [Scheele 1985, Rohr 1984], demonstrated that endogenous secretory proteins are externalized at different rates. These differences resulted from their different rates of intra-cellular transport which were determined by their rate of export from the ER. Similar observation were reported with closely related membrane bound histocompatibility antigens [Williams 1985] indicating that regulation of both secreted and membrane proteins transport already exists at the level of the ER. In addition, defined alterations - either single amino acid substitutions or small deletions - in proteins such as vesicular stomatitis virus glycoprotein [Ros 1983, Machamer 1985], the rous sarcoma virus [Wills 1984] or immunoglobulin light chains

[Wu 1983] block their export from the ER. The recent demonstration that addition of a specific tetra-peptide sequence (Lys-Asp-Glu-Leu) to the carboxy terminal end of a lysosomal protein results in its permanent retention in the ER [Munro 1987] indicates the existence of a highly selective recognition mechanism. Furthermore, the fact that the protein was not retained in the ER when the tetra-peptide was present in the center of the protein suggests that the mechanism involved not only depends on the presence of the tetra-peptide sequence but also the context in which it is presented. Thus it appears that ER to Golgi transport, which was thought to be a bulk-phase movement process, may be a critical step in protein sorting. Note however, ER selectivity has only been demonstrated for constitutively secreted proteins the sorting of which should logically take place at a site nearest to their synthesis.

Golgi transport and function: The Golgi apparatus is a multilamellar structure having a specific vectorial organization, designated cis to trans, which can be distinguished by both morphologic and biochemical criteria [reviewed in Orci 1984, Dunphy 1985a]. Freeze fracture electron microscopic imaging has demonstrated the existence of a cholesterol gradient within the Golgi cisternae in which there is a low concentration of cholesterol at the cis face membrane with increasing amounts toward the trans face [Orci 1981, 1982]. In addition, a membrane protein gradient orientated in the reverse direction has been demonstrated in the same Golgi cisternal membranes. Individual Golgi cisternae are also immu-

nohistochemically and biochemically distinguishable. Various processing enzymes including galactosyltransferases, mannosidase II, N-acetylglucosamine phosphotransferase, phosphoglycosidase and sialyltransferase, have been localized to specific areas of the Golgi stack [Roth 1982, Goldberg 1983, Dunphy 1985]. High speed centrifugation has separated at least two Golgi membrane fractions on the basis of their differential density [Fries 1980]. Furthermore, clathrin (a protein known to be important in secretory granule formation) appears localized at the trans Golgi face [Orci 1984a,b, 1985, Griffiths 1983,1985]. Thus, the Golgi apparatus is often thought of as a cellular distillation apparatus whose function is to modify, purify and target the various proteins to their correct destination [Orci 1984, Dunphy 1985, Farquhar 1981]. Although the processes by which this is accomplished remain unclear, the best characterized protein sorting mechanism is that described for targeting hydoxylase enzymes to the lysosomal compartment, which has served as a model for investigating similar mechanisms in other systems.

Sorting and targeting of lysosomal enzymes was shown to involve receptor recognition of a mannose-6-phosphate carbohydrate moiety attached to the peptide [Tabas 1980, Sly 1982]. Lysosomal hydroxylases contain asparagine residues that are cotranslationally glycosylated within the ER by the en bloc transfer of a preformed high -mannose oligosaccharide from a lipid-linked intermediate to the nascent polypeptide [reviewed in Hubbard 1981, Kornfeld 1985] . While still in the ER 1 mannose and 3 glucose units are removed, following which the

newly synthesized lysosomal enzymes transit from the ER to the trans face of the Golgi apparatus, where further oligosaccharide modification and phosphorylation of specific mannose residues takes place [Reitman 1981, Waheed 1981a,b]. Receptors for the mannnose-6-phosphate group in the Golgi cisternae bind the newly synthesized enzymes and are then translocated to the lysosomal compartment [Geuze 1984 a,b, Brown 1984a]. Within lysosomes the low pH environment causes the dissociation of the enzymes from the receptor and thus completes the delivery process [Gonzalez-Noriega 1980]. However, mannose-6-phosphate recognition cannot be the only mechanism involved.

Cells deficient in the mannose-6-phosphate receptor [Gabel 1983, 1984], or the transferase that specifically phosphorylates lysosomal enzymes [Waheed 1982, Owada 1982] continue to sort lysosomal enzymes to their correct destination, albeit somewhat less efficiently. Furthermore, in yeast, sorting of carboxypeptidases to the vacuole - a structure in many ways analogous to mammalian lysosomes - is directed by information residing in the amino acid sequence of the carboxypeptidase precursor, not the oligosaccharide [Schwaiger 1982, Johnson 1987]. Thus other targeting signals must also be present.

In the B-cell, sorting and processing of (pro)insulin were events primarily ascribed to Golgi and post Golgi compartments. This view was in large part due to the assumption that the Golgi complex was the first subcellular compartment along the secretory pathway at which protein sorting was necessary [Orci 1984d]. Macromolecullar intra-cellular movement

prior to this point was thought to occur by means of bulk-phase transfer - a concept presently being challenged as reported above.

Once present at the cis Golgi face proinsulin is translocated through the cisternae toward the trans face [Orci 1982]. This transport appears to be mediated by a new type of coated vesicle [Orci 1986a] which is distinct from the clathrin-coated vesicles previously implicated in subcelluar compartmental transfer [reviewed in Pearse 1981]. In addition, this transit mechanism is an energy dependent process that requires the participation of as yet unknown cytosolic protein(s) [Orci 1986]. It remains to be seen if proinsulin transport through the Golgi stack is a selective vesicular budding process or a result of bulk phase intra-cisternae vesicular translocation. At present a proinsulin-specifc receptor (analogous to the mannose-6-phosphate receptor) has not been demonstrated. Reports of membrane associated proinsulin immunoreactivity on Golgi membranes, but not secretory granule membranes [Orci 1984e], has been presented as evidence for the existence of specific proinsulin binding sites at the Golgi level. However, the possibility that this observation may merely reflect differences in non-specific association resulting from pH differences in these two compartments or just the greater amount of proinsulin present within the Golgi complex, also must be considered.

En route to the secretory granule compartment, proinsulin accumulates at the trans Golgi face - a terminal Golgi compartment distinguished by the characteristic presence of a

clathrin coat [Orci, 1985]. As with receptor mediated endocytosis [Farquhar 1981, 1985] clathrin is believed to play a critical role in selective vesicular budding [Orci 1982] and possibly (pro)insulin processing [Orci 1984a]. This analogy with receptor mediated endocytosis further supports the proposed existence of specific (pro)insulin receptors (or endocrine domain receptors) and the contention that sorting of (pro)insulin takes place primarily at the trans Golgi face [Orci 1984c, 1985, Rothman 1981, Dunphy 1985]. In addition to sorting proinsulin, mechanisms must exist to direct other proteins (converting enzymes and cofactors) which affect proinsulin modification to the conversion compartment. These mechanisms remain unclear, however, it appears that they may be regulated [Nagamatsu 1987].

Granule formation and maturation: The vesicular budding mechanism responsible for (pro)insulin containing secretory granule formation is believed to be clathrin mediated [Orci 1982, 1985] and requires ATP [Orci 1986b]. As suggested above, this process may be analogous to receptor mediated endocytosis. However, the observation that secretion continues in yeast mutants that are devoid of clathrin [Johnson 1987], indicates that another factor(s) or mechanism may regulate the exocytosis process. In the B-cell, coincident with proinsulin to insulin conversion, the newly formed vesicle undergoes a maturation process that is marked by the following characteristic changes: 1) decrease in the intra-granular pH, 2) loss of the clathrin coat on the cytosolic granule membrane face, 3) decrease in the amount of membrane associated insulin

immunoreactivity, 4) formation of a characteristic intragranular electron dense core [reviewed in Steiner 1984, Orci 1984f]. These changes have been implicated in proinsulin to insulin conversion, however, their importance in secretory granule secretion, storage and degradation remain to be determined.

Recent studies have demonstrated that inhibition of proinsulin to insulin conversion - by substituting protease resistant lysine, argenine analogs - prevented the maturation of coated granules into noncoated granules [Orci 1984c,d] but did not alter the secretion of the modified proinsulin [Halban 1982, Orci 1984d]. Autoradiographic analysis of freshly isolated pulse-chase islets determined that completion of granule maturation occurs by approximately 90-120 min post translational synthesis [Orci 1982, 1984f]. However, externalization of labeled hormone - which is primarily in the proinsulin form -began at approximately 30 min post synthesis. Thus, completion of proinsulin processing is not a prerequisite for secretion and exocytosis may be completely independent of secretory granule content. This last point is of particular importance in regards to protein targeting and vesicular transport within polarized B-cells [Bonner-Weir 1984]. It implies that sorting and determination must occur prior to granule formation. Note, however, maintenance of the physiochemical properties appears to be critical to this process. Dissipation of the proton gradient across the secretory granule membrane (by culturing islets in the presence of monensin), in addition to inhibiting proinsulin to insulin conversion, also blocks

secretion of newly synthesized and stored insulin [Gold 1984, Orci 1984a]. This suggests that the intragranular acidic environment is a requisite in the regulated secretory pathway.

Evidence has also been accumulating which suggests that islet secretory capacity undergos a temporal aging process. Following maturation, secretory granules exist in their characteristic condensed form awaiting either secretion or final degradation [Orci 1984c]. For many years it has been recognized that pancreatic tissue preferentially secretes newly synthesized insulin rather than the older stored insulin [Howell 1965, Sando 1972, Gold 1981]. This has lead to the proposal that if islets remain quiescent the newly synthesized secretory granules apparently senesce and lose their ability to be preferentially secreted. Although the mechanism involved in this process has not been determined one interpretation has been that "immature" secretory granules containing newly synthesized hormone are more secretable than older stored vesicles. The idea of granular senescence is also consistent with the kinetics of islet autodegradation of insulin [Halban 1980a,b, Orci 1984c].

Immunohistochemical studies indicate that a minimum of three hours is required before any of the newly formed hormone is present in multi-vesicular bodies [Orci1984c] - a lysosomal subcellular compartment thought responsible for intra-cellular crinophagy [Orci 1982]. However, the estimated rate of insulin degradation, in stimulated islets, is less than 1% per hour [Halban 1980a]. In addition it has been reported that crystallized insulin is resistant to degradation and investi-

gators have proposed that this may account for the "unusually slow rate of degradation of insulin within B-cells" and the presence of insulin immunoreactivity within lysosomal bodies [Halban 1987]. This suggests that islet insulin may exist in a nonsecretable compartment for long periods of time and that under stimulated conditions newly formed secretory granule contents are continually exocytosed so that degradation does not dramatically affect homeostatic pancreatic insulin content.

ENZYMATIC MODIFICATION

Commonality in endocrine cells: The presence of dibasic amino acids is a common feature of protein cleavage sites [reviewed in Steiner 1984, Loh 1986], however, not all dibasic sequences are cleaved indicating that context of the dibasic site (conformation) is also important. Although the nature of the enzyme(s) involved in prohormone conversion remains to be determined processing enzyme(s) isolated from different cells appear to share unique characteristics that differentiate them from other intra-cellular proteases. The enzyme(s) is a cystine proteinase, has a pH optimum of approximately 5, is membrane associated (in secretory granules) and probably requires segments of the prohormone for substrate recognition and selectivity. The observation that different cells of endocrine origin have the capacity to "correctly" process exogenously introduced proinsulin to insulin [Moore 1983] suggests that these same enzymes may be integral components of the regulated pathway in all endocrine cells [Kelly 1985]. However, demon-

stration of differential monobasic and dibasic amino acid cleavage site prohormone processing in different endocrine cells and individual cells of the same origin , indicates that a cellular specialization in prohormone converting activity must exist [Eppiers 1980, McDonald 1987]. One hormone for which differential cleavage has been demonstrated is the production of SRIF-14 and SRIF-28.

Somatostatins (SRIF): The somatostatins are peptides of 14 and 28 amino acids that are produced in a variety of endocrine and nonendocrine tissues, including hypothalamus, cerebral cortex, stomach and pancreas [Reuchlin 1983 review]. In mammals the two SRIF's are derived from the same prohormone precursor [Shen 1984, Montminy 1984, Tavianini 1984] which is cleaved at either a dibasic (SRIF-14) or monobasic (SRIF-28) amino acid site Thus, differential expression in various tissue results from alterations in post-translational processing. Because all cells appear to use the same exocytotic pathway, it stands to reason that the observed differences must be due to the presence or absence of specific enzyme(s) within each cell type necessary for appropriate cleavage. Therefore, it is postulated that determination of which SRIF is to be produced is not regulated by the primary gene product but rather the converting enzyme(s) which that particular cell expresses.

Anglerfish islets: Studies in pancreatic islets of anglerfish indicate that different enzymatic activities may exist within the same cell type [Noe 1981, 1983, 1986, Moore 1984]. In contrast to mammals, anglerfish islets contain two distinct SRIF's that are presumably products of different

genes (two different SRIF mRNA's have been cloned but their genomic DNA identity not determined [Hobart 1980]). Interestingly both prohormones contain cleavage sites that can potentially produce SRIF-14 or SRIF-28. However, in vivo, proSRIF-I is only cleaved at the dibasic (lys-arg) site resulting in synthesis of SRIF-14, while proSRIF-II (which contains a lys-arg cleavage site in the same location as proSRIF-I) is cleaved at a monobasic (arg) site and produces SRIF-28 [Noe 1984]. Histochemical analysis of anglerfish islets revealed an exclusive presence of either SRIF-14 of SRIF-28 within cells expressing SRIF - that is cells containing SRIF-14 did not contain SRIF-28 and vise versa [McDonald 1987]. These studies indicate the existence of heterogeneous populations of SRIF producing D-cells within islets.

Recently two distinct pools of cleavage activity (monobasic vs. dibasic cleavage), which differ in their physiochemical properties, have been demonstrated [Noe 1984] and isolated [Mackin 1987a] in anglerfish islets. These data have been taken as evidence that converting enzymes are segregated within specific cell populations. Although this conclusion is consistent with the proposed existence of a family of converting enzymes (having hierarchical substrate selectivity) that are expressed in a cell specific manner [Steiner 1984], the possibility that both cleavage activities were the result of the same enzyme, whose cleavage specificity was altered by its membrane association, or by some other factor(s) [Mackin 1987b] cannot be dismissed. Furthermore, these observations may also be explained by differential expression of the two

reputed anglerfish SRIF genes in different islet D-cell populations. Thus, as with the two rat insulin genes (discussed below) expression of the SRIF's may be a consequence of either cell specific expression of the hormone gene or the cell specific nature of converting enzyme activity.

Proinsulin processing: Proinsulin to insulin conversion requires the specific excision of the intramolecular "C-peptide" sequence [Steiner 1983]. This activity appears localized to clathrin coated Golgi and secretory granule elements [Orci 1985]. In both murine insulins the "C-peptide" sequence is 31 amino acids in length and is bracketed by dibasic amino acids lysine and arginine [Clark 1969, Markussen 1971]. As with conversion of other prohormones the enzyme(s) responsible remain to be characterized. Although non-specific peptidases (ie. trypsin) can cleave proinsulin to insulin-like peptides [Steiner 1974], this procedure is inefficient and generates numerous subfragments that are not detected within islets. At present it is believed that two enzymatic activities are necessary for correct processing [Docherty 1982b, 1984], The first requires the specific recognition and cleavage at the dibasic amino acids; the second is an exopeptidase activity which trims the A and B insulin chains at the cleavage site. It has been proposed that the endopeptidase activity is affected by a procathepsin B-like thiol protease [Docherty 1982a] and the final processing catalyzed by a carboxypeptidase enzyme [Docherty 1982b].

Note that in studies using the two conversion activities isolated from anglerfish islets [Mackin 1987a], which were

reportedly devoid of carboxypeptidase activity, only proSRIF-I to SRIF-14 converting activity (dibasic site cleavage) and not proSRIF-II to SRIF-28 activity (monobasic cleavage), would "correctly" process proinsulin to insulin (albeit less efficiently than proSRIF-I). This apparently contradicts the proposed role for carboxypeptidase in this process [Docherty 1982a, Mackin 1987b] - low level caboxypeptidase B-like contamination may be responsible.

REGULATION OF INSULIN BIOSYNTHESIS AND SECRETION

EFFECTS OF GLUCOSE STIMULATION

Glucose is the primary physiologic stimulator of insulin synthesis and secretion in pancreatic islets. The B-cell is exquisitely responsive to minor changes in glucose concentration and is thus able to maintain fairly constant *in vivo* glucose levels. Isolated islets exhibit a sigmoidal glucose response curve for both insulin synthesis and secretion [Maldanato 1977, Grodsky 1963]. The glucose threshold for insulin synthesis is 3 mM and that for secretion is 5 mM while the approximate Km's are 6 mM and 8 mM respectfully. In addition to kinetic differences, the effect of glucose on insulin synthesis and secretion has different ionic requirements; synthesis requiring extracellular magnesium [Lin 1973] while secretion is inhibited by magnesium but requires calcium [Grodsky 1966] and is associated with plasma membrane depolarization [Atwater 1976]. Thus, although glucose stimulates both synthesis and secretion the actual intra-cellular affector mecha-

nism for the two processes may be different.

Perfused rat pancreas and perifused isolated islets exhibit a biphasic insulin secretory response to glucose [O'Conner 1980]. Within several minutes following elevation of the glucose concentration in the milieu there is an initial burst of insulin release lasting 2-5 min followed by a brief nadir. This is followed by a second ascending secretory phase which reaches maximum between 2-4 hours. During this period removal of glucose results in a rapid diminution of secretion and subsequent readdition of glucose produces a time-dependent potentiation of B-cells resulting in hypersecretion to a subsequent stimulus [Cerasi 1975, Grill 1978, Grodsky 1969]. This potentiation is time and dose dependent having a memory of approximately 30 min and appears to be independent of secretion during the priming period [Grill 1981]. Continued incubation under specific glucose stimulating conditions (>4 hrs) results in a gradual decrease in secretion rate with no apparent decrease in cell imuunoreactive insulin content [Hoenig 1986, Bolafi 1986]. Stimulating cells with higher glucose concentration or other secretogogoues (ie. KIC, IBMX) results in enhanced secretion indicating the islet secretory mechanism continues to function. This observation has been interpreted as evidence for "desensitization" of islets to a specific secretory signal [Bolafi 1986b]. Alternatively these data may represent evidence for "exhaustion of a labile secretory pool" within islets [Hoenig 1986].

The effect of glucose has been demonstrated to specifically stimulate insulin synthesis by increasing transcription

and translation [Permutt 1972, 1974, Giddings 1981, 1982]. Within minutes of glucose stimulation the B-cell increases insulin biosynthesis 5 to 10 fold - as measured by radioactivity incorporation into proinsulin [Itho 1980]. This effect has been shown to be independent of mRNA synthesis -occurs in the presence of actinomycin D, an inhibitor of transcription [Kaelin 1978]. Furthermore recent studies suggest that glucose stimulates translational insulin synthesis by: 1) stabilizing insulin mRNA [Welsh 1986], 2) stimulating initiation rate of insulin synthesis [Nielsen 1985], 3) stimulate elongation rates of nascent preproinsulin [Welsh 1986] and 4) increase association rate of cytoplasmic insulin mRNA molecules with the ER membrane [Welsh 1985] by as yet undetermined SRP-mediated mechanism. The effect of glucose on transcription requires approximately 60 min of stimulation and appears to achieve maximal levels by 4 hrs of stimulation [Itho 1980].

RECOGNITION OF THE GLUCOSE STIMULUS

Upon addition of glucose to the media the B-cell responds with a multitude of physiochemical affects, including increases in ATP, cAMP, phsopholipid turnover and various ionic changes. Two models have been proposed to explain the mechanism by which glucose is recognized as a signal by the B-cell [Ashcroft 1980 review]. The first is analogous to the classic peptide hormone receptor model. It envisages a protein located on the cell membrane which somehow (presumably through binding) recognizes the glucose molecule and upon binding initiates a signal within the cell. The second model proposes

that metabolism of glucose results in the formation of one or more intermediates, or products there of, that are responsible for initiating the response. This model is supported by studies demonstrating that compounds chemically related to glucose, or its triose metabolites, stimulate synthesis and secretion in proportion to their rate of metabolism. In addition, other physiologic agents (ie. amino acids) and their metabolites (KIC) also stimulate insulin secretion. However, stimulation with these agents alone, does not produce the characteristic biphasic secretory response observed with glucose. Although the glucose recognition mechanism remains to be elucidated some investigators have proposed that glucokinase, found in the islets, is the primary mediator (receptor) of the glucose stimulus [Meglasson 1983].

INTRA-CELLULAR REGULATORY SIGNALS

Intra-cellular protein phosphorylation has long been thought to be part of the mechanism for B-cells response to glucose [McDonald 1984 review]. At least three intra-cellular phosphorylation mechanisms have been described. All three mechanisms require calcium for their effect and all have been identified in islets.

Glucose stimulation of B-cells is associated with elevating cellular content of cAMP. However, agents that raise cellular cAMP require a threshold concentration of glucose to stimulate secretion [Charles 1975, Maldonato 1977]. These agents include theopheline, IBMX, forskolin, glucagon, gut hormones and cAMP derivatives. Although these agents enhance

the magnitude of the glucose stimulated secretion, controversy exists as to their effect on (prepro)insulin synthesis [Wollheim 1981 review]. The ability of cAMP generating agents to enhance insulin release at maximal glucose stimulatory concentrations suggests that cAMP effects the Vmax rather than the Km of the glucose response mechanism [Brisson 1972].

Calmodulin activity, a ubiquitous Ca binding protein [reviewed in Valverde 1984] has been demonstrated in islets [Sugden 1979, Valverde 1979] and isolated from insulinoma cells [Wollheim 1981]. Trifluoperazine, which binds to the Ca-calmodulin complex and inhibits a Ca-dependent phosphodiestrase, in vitro inhibits insulin release [Tomlinson 1980, Wolff 1979] Elevated glucose increases the amount of Ca-activated calmodulin without increasing the actual amount of calmodulin itself [Chafouleas 1979]. The apperent activation of acenylate cyclase by calmodulin has further implicated it as a regulator of the secretory process [Valverde 1979].

Glucose stimulated insulin release has long been associated with accelerated phospholipid turnover [reviewed in Best 1984], including hydrolysis of inostitol phospholipids, resulting in the formation of diacylglycerol [Michell 1982, Schrey 1983, Nishizuka 1984]. This compound may have profound effects on cellular metabolism by affecting membrane fluidity [Hawthorne 1982, Allan 1976]. The recent identification of a Ca-dependent diacylglycerol activated protein kinase, C-kinase [Kishimoto 1980, Kuo 1980] and its presence in islets [Tanigawa 1982, Sano 1983], suggests a possible mechanism of how phospholipids may be involved in insulin secretion. C-kinase

has been shown to be involved in thrombin induced release of seratonin in human plateletes [Takai 1982] and proposed as an effector in other secretory cell systems - including GnRH induced LH release from pituitary cell cultures [Conn 1985] and insulin release from the B-cell [Hubinoit 1984]. The tumor promoter phorbol ester TPA has been shown to stimulate C-kinase activity by substituting for diacylglycerol and bind to the molecule [Castagna 1982, Niedel 1983, Kraft 1983]. In addition to being a potent insulin secretogouge [Virji 1978, Deleers 1981, Malaisse 1983, Pace 1985], TPA has been observed to have a multitude of cellular affects - including mitogenesis [reviewed in Whitman 1986], phosphorylation of various protein including the glucose transporter [Witters 1985], differential effects on specific protein synthesis [Osborne 1981, Ullrich 1983, Pruss 1985] and secretion [Rebois 1985, Aizawa 1985]. Recent evidence suggests that cAMP -through its effect on phosphotidylinositol cycle - may exert a regulatory function on C-kinase activity. The observed enhancement of both glucose-induced and TPA-induced insulin secretion following perifusion with forskolin (Zwalich, 1986) suggests that in islets a coordinate activation rather than counteraction of the two systems occurs.

MURINE INSULINS I AND II

Unlike most animals studied, rats and mice (as well as three species of fish, [Steiner 1986]), synthesize two distinct insulins [Smith 1966]. The two murine insulins (rat

insulin I & II, [Clark 1969]) , which are products of non-allelic genes [Cordell 1979, Lomedico 1979], have similar amino acid structure [Villa-Komaroff 1978, Chan 1976] but differ in their genomic location. Through various stages of development -from embryonic pancreatic rudiments [Rall 1979] to adult pancreatic islets [Clark 1969, Tanese 1970] - the two insulins appear to be present in approximately 60% insulin I, 40% insulin II proportion. However, alterations in this distribution have been reported in islets and insulin secreting tumors (discussed below). These observations suggest that biosynthesis of the two insulins may be differentially regulated.

INSULIN GENES

In rats the two genes are located approximately 100 kb apart on chromosome 1 [Soares 1985]. In mice the two genes are on separate chromosomes; the insulin I gene is located on mouse chromosome 6 while the insulin II gene is on chromosome 7 [Lalley 1984, Wentworth 1986]). For purposes of clarity, further discussion will focus on the two rat genes and specific comments will be made in reference to the mouse genes when appropriate. Although rat insulin I and II genes share greater than 90% sequence similarity ("homology") in their coding and 5'-flanking sequences, their genomic architecture is different. Arrangement of the rat insulin II gene is similar to that observed in most animals studied. DNA sequence analysis of phylogenetically diverse insulins (hagfish to human) reveal that most preproinsulin genes contain 3

expressed sequences (exons) separated by two intervening sequences (introns) which do not appear in the mature preproinsulin mRNA [Steiner 1984 review]. Although intron length and sequence are variable, the location of both introns is highly conserved across phylogeny. The first intron is located on the 5' end within what becomes the 5' untranslated region of the mRNA. In both rat genes this intron is 119 base pairs long and located 16 bases 5'-upstream of the methionine encoded translation start site [Lomedico 1979,Sores 1985, Cordell 1979]. The second intron is positioned within the C-peptide region, interrupting the sequence coding for amino acids 6 and 7. In rats this intron is 499 base pairs in length and present only in the rat insulin II gene [Lomedico 1979]. Similarity between the genomic architecture of the rat insulin II gene and other insulin genes throughout phylogeny, suggests that it may be the "older" of the two rat insulin genes. Comparison of the 5'- and 3'-flanking sequences in both genes and the absence of the second intron in the rat insulin I gene support the hypothesis that the rat insulin I gene arose as a gene duplication product of the insulin II gene [Soares 1985]. Investigators have proposed that the not fully processed, insulin II gene integrated back into the genome through an aberrant retroviral transposition event, where it continued to function.

Regulation of gene transcription

Flanking sequence: In addition to the similarity of the transcribed portion of the various insulin genes, the 5' non-transcribed flanking region also retains a great deal of

homology suggesting its probable importance in regulating expression [Steiner 1985]. Studies using the 5'flanking DNA of diverse mammalian genes have demonstrated that this region contains not only the transcriptional promoter, but also elements which regulate cell specific expression [Walker 1983, Edlund 1985]. Deletion studies within the 5'-flanking regions of the rat insulin genes have identified two distinguishable DNA elements involved in cell specific expression: 1) a cell specific enhancer located -103 to -335 bases upstream of the transcription start site and 2) a fragment devoid of enhancer activity whose position at +1 to -113, suggests its integral association with the promoter [Walker 1984, Edlund 1985]. Both elements are situated within the previously described tissue-dependent DNAase I hypersensitivity domain of the rat insulin II 5'-flanking DNA [Wu 1981] (the two rat insulin genes share a >95% sequence homology in this region [Soares 1985]). This sensitivity is thought to be confined to regions of actively expressing genes [reviewed in McGhee 1980] and appears to correlate with binding of trans active factors regulating expression [Edlund 1985]. DNAase digestion protection studies (footprinting) have demonstrated the presence of what appears to be a cell specific nuclear factor(s) that binds to the enhancer element [Ohlsson 1986]. Furthermore, investigators have reported that cAMP regulates insulin mRNA levels possibly at the transcriptional level [Nielsen 1985, Hammonds 1987]. Although this has not been confirmed in normal b-cells, the recent identification of a specific cAMP regulated bindng region on the rat SRIF gene [Montminy 1987] dem-

onstrates the existence of a phophorylation dependent element in transcriptional regulation and suggests that a similar mechanism(s) may participate in regulating insulin gene expression. Recent reports have also indicated the presence of a transcriptional "silencer" element located between 2.0 and 4.0 kb upstream of the rat insulin I gene transcription initiation site [Laimins 1986]. Thus the 5'-flanking region may be responsible for a multifactorial hierarchical regulation of the insulin gene.

DNA methylation: Several studies have reported a correlation between the methylated state of a gene and its transcriptional activity [reviewed in Felsenfeld 1982], which has led to the hypothesis that methylation may regulate gene expression. Studies of the methylation patterns at the 5'-flanking region of the two rat insulins in various tissues and insulin secreting tumors [Cate 1983] have proved inconclusive. Although, both genes were apparently undermethylated within insulin-producing tissue [Cordell 1982, Chick 1983], the two genes were found to have very different patterns of methylation [Chick 1983]. Furthermore, neither the general level of methylation or site specific methylation correlated with gene expression suggesting that methylation does not exert a specific control on rat insulin I & II gene expression.

POST-TRANSCRIPTIONAL REGULATION

The primary transcript of the preproinsulin gene results in the formation of heterogeneous nuclear RNA (hnRNA), containing both introns and exons, which is then processed to

mature mRNA [Reed 1985 review]. Intron excision appears to be a rapid process which along with addition of a poly adenylated tail and 5' methyl capping convert hnRNA to mature translationally competent preproinsulin mRNA which then leaves the nucleus and appears in the cytoplasm. Each step in the modification of hnRNA can potentially regulate the levels of insulin mRNA and insulin biosynthesis by affecting the rate of synthesis, stability or translational efficiency. Studies in several rat insulinoma cell lines have indicated that alterations in this process are responsible for the observed differential expression of the two insulin genes (discussed below). Furthermore, it has been proposed that both glucose and cAMP regulate this process [Hammonds 1987, Nielsen 1985].

ALTERED INSULIN I AND II LEVELS

Insulinoma: Preferential synthesis of rat insulin I has been observed in several rat insulin secreting tumors. The apparent differences have so far all been linked to ambiguities in the mRNA of rat insulin II. In an X-irradiation induced tumor, which synthesized 10 times greater amounts of rat insulin I than II, both mRNA's were present in equal amounts [Cordell 1982]. However, the translational efficiency of the two insulin mRNA's was dramatically different; this difference was apparently due to a structural modification at the 5'-terminus of the rat insulin II mRNA. In tumors of different origin, which also exhibit augmented rat insulin I synthesis, the level of the individual insulin mRNA was reported to be altered [Giddings 1986]. This alteration was not due to

differences in transcription of the two genes but resulted from the subsequent processing of the respective hnRNA. Thus, the molecular basis for the disproportionate expression of the two insulins in tumor tissues is rather varied and appears to take place prior to the actual synthesis of the protein. Note that a characteristic feature of insulinomas is their non-regulated (constitutive) pattern of insulin synthesis and secretion. Therefore, any regulation of this type of expression would have to be exerted at the DNA or RNA level.

Islets: Using electrophoresis to separate the two insulins on the basis of their differences in charge, investigators have reported dramatic differences in the levels of insulin I and II under several conditions. Pancreatic islets isolated from hyperinsuliniemic Wistar-Furth rats that bear growth hormone secreting tumors were reported to contain 5 fold greater amounts of rat insulin I than II [Kakita 1982a,b]. In the same studies freshly isolated islets from normal Sprague-Dawley rats - which normally contain the two insulins in a 60% insulin I, 40% insulin II distribution -were reported to synthesize 10 fold greater amounts of rat insulin I than II following 4 hrs culture in media containing 16 mM glucose. These same investigators also reported augmentation of insulin I levels in mouse islets similarly treated [Kakita 1982b]. In contrast, other investigators reported that glucose stimulated the synthesis of rat insulin I and II to the same extent in islets following culture for over 18 hrs in 8 mM glucose [Rhodes 1987].

The effect of prolonged glucose exposure in vivo on the

level of rat insulin I and II mRNA has also been studied. Islets isolated from rats in which hyperglyciemia was induced and maintained for 4 days contained similar amounts of rat insulin I and II mRNA as found in euglycemic controls [Giddings 1986]. Taken together these data suggest that islets may preferentially synthesize rat insulin I in response to acute glucose stimulation and in contrast to insulin secreting tumor cells this effect must be a post-translational phenomena.

PROPERTIES OF THE HORMONE RESERVOIR

CONCEPTUAL MODELS

The endocrine pancreas contains a large reservoir of stored insulin which it is capable of releasing upon stimulation. Understanding the characteristics of this reservoir is critical for our determining the molecular basis of the glucose stimulated insulin secretory response. This reservoir can be conceptually organized in terms of three configurational models (figure 1).

The first model postulates that the hormone reservoir exists as a homogeneous pool, such that, all the hormone present would have the same probability of being released (figure 1{I}). According to this model newly synthesized hormone would flow into this large pool where it readily mixes with the hormone already present. Upon stimulation both the newly synthesized and older hormone would be secreted in proportion to their content within the total pool.

The second model proposes that the hormone reservoir is segregated into compartments having different response characteristics (figure 1{II}). This model is predicated on the existence of at least two hormone compartments, labile and stable, that differ in their rates of secretion. In addition the character of each compartment would be determined by: 1) the individual secretory rates, 2) the relative size of each compartment and 3) the contribution (distribution) of newly synthesized hormone to each compartment.

The third possibility is an extension of the second model, the axiom here being that a large portion of the total hormone exists in a relatively inert non-secretable compartment (figure 1{III}). Accordingly hormone synthesis and secretion would involve only the labile compartment. Therefore, if the size of the labile pool is <50% of the total hormone content, this model predicts that newly synthesized hormone would apparently always be secreted in preference to the older hormone.

To elucidate the character of the hormone storage pool, several laboratories performed pulse-labeling experiments using pancreatic tissue slices and isolated islets [Howell 1965, Creutzfeldt 1973, Gutman 1973, Gold 1981]. These studies demonstrated that in response to glucose stimulation, radioactive insulin (newly synthesized) was secreted at a higher fractional rate than total immunoreactive insulin (stored). This in turn resulted in secreted insulin having a higher specific activity than cellular insulin. Based on these observations, the investigators concluded that the large

hormone reservoir does not represent a homogeneous pool, but appears to exhibit a compartmental character as supposed by the latter two models (figure 1 II,III).

Conformational states:It is important to note that within the context of each model, the hypothetical compartments comprising the hormone pool may exist in different states. In addition the various states may represent a dynamic continuum between extremes, rather than a static steady state condition. Figures 2 and 3 illustrate several possible states for each model and indicate how changes in compartmental properties affect heterogeneous secretion during a pulse chase. Although these representation define the hormone reservoir in terms of two compartments set at extreme limits, one can envisage the existence of a continuum rather than a discreet set of determinants.

For purposes of comparison in each example the following remained constant: 1) the total amount of radioactive hormone synthesized (1000 cpm), 2) the total amount of hormone present initially (100 ng) and 3) the total amount of unlabeled hormone secreted (5 ng). In these derivations the following aspects were variables: 1) the distribution of the radioactive hormone (newly synthesized) among the compartments, 2) the relative size of the compartments and 3) the individual secretory rate of each compartment.

The main feature of the second model is that compartments have different rates of hormone release. According to this hypothesis, preferential release would result if newly synthesized insulin bypassed and/or equilibrated very slowly with

the major portion of the stored insulin reservoir. As illustrated in figure 2a, when the compartments are of equal size and newly synthesized insulin is equally distributed, the fractional release of total radioactive and immunoreactive hormone would be the same. With the resulting secreted and cellular specific activities being identical. Therefore, although compartmental heterogeneity exists, the insulin reservoir would appear to be homogeneous.

Figure 2b illustrates that alteration of the radioactive hormone distribution has a profound effect on the observed compartmental properties. As in figure 2a, equal compartment sizes were assumed, however the newly synthesized hormone is primarily sequestered within the labile pool. The fractional release of radioactive hormone is therefore greater than that for immunoreactive hormone and newly synthesized insulin appears to be preferentially secreted, as evidenced by the secreted insulin having a higher specific activity than cellular insulin. Under these conditions (figure 2b) the differential compartmental properties of the hormone pool would be observed.

Variations in the compartment size also have a dramatic effect on the heterogeneous secretory response. Figures 2c and 2d depict states in which the labile compartment comprises only 5% of the total hormone reservoir and the radioactive hormone flows primarily into the labile pool. Figure 2c represents a state in which the major portion of the secreted hormone originates from the labile pool. In this instance radioactive hormone is released at a much faster fractional

rate than immunoreactive hormone. Under the conditions presented, this results in a >30 fold higher specific activity of the secreted hormone than that observed in the remaining reservoir.

In contrast to all previous schemes the labile pool represented in figure 2d contributes only a minor portion of total released hormone (20%). However the secretory rate from this compartment is 4 times greater than for the stable pool. In this state radioactive hormone would also be secreted at a greater fractional rate than immunoreactive hormone, so that based on the differences in the specific activities between the secreted and remaining hormone reservoir, newly synthesized insulin would apparently be preferentially released, but most of the total insulin would be released from the stable compartment.

Interesting to note that with the exception of the scheme represented in figure 2c, changes in the various parameters had relatively little effect on the actual specific activity of the remaining hormone reservoir.

The third model (figure 1{III}) supposes that the major portion of the hormone reservoir remains dormant and is not affected by hormone synthesis or release (maintains a constant size >50% of the total hormone reservoir). With the imposed constants, the only variable that can change in this model is the size of the labile pool. Decreases in the size of the labile compartment, illustrated in figures 3A,B and C, result in elevation of the secreted insulin specific activity with a concomitant decrease in the specific activity of the hormone

reservoir. As evidenced by the various states in this model, preferential release of newly synthesized hormone will always be observed and as the size of the labile pool diminished, the cellular specific activity will also decrease.

MECHANISMS FOR COMPARTMENTAL RELEASE

Based on the various states within these models, three possible mechanisms can account for preferential release of newly synthesized insulin: 1) intra-cellular segregation -differences between secretory granule populations and differences in secretory products (insulin I vs. II), 2) inter-cellular compartmentalization - different B-cell populations within islets, and 3) inter-islet differences within the pancreas - variations based on islet composition, primordial origin, size, etc.

In the rat pancreas, at least two different populations of islets have been identified. These islets differ not only in their topography within the pancreas -head vs. tail - but also in their primordial origin and cellular composition [Baetens 1979]. Islets located in the "head" of the pancreas develop from the ventral primordium and are composed of 90% B-cells. Islets isolated from the tail of the pancreas are of dorsal primordial origin and a greater proportion of their mass consists of glucagon containing A-cells [Trimble 1981, 1982]. Furthermore, B-cells within each islet represent a geographically and morphologically heterogeneous cell population [reviewed in Pipeleers 1987].

Recent evidence indicates that individual B-cells exhibit

a graded stimulus responsiveness so that each B-cell has a specific stimulus threshold at which it may be recruited [Solomon 1986]. Furthermore, cell-cell communication, via direct inter-cellular junctions [Meda 1980, 1984] or paracrine effectors [Bonner-Weir 1982, Pipeleers 1985] and autocrine mechanisms have been described as possible regulators of the insulin secretory response [reviewed in Orci 1982]. This has lead to the concept of "biosociology" of pancreatic B-cells within islets which postulates a requisite synergistic interaction to environmental signals [Pipeleers 1987].

NON-INSULIN SECRETING CELLS

Preferential release of newly synthesized protein has also been reported in several other "regulated" secretory cell systems including those secreting prolactin [Swearigen 1971], parathyroid hormone (PTH) [MacGregor 1975], pancreatic amylase [Salby 1976], gonadotropin [Hoff 1977], vasopressin [Sachs 1969] and growth hormone [Stachura 1985]. One of the better characterized heterogeneous secretory cell systems is PTH secretion from parathyroid cells [reviewed in Cohn 1983].

Double isotope pulse-labelling experiments, using dispersed porcine parathyroid cells, demonstrated that decreasing calcium concentration (the physiologic regulator of PTH secretion) inhibited the rapid rate of PTH degradation and stimulated secretion of preexisting and newly synthesized PTH to the same degree [Morrissey 1979]. In contrast, dibutyryl-cAMP and isoproterenol, preferentially enhanced secretion of preexisting ("stored") hormone. On the basis of these observations

it was proposed that parathyroid cells contain a heterogeneous population of secretory granules. In the unstimulated state, the time between PTH synthesis and degradation is short, so that secretory granules apparently senescence at a rapid rate and may therefore exist in various states of secretability which constitute different granule populations. It was suggested that differential recruitment of preexisting vs. total (newly synthesized and preexisting) hormone was a result of physiochemical and/or spatial differences between these granule populations. Although these investigators demonstrated that the parathyroid gland contains two compartments of secretable PTH they could not eliminate the possibility that these differences may be due to differences in cellular characteristics within the gland rather than the proposed differences in secretory granules within each cell.

RATIONAL

Exposure of pancreatic islets to glucose results in a dramatic increase of insulin secretion, which under maximal secretory conditions is approximately 3-5 fold greater than the maximal insulin synthetic rate. This ability to exceed the synthetic rate is a direct consequence of the presence of a large stored hormone pool. Based on the absolute size of this reservoir islets can theoretically maintain a maximal glucose stimulated secretory rate for over 12 hrs in the absence of any additional hormone synthesis. Experimentally, however, it appears that elevated secretion can only be maintained for approximately 6 hours, reaching a maximal level by about 4 hours and significantly diminishing thereafter. This implies that the islet hormone reservoir exists in various states of secretability. Therefore, understanding the secretory and storage character of the hormone reservoir is critical in determining the mechanism responsible for dynamics observed in the glucose stimulated insulin secretory response. In these studies hormone (insulin) synthesis, processing, secretion and storage were evaluated in murine islets and tumor cells, and their relation in determining the configuration of the hormone reservoir investigated. Furthermore, murine islets synthesize two distinct insulins (I,II), their individual regulation and distribution within the islet hormone pool were also investigated.

Aims:

1) determine the steady state conditions for insulin synthesis,

2) evaluate the effect of environmental conditions on pro-insulin to insulin processing,

3) characterize the storage and secretory properties of insulin secreting cells,

4) determine the effect of environmental conditions on the configuration of the hormone reservoir,

5) evaluate possible mechanisms responsible for the nature of the hormone reservoir,

6) determine if differences in the two non-allelic murine insulins result in their different expression in islets.

PROINSULIN TO INSULIN CONVERSION

Prior to evaluating secretory and storage properties of islets it was necessary to determine the hormone synthetic and processing characteristics under the conditions to be investigated.

SYNTHESIS

To minimize variability due to differential rates of (pro)insulin synthesis initial studies were performed to determine "steady state" conditions for maximal glucose (20 mM) stimulated insulin synthesis. Other laboratories have demonstrated that glucose initially stimulates insulin synthesis at the translational level and only after approximately 60 min of exposure is there a detectable effect on transcription of insulin mRNA [Itoh]. We determined that insulin synthesis (as measured by the amount of radioactivity incorporated into proinsulin + insulin following a 15 min ^{3}H-leucine pulse) reached maximum by 45 min preexposure to 20 mM glucose and remained at this level for up to 225 min. Recent evidence in our laboratory further indicates that in response to a particular glucose concentration, maximal stimulation of (pro)insulin synthesis is achieved by 45 min of exposure and remains at this level for 24 hrs [Nagamatsu 1987]. Thus (pro)insulin processing and secretion can be investigated independent of synthesis.

ACUTE GLUCOSE PREINCUBATION: [J. Clin. Invest. 69:554, 1982]

Having established "steady state" conditions for proinsulin synthesis, we investigated the kinetics of proinsulin to insulin conversion. In all studies to be presented insulin synthesis was maximized by preincubating islets for a minimum of 45 min in 20 mM glucose prior to pulse. Following the 45 min preicubation with 20 mM glucose islets were pulsed 15 min with ^{3}H-leucine, then cultured for up to 160 min in media containing 20 mM glucose. Samples were taken at specificed times during the chase; media and islets were not segregated but analyzed together at each time point. Figure 4 illustrates the effect of time on the processing of newly synthesized proinsulin to insulin in islets. Consistent with other reports [Steiner 1972, Gold 1980], proinsulin to insulin conversion did not begin until 30 min after pulse initiation, then followed pseudo-first-order kinetics with a $t_{1/2}$ of 50 min. Under these conditions by 2 hr approximately 75% of radioactive proinsulin was converted to insulin. Disappearence of proinsulin was matched by a corresponding increase in the presence of insulin (figure 5). The amount of radioactive proinsulin + insulin recovered at each time in the combined samples was not statistically different indicating that, in glucose stimulated islets, conversion was highly efficient and digestion of newly synthesized (pro)insulin in islets or in the incubation buffer was undetectable during a 2 hr period (figure 5).

Note: the radioactivty in insulin and proinsulin for this and all subsequent data was corrected for the leucine content of each protein as described in methods. Furthermore,

all data presented, except that specifically reported in discussion of rat insulin I & II, does not make the distinction between the two insulins. In subsequent studies incubation media (secreted) and cellular (pro)insulin content were segregated and individually analyzed. Data will be presented so as to indicate this distinction when appropriate.

ALTERATION OF POST-TRANSLATIONAL CONDITIONS

We next investigated the post-translational effect of various agents on proinsulin to insulin processing. Following the pulse, islets were cultured in either K+, TPA, 2 mM glucose or 20 mM glucose. As illustrated in figure 6, under all conditions studied, the rate of proinsulin to insulin conversion remained unaltered. In these studies media and islets were analyzed seperately; the data depicted in figure 6 represents percent of intact proinsulin remaining in the islet sample only at each time - proinsulin measured in media paralleled that observed in islets. Thus it appears that once proinsulin is synthesized, conversion to insulin is insensitive to its surrounding milieu -independent of the secretory state and to a certain extent the metabolic condition of the islet.

ACCELERATED PROINSULIN TO INSULIN CONVERSION

<u>In vivo</u> [Diabetes 35:6, 1986]: Studies with islets from rats made hyperglycemic by glucose infusion were reported to have decreased insulin content and possibly accelerated the rate of proinsulin to insulin conversion [Logothetopoulos 1980]. We investigated if acceleration of proinsulin process-

ing could be affected by pharmacological manipulations *in vivo*. Tolbutamide (an insulin secretagogue) was orally administered to rats for three days, after which time their islets were isolated and studied for proinsulin synthesis and processing. Although this treatment resulted in a >75% decrease in the amount of insulin contained in each islet (control = 38 + 1.2 ng IRI/islet, tolbutamide treated = 7.9 + 1.2 ng IRI/islet), the fed plasma glucose levels of the treated animals were not significantly different from those of the control group (figure 7).

Consistent with previous reports [Lee 1970, Morris 1970, Sodoyez 1970, Levy 1975, Schatz 1978], tolbutamide pretreatment did not alter insulin synthesis - as determined by ^{3}H-leucine incorporation into proinsulin and insulin (figure 8). Pulse-chase experiments revealed that the onset time of proinsulin to insulin conversion was approximately the same in both groups of islets (figure 9). However, the overall rate of proinsulin processing was dramatically accelerated in islets isolated from tolbutamide pretreated rats (control $t_{1/2}$= 36 min, tolbutamide $t_{1/2}$= 20 min; figure 9). These data demonstrate that *in vivo* manipulation, which apparently maintains the glycemic state of the animal, can differentially affect proinsulin synthesis and processing. Furthermore, the observed accelerated rate of proinsulin conversion does not result from a decrease in the minimum time necessary for transport to the conversion compartment.

In vitro: To determine if accleration of proinsulin processing could be induced *in vitro*, prior to initiation of

the pulse, islets were cultured for either 45 min or 225 min in media containing 20 mM glucose. Following the 15 min pulse, islets were chased for up to 150 min in KRB containing 2 mM glucose. These chase conditions were chosen to minimize loss of radioactive proinsulin from islets during the sample collection period. As previously discused, the same amount of radioactive hormone (proinsulin + insulin) was recovered following both preincubation periods, indicating that by 45 min of glucose stimulation proinsulin synthesis was at maximum.

As shown in figure 10 preincubating islets for 225 min vs. 45 min in 20 mM glucose results in a dramatic acceleration of proinsulin to insulin conversion with no apparent change in the time of onset (45 min exposure $t_{1/2}$ = 41 min, 225 min exposure $t_{1/2}$ = 20 min). Others in our laboratory have reported that the <u>in vitro</u> effect of glucose on accelerating proinsulin to insulin conversion is dependent on active protein synthesis during the incubation period [Nagamatsu 1987]. In addition, preincubation of islets with metabolizable agents other than glucose (ie. leucine) for similar time periods, also accelerated proinsulin conversion [Nagamatsu personal communication]. These data suggest that the metabolic or secretory state of the islet, prior to the actual translational synthesis of the hormone appears to play a critical role in determining the rate of proinsulin to insulin conversion.

<u>Mathematical model:</u> Prohormone modification requires that proinsulin be transported to the conversion compartment (clathrin coated compartment) where actual enzymatic modification

can take place [Orci 1982]. To evaluate the contribution of each of these steps in altering proinsulin processing kinetics a mathematical model was developed. This model allowed independent variation of transport and cleavage activity and calculated their resultant effect on overall prohormone processing rate.

As previously reported, under non-accelerated conversion conditions the observed proinsulin to insulin processing rate has a half life of approximately 50 - 60 min. Other laboratories have determined that under these same conditions proinsulin transport to the clathrin coated compartment occurs with a half life of 10 - 15 min. According to this model, with a set transport rate of 10 min, the enzymatic activity should have a half life of 50 - 60 min to result in a proinsulin processing rate of 50% per hour. Because of the great differences in the two rates, variations in the rate of transport to the conversion compartment ($t_{1/2}$ = 5 min, 10 min and 20 min) have little effect on proinsulin processing (figure 11a). In contrast, when transport was held constant ($t_{1/2}$ = 10 min) variations in the rate of enzymatic activity had profound effects on the calculated proinsulin conversion rate (figure 11b) Comparison of the various computed curves with experimental data (figure 10) indicated that modification in the rate of enzyme activity could result in the dramatic acceleration of proinsulin processing. However, alterations in the transport rate had relatively little effect on conversion. Thus based on these mathematical calculations the molecular basis for accelerated conversion appears to be related to an increase in

enzyme cleavage activity.

INSULIN SECRETING TUMORS [Diabetes 33:556, 1984]

Proinsulin to insulin processing was also assessed in insulin secreting tumors. A transplantable rat insulinoma (initially induced by X-irradiation [Chick 1977]) was studied according to the protocol schematically represented in figure 26. Consistent with other laboratories investigating this [Sopwith 1981] and other tumor cell lines [Praz 1983, Nielsen 1985, Hammonds 1987, Ashcroft 1987] we determined that (pro)insulin synthesis and secretion in these cells was unresponsive to variations in the glucose concentrations (2 vs. 20 mM; figures 27, 28).

These tumor cells externalized a greater relative percentage of labeled proinsulin than that secreted by freshly isolated islets from normal rats (Table 4). The high level of proinsulin externalization suggests that a portion of the newly synthesized proinsulin either bypasses the conversion-storage compartment or transits through this compartment at an accelerated rate which limits the exposure to the proteolytic mechanism. However, at the termination of the experiment (152 min), rat tumor cells and normal islets contained a similar proportion of intact labeled proinsulin (tumor cells 19.6%, islet cells 17.4%; Table 4). These data contradict previous reports [Patzelt 1978] and indicate that for hormone retained intracellular, proinsulin processing kinetics appear to be similar to that observed in islets.

CHARACTERIZATION OF THE INSULIN RESERVIOR

Pulse-labelling experiments were designed to quantitatively characterize the hormone reservoir in islets and insulin secreting tumor cells. The analysis was based on the quantitaion of specific activities (cpm/ng) of purified insulin in media and islets; due to the unavailability of rat proinsulin standards, prohomone was excluded from the specific activity calculation. All studies were conducted under conditions of maximal glucose stimulated insulin synthesis and defined proinsulin processing kinetics, as described above. The common paradigm used involved 45 min preincubation and 15 min pulse in the presence of 20 mM glucose. With the exception of prolonged glucose incubation studies, alterations in experimental conditions all occured after the pulse period.

COMPARTMENTAL PROPERTIES OF ISLETS

Kinetics of preferential release [J. Clin. Invest. 69:554, 1982]: Initial studies evaluated insulin secretory and storage characteristics in tthe presence ofcontinued glucose stimulation (20 mM). Following the pulse islets were cultured for up to 165 min during which time radioactive and immunoreactive insulin were measured in individual secreted and islet cell samples. Throughout the chase period, islet immunoreactive insulin content (35 ng/islet) and the fractional rate of immunoreactive insulin secretion (2%/20min) remained relatively constant (figures 12, 13 respectively). However, the radioactive hormone distribution was markedly

different.

Islets exhibited a progressive and significant decrease of recovered radioactive insulin and proinsulin with time. More than half of the newly synthesized hormone flowed through the B-cells during these incubations (figure 12a). Note that the loss of total radioactive proinsulin plus insulin far exceeded the loss of immunoreactive insulin from these same islets.

Hormone released into the incubation buffer is considered in figure 13, which plots fractional secretion rates of immunoreactive insulin and of total radioactive hormone plus prohormone. Although fractional rates depend on equivalent yields of both secreted and cellular hormones for accuracy, fractional rather than actual rates were plotted because the isotope content of islets changed significantly with time (figure 12a). Rates were calculated from the quantity of hormone secreted during a 20 min interval; as an example, the rate at the 30 min time point was calculated from hormone secreted between 20 and 40 min. For the first 30 min there was almost no radioactive hormone secreted (figure 13). Thereafter, fractional secretory rates of nelwy synthesized hormone increased rapidly and clearly exceeded the fractional secretion rate of immunoreactive insulin. Thus after the first hour newly synthesized hormone (radioactive) was clearly being preferentially secreted. During the earliest interval, secreted newly synthesized hormone was rich in prohormone and contained more radioactive proinsulin than insulin (table 1); note that it was not quite as rich in newly synthesized proin-

sulin as the hormone contained in the islets. After 60 min, secreted radioactive hormone generally mirrored the extent of completion of the cellular conversion of newly synthesized proinsulin to insulin.

These data indicate that during glucose stimulation a major portion of the newly synthesized hormone is present in a hypothetical labile compartment resulting in its preferential secretion relative to the older stored hormone. This confirms the existence of at least two hormone pool within islets (labile and stable) and is consistent with both models describing the compartmental character of the hormone reservoir discused earlier (figures 2,3). Furthermore, these observations demonstrate that completion of proinsulin processing is not a prerequisite for secretion.

Size of the labile compartment [J. Clin. Invest. 69:554, 1982]: To predict how the size of a hypothetical, glucose-labile insulin secretory compartment affects preferential secretion, a simplified mathematical expression was formulated, permitting integration of all the different time windows of sampling.

The mathamatical derivation was based on the schematic representation of insulin biosynthesis coupled to heterogeneous storage and secretion shown in figure 14. This illustration is a more detailed representation of the compartmental models discused in the introduction (figure 1). Furthermore, it supposes that the labile compartment is smaller than the stable insulin pool and that the flow of newly synthesized hormone is directed predominantly into the labile compartment.

Both assumptions appear to have merit as discused in the introduction. In figure 14, proinsulin and other constantly turning over insulin precursors are represented by P, and insulin is represented by I. Lower case letters are rate constants; a_1 - a_3 provide for the transit time of the precursors (ER - Golgi -secretory granules), which occurs prior to initiation of conversion. The continuous biosynthesis to secretion sequence represents the glucose-labile secretory compartment or "channel" of newly synthesized insulin; the lower box represents the remainder of the stored hormone. Both storage compartments contain proinsulin, which is secreted by the same mechanism as insulin (not constitutive vs regulated). Proinsulin is represented in dynamic equilibrium with insulin in these compartments only as a mathematical convenience to describe a proteolytic reaction that does not go to completion.

Equations written for insulin synthesis and secretion at steady-state rates (not shown [Landahl 1982]) were used to predict changes in the specific activity of cellular and secreted insulin with time. Based on experimental data, assuming steady state conditions for hormone synthesis was appriopriate. However, because hormone processing and secretion continued to change with time, this compartmental model is only justified for the conditions which exhibit similar temporal kinetics (45 min min preincubation and 15 min pulse in the presence of 20 mM glucose). As discused in the introduction, with only one homogeneous compartment, the specific activity (defined as insulin cpm/ng insulin) of stored and

secreted insulin would always be identical. With heterogeneous storage, as the size of the labile compartment gets smaller and smaller, the specific activity of secreted insulin would exceed that of the cellular insulin more and more.

Hypothetical curves for different sizes of labile compartments were computer drawn in figure 15a. In No. 1 the labile compartment was 33%; in No 2, it was 20%; and in No 3, it was 11% of the total islet insulin. The relative size of the labile compartment can also be estimated visually because the approximate size of the compartment is inversely related to the ratio of maximal peak heights. The experimentally observed changes with time of the specific activities of secreted and cellular insulin are shown in figure 15b. After the 15 min exposure to ^{3}H-leucine, the specific activity of cellular insulin continued to increase for 2 hr, due to the slow cellular conversion of proinsulin to insulin. In agreement with previous reports [Sando 1972, 1973], after 1 hr the specific activity of secreted insulin markedly exceeded that of the average cellular insulin. A comparison with the family of hypothetical curves in figure 15 indicated that these experimental data most closely resembles that predicted for a glucose-labile compartment containing 33% of the total islet insulin.

Stimulation Sensitivity of the Labile Compartment [J. Clin. Invest. 69:554, 1982]: To evaluate the effect of other agents on mobilization of insulin storage compartments, after the pulse islets were cultured in KRB containing 20 mM glucose till minute 90 (75 min post pulse), following which secreted

hormone was sampled in two sequential 20 min intervals between 90 and 132 min. This paragdim was adopted to insure the transport of the radioactive hormone into the secretory granules and allow for the completion of at least two half lives of proinsulin to insulin conversion. In control experiments 20 mM glucose remained the secretogogue throughout this period.

As shown in figure 16, regardless of secretogogue or secretory rate the specific activity ratio of secreted to cellular insulin was identical in each secretion interval. Test secretogogues: 2 mM glucose + 50 mM ^{+}K, induced secretion by depolarizing the plasma membrane; 20 mM glucose + IBMX, potentiates secretion by decreasing c-AMP degradation thereby increasing cellular c-AMP levels. Preliminary experiments indicated that IBMX induced major potentiation of the secretion rate. Thus the observation time period was shortened to reduce excess loss of isotope and to provide actual amounts of released insulin similar to the other secretogogues.

The effect of other agents and conditions were also evaluated (Table 2). With these very different stimuli, secretory rates differed by more than threefold (figure 16c). However, the specific activity ratios between secreted and average cellular insulin always remained the same, indicating the same islet compartments were being mobilized in response to acute stimulation with each of these agents.

Role of Islet Composition and Geographic location on Compartment Size [J. Clin. Invest. 69:554, 1982]: Islets from the dorsal and ventral primordia of the pancreas were prepared

in order to determine if preferential secretion of newly synthesized insulin would be comparable or different in islets with established differences in cellular composition [Baetens 1979]. Islets from both regions contained identical amounts of insulin (figure 17), but those from the ventral primordia contained significantly less glucagon, as reported [Trimble 1981, 1982]. After continuous incubation in 20 mM glucose, the fractional secretion rates of immunoreactive insulin from both groups of islets were identical (figure 17c). The specific activity ratios between secreted and cellular insulin were comparable and the same as seen in experiments with total pancreatic islets (figure 16, Table 2). Thus, under these conditions, the hormone reservoir in islets from different pancreatic locations and cellular composition, exhibit similar compartmental characteristics.

Microtubule Involvement [The Importance of Islets of Langerhans for Modern Endocrinology, p. 27, 1984; Federlin, Scholtholt eds., Raven Press]: Microtubules have long been implicated in the B-cell secretory mechanism [Malaisse-Lagae 1979]. To determine if a sustained interaction between nascent secretory granules and a stable microtubule network was required for preferential release, experiments were performed according to the pargdim in figure 18. At a time coincident with practically all of the labeled hormone being present within secretory granules (110 min), assembled microtubules were disrupted with 0°C temperature, followed by sufficient time at 37°C for reestablishment of microtubular structures [Means 1980].

Analysis of secreted hormone before and after treatment showed that insulin with similar specific activity was being released at both times (no alteration in the ratio of secreted/cellular insulin specific activities; figure 18). Thus, if specific sets of microtubules mediated preferential release of newly synthesized insulin, dissolution and reformation of microtubules should randomize secretion; however, cold treatment had no randomizing effect. These data imply that intra-cellular segregation of secretory granules may not be involved in determining the compartmental properties of islets. Furthermore, this is consistent with inter-cellular differences being responsible for the insulin reservoir heterogeneity.

Role of Calcium in Regulated Heterogeneous Secretion [J. Clin. Invest. 69:554, 1982]: For these investigations, pulse-labelling experiments were conducted according to the protocol depicted in figure 19. Following the pulse islets were incubated in KRB made with 20 mM glucose but without added calcium (a cation required for regulated secretion [reviewed in Prentki 1984]). The fractional secretory rate of insulin dropped dramatically without calcium in the buffer (figure 19a). Instead of a higher specific activity, as observed with the other stimuli, the low amounts of secreted insulin in the absence of calcium, had a lower specific activity than the average cellular insulin (figure 19b). Thus, under these conditions islets apparently preferentially released older insulin. Upon readdition of KRB containing calcium, the secretion rate and the specific activity ratio increased significantly,

indicating that the labile compartment remained viable and responsive to glucose. These data demonstrate that heterogeneous insulin release is a calcium dependent phenomenon and that in islets externalization of newly synthesized insulin does not occur via the constitutive pathway (at this time) [Kelly 1985].

Non-metabolizable Effectors of Heterogeneous Secretion: The differential externalization of newly synthesized vs. stored insulin in the presence and absence of calcium (respectfully) suggested that compartmental recruitment of secreted insulin may be a regulated process. Pulse-chase experiments were performed to evaluate the effect of post-translational incubation conditions on the preferential release of newly synthesized insulin.

Following the pulse, islets were incubated for up to 165 min in media containing 20 mM glucose, 2mM glucose + 50 mM K^+ or 0 mM glucose + 100 nM Phorbol-12-Myristate 13-Acetate (TPA), during which time secreted and islet cell samples were collected and analyzed for their radioactive and immunoreactive insulin content (figure 20).

These agents effect insulin secretion in the following manner: K^+ is a non-specific stimulator of insulin secretion which acts by depolarizing the cell and TPA is a potent insulin secretogoge believed to exert its action through activation of protein kinase C.

Figure 20 illustrates the effect of time on the specific activity of secreted and cellular insulin during incubation with each agent. Under each condition studied the specific

activity of the secreted insulin was greater than that of the cellular insulin. These data demonstrate that preferential release of newly synthesized insulin is not a stimulus specific phenomena.

Effect of secretory state: To determine if different secretory states can affect quantitative differences in preferential release, islets were incubated for the 115 min post-pulse period, in media containing 2 mM, 5 mM, 10 mM or 20 mM glucose. These concentrations encompass the glucose stimulated dose response curve for insulin secretion. During the last 20 min of incubation, secretion samples were collected (figure 21) and the radioactive and immunoreactive insulin content determined in both secreted and final cell samples. As illustrated in figure 21 increases in the glucose concentration resulted in concomitant elevations of insulin secretion and increases in the specific activity of the secreted insulin relative to cellular insulin - as evidenced by the ascending secreted/cellular insulin specific activity ratio with elevation in glucose concentration. These data demonstrate that different rates of insulin secretion result in greater release of newly synthesized insulin suggesting that the size of the "labile" compartment may be determined by the secretory state (rate) of the islet.

The effect of prolonged 2 mM glucose preexposure on a subsequent glucose stimulated secretory response was also investigated (figure 22). Following the pulse islets were maintained for 60 - 250 min in 2 mM glucose, then stimulated with 20 mM glucose for either 20 or 40 min (two sequential 20

min periods). As illustrated in figure 22 maintaining islets in 2 mM glucose (which is an extreme hypoglycemic condition) has little effect on the secreted/cellular insulin specific activity ratio. However, these same conditions result in a progressive decrease in the amount of total immunoreactive insulin released in response to maximal glucose stimulation. Interestingly, the decreased secretory responsiveness exhibited kinetics similar to that observed for islets maintained in media containing elevated glucose concentrations (Matchinsky, Bolaffi).

Taken together these data indicate that preferential release of newly synthesized insulin is an inherent consequence of stimulated insulin secretion from islets. Furthermore, this process does not appear to be stimulus specific but does exhibit a temporal component.

First and Second Phases of Insulin Release: To determine the contribution of newly synthesized insulin during the first and second phases of insulin secretion experimental protocols were modified. As illustrated in figure 23, following the pulse islets were incubated in 2 mM glucose for 75 min, then stimulated with 20 mM glucose for three sequential periods of 10, 20 and 20 min. Based on observations from this and other laboratories, this paragdim would result in the first phase of release being segregateed into the initial 10 min stimulatory period while the ascending second phase of insulin secretion would be distributed in the subsequent periods. In addition, this extended culture time would minimize any possible secretory response alterations due to the potentiating effect of

prior glucose exposure on subsequent glucose stimulated insulin release [Grodsky 1969, Cerasi 1975, Grill 1978].

Depicted in figure 23 are the fractional secretory rate and the secreted/cellular insulin specific activity ratio for each period. As expected the rate of insulin secretion increased throughout the duration of glucose stimulation. The first phase of insulin secretion was marked by a low level of immunoreactive release and a secreted/cellular insulin specific activity ratio of less than unity. In each subsequent stimulatory period there was a dramatic increase in the immunoreactive insulin secretory rate and a coincident elevation in the ratio of secreted/cellular insulin specific activity. These data are consistent with the previous observations indicating that greater stimulation of insulin secretion is associated with a greater release of newly synthesized insulin. Furthermore, these increases in secretory rate and specific activity appear to represent a potentiating effect of glucose on insulin secretion. In terms of the insulin reservoir model presented, these data suggest that first phase insulin release may: 1) recruit from a relatively small third secretory compartment close to terminal release that still contains older insulin that must be released (depleated) prior to secretion of the newer insulin, or, 2) occurs from both the labile and stored compartment to the same extent.

Tolbutamide Pretreatment in vivo [Diabetes 35:6, 1986]: To determine the effect of diminished islet insulin content -induced in vivo - on the secretion of newly synthesized insulin, normal rats were treated for 3 days with high doses

of tolbutamide after which islets from treated and control animals were isolated and their insulin secretory characteristics investigated. As previously described, tolbutamide treated islets exhibited decreased islet insulin content and accelerated proinsulin to insulin conversion but the (pro)insulin biosynthetic rate remained unaltered (figures 7).

Initial pulse-chase experiments conformed to the parigdim described by figure 26. As illustrated in figure 24, islets from tolbutamide pretreated rats exhibited a significantly decreased glucose-stimulated insulin secretory response during the observation period (90-130 min). During the same period, the secreted/cellular insulin specific activity ratio was much lower in pretreated islets than that calculated for controls.

Analysis of the temporal distribution of newly synthesized insulin revealed that prior to the 100 min time point, the specific activities of the secreted and cellular insulin from treated islets were almost identical and diverge relatively little thereafter (figure 25). Furthermore, at each time measured the specific activities of secreted and islet insulin were 4 - 5 times greater in islets from tolbutamide-treated rats than those of untreated animals (figure 25 vs. 20). It is apparent from visual inspection of figure 25 that the secreted/cellular insulin specific activity ratio remains relatively constant throughout the chase period and does not significantly deviate from unity -indicating that islets pretreated with tolbutamide secrete insulin in a homogeneous pattern.

In contrast to untreated islets (figure 15, 20), these

data demonstrate that tolbutamide administration *in vivo* apparently hyperstimulated the islets insulin secretory response and dramatically depleted the stored insulin reservoir. These alteration were associated with the following *in vitro* observations: 1) acceleration of proinsulin to insulin conversion, 2) decreased amount of insulin secreted with no alteration in the fractional rate of release, 3) dissolution of the compartmental secretory properties of islets (the diminished insulin reservoir appeared homogeneous and no longer conformed to the model state proposed earlier for normal islets), and 4) a direct dependence of the insulin secretory rate on the (pro)insulin synthetic rate as evidenced by the high specific activity of the secreted insulin.

The dramatic depeltion of the insulin stores agrues against the presence of a massive inaccessible pool of stored hormone and is inconsistent with the proposal that a large portion of the insuilin exists in an inert compartment.

Prolonged glucose stimulation: To evaluate the effect of prolonged glucose exposure *in vitro* on the compartmental properties of islets a different experimental protocol was introduced. Prior to pulse, islets were preincubated for 225 min under three different conditions: 1) 20 mM glucose for 225 min, 2) 2 mM glucose for 180 min, then 20 mM glucose for 45 min, or 3) 2 mM glucose for 225 min. Following the 15 min pulse in 20 mM glucose, islets were maintained in media containing 20 mM glucose thereafter (till min 130). A 20 min secretion sample was collected between minutes 110 - 130 post pulse.

As illustrated in table 3, the fractional secretory rate of immunoreactive insuiln during the 20 min interval (110-130 min) was similar under all three conditions. However, the actual amount of insulin secreted per islet differed dramatically. Consisted with the reported kinetics of the onset of third phase insuiln release ("desensitization"), islets continuously exposed to 20 mM glucose throughout the experiment secreted less insulin during the collection period than either, islets preincubated for 225 min in 2 mM glucose or those cultured 180 min in 2 mM glucose then 45 min in 20 mM glucose prior to pulse (0.46, 0.96. 1.00 ng/islet x 20 min respectfully). This incongruity is partially due to the decreased islet insulin content following 355 min of culture in 20 mM glucose (26.64 ng/islet vs. 36.3 ng/islet in controls). The culturing of islets for just under 6 hrs in 20 mM glucose also resulted in a dramatic elevation in the specific activity of the secreted insulin when compared to the other two conditions (Table 3), or to the insulin secreted by fresh islets preincubated for only 45 min in 20 mM glucose prior to pulse (presented above). Furthermore, following continued glucose exposure (6 hrs), the calculated secreted/cellular insulin specific activity ratio was approximately three times greater than that observed for any other conditions investigated.

These studies demonstrate that prolonged exposure to glucose alters the compartmental character of the islet insulin reservoir, so that newly synthesized insulin is secreted with an even greater preference than observed following more acute

glucose stimulation. Thus, based on the two compartment insulin reservoir models, "chronic" stimulated secretion apparently depletes the stimulus defined "labile" secretory pool resulting in less dilution of newly synthesized hormone by residual older insulin in the labile compartment. This suggests that the decrease in insulin secretion during the "third" phase of insulin release may be a consequence of "compartmental exhaustion" resulting in a more direct coupling of the secretory rate and the rate of insulin synthesis.

INSULIN SECRETING TUMORS [Diabetes 33:556, 1984]

A common characteristic of insuilnomas is their inability to repond approriately to changes in glucose concentration. To determine if this attenuated responsivness corresponds to alteration of the hormone reservoirs compartmental properties, insulin secretion and storage were evaluated in insulin secreting tumors. Tumors were excised from carrier rats and treated according to the paragdim illustrated in figure 26.

Consistent with other reports [Sopwith 1981, Patzelt 1978] these tumors demonstrated a continuous elevated fractional rate of insulin release that was unresponsive to changes in glucose concentration and showed only a minor augmentation of insulin secretion in response to leucine and IBMX (figure 27, 28). At the termination of the experiment (152 min) the specific activity of insulin present within tumor cells was three times greater than islets identically treated (34 cpm/ng vs. 11 cmp/ng; Table 4). Furthermore, under all conditions studied these insulinomas secreted insulin in a

homogeneous manner (figure 29), as evidenced by secreted/cellular insulin specific activity ratios of approximately one.

Note; these experiments cannot exclude the possibility that incorporation of ^{3}H-leucine in the insulinoma may have occurred from a cellular leucine pool that was not identical to that in normal islets. However, a threefold more rapid replacement of stored insulin by de novo synthesis appears to be more consistent with the data. On a cellular basis, a threefold more rapid replacement of cellular insulin could indicate either that rates of proinsulin biosynthesis are higher than rates in maximally glucose-stimulated islets or that insulin storage capacity is decreased in these cells (or possibly both).

These studies demonstrate that hormone secretion and storage by insulinomas does not exhibit the heterogeneous compartmental character of normal islets. It appears then that under conditions of continuous secretion and depeleted hormone storage capacity (as also observed in tolbutamide pretreated islets), the hormone reservoir loses its compartmental character and becomes a homogenous pool (figure 1{I}). Furthermore these investigations confirm that heterogeneous hormone release can only be affected in a regulated secretory cell system.

INSULINS I AND II

SYNTHESIS

Effect of glucose stimulation: To determine the effect of glucose on insulin I and II synthesis islets isolated from normal Long Evans rats, were pulse-labelled as illustrated in figure 30. Islets were preincubated for 225 min in either 2 or 20 mM glucose prior to pulse, then maintained in 20 mM glucose thereafter. Because methodology for the quantitative separation of the two proinsulins did not exist experiments were not terminated immediately post-pulse, rather, a 95 min chase period was introduced prior to any sample collection. As previously determined, this length of time even at low glucose preincubation conditions allowed for the completion of at least 2 half lives of proinsulin to insulin conversion. Just prior to the termination of the experiment, a 20 min secretory period was collected and both secreted and islet samples analyzed for their radioactive and immunoreactive rat insulin I and II content.

As expected, preincubation in 20 vs. 2 mM glucose resulted in a dramatic increase of newly synthesized insulin, demonstrated by the significant differences in the amount of radioactive insulin in secreted and islet samples (figure 30). However, as can be seen by the percentage of rat insulin I above each set of bars the relative distribution of radioactive and immunoreactive rat insulins I and II by this time was approximately 60:40% insulin I to II. Furthermore, although prolonged prior glucose exposure resulted in a significant decrease in the actual amount of islet and secreted insulin immunoreactivity (as previously described), no alteration in the percent of insulin I and II were observed. These data dem-

onstrate that glucose stimulated the synthesis of both insulins in proportion to their immunoreactive content and reported mRNA levels [Giddings 1986]. In addition, under these conditions, which we previously demonstrated alter the character of the insulin reservoir, islets continued to secrete the two insulins in approximately 60:40%, insulin I:II. Thus the relative synthesis and secretion of rat insuiln I and II appear to be coordinately regulated by glucose and independent of the compartmental state of the islet.

Regional differences: To determine if islets from different primodial origins and geographic regions differentially synthesize rat insulin I & II, islets from the "head" and "tail" of the pancreas were isolated and cultured separately for 6 hrs in media containing 20 mM glucose in the presence of ^{3}H-leucine. Under these conditions the radioactive content of rat insulin I:II in islets isolated from the "head" was 62:38%, while that in islets isolated from the tail was 72:28%. Thus in both groups of islets, during prolonged glucose exposure the two insulins were still synthesized in approximately 60:40% insulin I:II.

Mouse islets: In mice the two insulins have been localized on two separate chromosomes [Lalley 1984]. To determine if differences in genomic location have an effect on glucose stimulated insulin synthesis, pulse-labelling experiments, similar to those previously described for rats, were performed using mouse islets. Groups of 100 islets were incubated for 45 min in 20 mM glucose or 225 min in 2 or 20 mM glucose, then pulsed 15 min and cultured for 135 min in media containing 20

mM glucose (figure 31). Although there was greater variation in the data (when compared to rat islet experiments), under all three conditions the relative distribution of radiolabelled rat insulin I:II was similar to that observed in rat islets, approximately 60:40% (figure 31). Immunoreactive rat insulin I and II measurements of the same samples contradicted the radioactivity distribution data and suggested that mouse islets contained greater amounts of immunoreactive rat insulin II than I. Optical density measurements of rat insulin I and II column elution profiles, of these samples were more consistent with the radioactivity distribution data indicating that for these series of experiments measurements of immunoreactivity were inaccurate. In contrast to rats, mouse islets secreted very little insulin during the observation period (<1% of insulin content). However, the labelled rat insulin I, II which was externalized was in the same relative proportion as observed in islets. Thus in mouse and rat islets glucose apparently stimulates the overall synthesis and secretion of both insulins to the same extent.

PROCESSING

Initial characterization: Experiments were performed to determine how differences in the amino acid structure of the two rat insulins affect their processing. Islets were preincubated for 45 min in 20 mM glucose, pulsed for 20 min and to minimize hormone externalization chased for up to 250 min in 2 mM glucose (figure 32). Under these conditions the relative appearance of labelled rat insulins I and II occurred with

different kinetics (total mean proinsulin to insulin conversion half life was approximately 50 min).

At early chase times, rat insulin I was the predominant radioactive insulin; prior to the 60 min time point it accounted for greater than 85% of the radioactive insulin present. At later chase times, with continued proinsulin to insulin conversion, the relative proportion of rat insulin II increased so that by 150 min chase, when insulin maturation neared completion, the intra-islet radioactive insulin distribution approached the IRI distribution (60:40%) found in freshly isolated untreated islets (insulin I = 58.4%, insulin II = 41.6%, SEM + 2.8%). Furthermore, similar amounts of total radioactive IRI in species I and II (insulins + proinsulins, after correction for loss due to C peptide excision) were recovered at the beginning and end of these experiments (3.3×10^{-3} cpm/islet at 20 min, 3.5×10^{-3} cpm/islet at 290 min), indicating that the proinsulins must have originally been synthesized in a 60:40% proportion and that both insulins remain stable for over 4 hours in islets. Thus differential biosynthesis or degradation of rat insulin I or II cannot explain the observed differences in the disproportionate amounts of the two insulins at early chase times.

Accelerated conversion: We previously demonstrated that in addition to stimulating insulin synthesis, preincubating islets for over 3 hours in high glucose media accelerates the proinsulin to insulin conversion rate. To determine what effect glucose induced processing has on the temporal appearance of rat insulin I and II, following culture under acceler-

ated and non accelerated conditions, islets were pulsed for 20 min and chased for various times. Figure 33 illustrates the actual number of counts incorporated into rat insulins I & II under non-accelerated and accelerated conversion conditions, at the designated chase times. Consistent with our previous observation, 225 min culture in media containing 20 mM glucose resulted in the appearance of greater amounts of radioactive insulin at earlier times. At early chase times under accelerated conversion conditions, there was a dramatic increase in the presence of both radioactive insulins. However, the relative percent of newly synthesized rat insulin I at each time under accelerated conversion conditions was approximately the same as that observed under non-accelerated conversion conditions (represented by the percentages above each set of bars). Thus, although at early chase times processing kinetics of rat insulin I and II appear to be different, glucose accelerates the conversion of both proinsulins to the same extent.

Intra-cellular transport: To determine if differences in intra-cellular transport of rat insulin I and II could account for their differential processing, pulse-labelling experiments were performed in which transport from ER to conversion compartment was terminated at various times during the chase, but the actual enzymatic cleavage of proinsulin to insulin was allowed to continue to completion. Transport was inhibited by culturing islets in media containing antimycin A, a metabolic poison previously shown to terminate intra-cellular transport with no apparent effect on enzymatic conversion [Orci, Stiener]. Other laboratories have further reported that

cytosolic ATP is probably not required for the enzymatic conversion per se, but appears to be important in setting up the intra-granular milieu necessary for processing [Rhodes et al. 1987 JBC 262:10712]. This implies that once the prohormone is sequestered within the conversion compartment and the correct pH extablished, enzymatic activity in not effected by alterations in the extra-compartmental environment.

Islets were pulsed 5 min and chased for 175 min in KRB containing 2 mM glucose in the absence (control) or presence of 50 uM antimycin A (figure 34). Another group of islets were cultured for 15 min following the pulse in 2 mM glucose (till min 20) after which they were maintained in media containing 2 mM glucose + 50 uM antimycin A for the remainder of the experiment. The addition of antimycin A at 5 and 20 min, resulted in 89% and 31% of the labelled proinsulin remaining intact respectively vs. 3% in controls (figure 34). Under the same culture conditions rat insuiln I accounted for approximately 95% and 85% of the radioactive insulin present respectively. At the termination of the experiment control islets contained <5% labelled proinsulin intact and rat insulin I:II were present in 56:44% distribution. Note, the same actual amount of labelled rat insulin I was present by 180 min in control islets and those treated with antimycin A at 20 min (71 cpm insulin I/islet, 74 cpm insulin I/islet respectively). In contrast the amount of radioactive rat insulin II was dramatically different under the same conditions (58 cpm insulin II/islet, 18 cpm insulin II/islet respectively). These data indicate that transport of rat insulin I to the conversion

compartment is completed by 20 min after initiation of the pulse and apparently occurs at a faster rate than transport of rat insulin II.

SECRETION

Effect of secretogogues: The effect of non-metabolizable secretogogues and post-pulse secretory conditions on rat insulin I & II processing and secretion were further investigated. After the pulse in 20 mM glucose, islets were chased for 75 min in either 0 mM glucose, 0 mM glucose + 25 uM forskolin, 0 mM glucose + 100 nM TPA or 20 mM glucose (figure 35). Following the chase period, islets were stimulated with 20 mM glucose for the test periods previously described. As demonstrated in figure 35, treatment with these agents resulted in vastly different secretory rates during the chase period (1.9% in 0 mM glucose to 21.5% in 100 nM TPA). However, no significant alteration in the relative content of rat insulins I & II in the post-chase secreted and islet samples was observed. These data suggest that in islets, once the proinsulins have been synthesized and transported culture conditions do not differentially regulate processing or secretion of the two insulins.

In the same experiments the contribution of each newly synthesized insulin to the secretory pool was assessed. Individual rat insulin I and II specific activities in secreted and islet samples were calculated (figure 36). As expected the specific activity of both insulins was at least two fold greater in secreted samples than that found in

islets. However, the ratio of rat insulin I to II specific activities was almost identical for secreted media and islet content (1.3 and 1.4 respectively). Therefore, heterogeneous secretion is not a result of differential externalization of the two insulins.

Stability and secretability: The effect of time on stability and secretability of newly synthesized rat insulins I and II was also evaluated. Following the pulse, islets were cultured in 2 mM glucose for up to 250 min (figure 37). Because culturing islets in 2 mM glucose in effect shuts off insulin release, secretion data were collected by exposing islets to 20 mM glucose for 20 min just prior to the collection of islet tissue. To allow for completion of newly synthesized hormone transport into secretory granules and minimize the potentiating effect of prior glucose exposure on subsequent glucose stimulated insulin release, collection of secreted samples (reexposure to 20 mM glucose) began at minute 50.

Figure 37a depicts the relative percentage of radioactive rat insulin II ($\text{Rat II}/_{\text{Rat I+II}} \times 100$) in the secreted media and islet samples. These data demonstrate that at all times measured the two rat insulins are secreted in relative proportion to their islet content, indicating that both newly synthesized insulins remain stable for over 4 hrs after synthesis.

Under these extended culture conditions there was a marked decrease of insulin secretion in response to glucose stimulation (reexposure). Similar observations have been

reported in studies characterizing potentiation of glucose stimulated insulin secretion, however, the kinetics of this decline (figure 37b) were strikingly similar to that described for the third phase of continuous glucose stimulated insulin release [Matchinsky 1986, Bolaffi 1986b].

These data demonstrate that, rat insulin I and II remain stable in islets and appear to contribute to the storage and glucose stimulated secretory pool in proportion to their synthesis and immunoreactive content throughout all phases of glucose stimulated insulin release.

DISCUSSION

Islets contain a large reservoir of insulin capable of sustaining continuous elevated secretion for over 12 hrs. Experimentally, however, glucose stimulated insulin release starts to fall off by 4 hr of exposure [Gold 1976, Matchinsky 1986, Bolaffi 1986]. Furthermore, islets stimulated with glucose apparently secrete newly synthesized hormone in preference to the large amount of stored insulin. Both these phenomena indicate that the large amount of insulin present in islets does not represent a homogeneous pool. As described in the introduction, two general models have been proposed to account for the observed phenomena: 1) the presence of a labile and stable pools that differ in their synthetic and secretory properties, 2) the presence of active and dormant pools with all insulin synthesis and secretion taking place only in the active compartment. Note that these models are described in terms of two compartments for the sake of clarity and may actually be thought of more as a continuum between the two compartments described in each model. Furthermore, within the context of each model multiple states are possible which may change with respect to time (figures 2a-d, 3a-c). The actual state of the islet hormone pool is thus determined by: 1) hormone synthesis, 2) size and distribution of the hormone pool, and 3) hormone secretion. To understand the secretory and storage properties of islets, it is therefore important to describe the nature of this hormone reservoir and evaluate the various parameters that determine its character.

Our investigations have confirmed that the insulin reser-

voir represents a heterogeneous pool. Under all enhanced secretory conditions studied (using various agents) islets secreted newly synthesized insulin at a greater rate than immunoreactive hormone; evidenced by the secreted insulin having a higher specific activity than islet insulin resulting in specific activity ratios of secreted/islet insulin being greater than unity. This implies that in islets, newly synthesized insulin is distinguished from the major portion of the stored hormone and targeted for preferential release. Studies performed in media devoid of calcium demonstrated that this heterogeneous release is not a consequence of coordinate hormone externalization by different pathways (regulated vs. constitutive), but rather may be an intrinsic property of regulated secretion in normal islets. The homogeneous secretory pattern observed in the insulinomas studied supports this hypothesis; a characteristic of insulin secreting tumors is their constitutive release of hormone and insensitivity to glucose stimulation [Chick 1977, Praz 1983, Ashcroft 1986]. In addition heterogeneous secretion, as evaluated in these studies, depended on the presence of a large amount of presynthesized (stored) hormone; when the hormone reservoir was depleted (tolbutamide treated islets and tumor cells) homogeneous secretion was observed. Thus preferential release of newly synthesized insulin is not an inherent property of all insulin secretory cells, but rather, a consequence of stimulating regulated secretion in normal islets that contain a large stored hormone pool.

Quantitative evaluation of the storage and secretory

characteristics of the insulin reservoir allowed us to conceptualize the hormone reservoir in terms of a two compartment model (illustrated in figure 14). Initially the islet hormone pool was defined under conditions of continuous exposure to 20 mM glucose for a total of 230 min (which included a 45 min preincubation period). The insulin secreted in these studies had a 3 fold higher specific activity than that remaining in islets. This was interpreted as demonstrating preferential release of newly synthesized insulin which we proposed resulted from the hormone pool consisting of at least two hypothetical compartments - labile and stable. Comparison of the data with that predicted by a rigorous mathematical model confirmed that under these conditions the maximal size of the hypothetical labile compartment approximated 33% of the hormone reservoir (figure 15).

Maintaining the same synthetic and processing conditions we next evaluated the stimulus sensitivity of the individual compartments. These studies determined that preferential release of newly synthesized hormone was not a stimulus specific phenomena. Furthermore, in other experiments, kinetics of release were similar for all stimuli (figure 20). These data implied that all stimuli access similar compartments and the degree of preferential secretion of newly synthesized hormone indirectly correlated with the islets secretory state.

Our studies further demonstrated that the compartmental configuration (state) of the hormone reservoir did not remain constant, but responded to environmental stimuli. Following >5 hrs of continuous glucose exposure the islet insulin con-

tent and the actual amount of immunoreactive insulin secreted decreased. However, newly synthesized insulin was secreted at an even greater fractional rate than previously observed; evidenced by the secreted insulin having a >9 fold higher specific activity than islet insulin. This indicated that in response to continued stimulation, newly synthesized insulin represented a greater proportion of the secreted hormone. One interpretation of these data is that continued stimulation, decreased the size of the labile pool, so that, radioactive hormone mixed with a smaller amount of preexisting insulin. Alternatively, if the size of the labile compartment were initially very small, it could remain unchanged as it was fed by synthesis, while the "stable" pool of insulin was not replaced and therefore decreased. In this arrangement secretion from the labile compartment would represent a relatively minor portion of the total secreted insulin while most of the secreted insulin would originate from the much larger, but slower releasing stable pool. Although either interpretation or combination of both is possible, our experimental data do not support the latter proposal being solely responsible for this phenomenon; the alteration in the size of the stable compartment necessary to account for the increased specific activity ratio observed following prolonged stimulation, would be far in excess of that demonstrated in these experiments.

The basis for heterogeneous hormone release and its role in islet physiology remain areas of debate. At the inception of our study it was realized that heterogeneous release from islets may be due to: 1) differing populations of islets, 2)

secretion of different secretory products (insulin I vs. II), 3) presence of differentially responding B-cells within the islet (i.e. core vs. peripheral cells), or 4) different secretory granule pools within the B-cells themselves. Although results from our investigations can support either of the latter two hypothesis (3 or 4), our data is more consistent with heterogeneous secretion being a consequence of differentially responding B-cells within islets.

Two subpopulations of islets, differing in primordial origin (dorsal vs. ventral), pancreatic location (head vs. tail) and cellular composition (differing in proportional amounts of individual cell types A-cells, B-cells and D-cells), have been identified [Orci 1982]. These differences suggested an obvious source for heterogeneous responsiveness, however, when segregated and independently tested in parallel, islets from both groups preferentially secreted newly synthesized insulin during 20 mM glucose culture in almost identical fashion. Thus, islets with such gross differences, and response characteristics similarly exhibit heterogeneous secretion, demonstrating that preferential release of newly synthesized insulin is not restricted to a specific subset of islets.

Rat islets synthesize two insulins (I,II) that are products of non-allelic genes. It was reported that freshly isolated islets from normal rats contained similar amounts of the two insulins (60:40%, insulin I:II), but glucose exposure resulted in a 10 fold greater synthesis of insulin I than insulin II [Kakita 1982]. These observations implied that the

two insulins were compartmentally segregated and therefore differentially secreted.

Our investigations demonstrated that synthesis, processing and secretion of the two murine insulins was coordinately regulated by glucose. Although maturation of insulin I and II occurred with different kinetics (discused below), at all times and conditions investigated, secretion of the two insulins reflected their distribution within islets. Thus, heterogeneous secretion is not a consequence of differential segregation of insulin I and II into the hypothetical labile and stable pools.

It has long been recognized that islets are composed of different cell types. Analysis of islet morphology has demonstrated that B-cells exist in differential cellular environments in which they are juxtaposed not only with other B-cells but also other hormone secreting cells. In this organizational grouping the various cells communicate by direct contact interaction (through gap junctions [Meda 1980]) and paracrine effector systems, the net result being a "biosociological" regulation of islet physiology [reviewed in Pipeleers 1987]. Recently this organizational heterogeneity has been extended to the characterization of individual B-cells.

Experiments <u>in vivo</u> have shown that under continued glucose stimulation B-cells located central within the islet degranulate sooner than those at the periphery [reviewed in Orci 1984]. This implies that B-cells located in the islets core either secrete insulin at a faster rate or are recruited sooner than cells at the islet cortex. This was the first

indication that differences in cellular topology correlated with functional response differences. In addition studies using dispersed islet cells, demonstrated that individual B-cells exhibit differential secretory responses [Meda 1984]. From these studies it is apparent that individual B-cells constitute a heterogeneous cell population which differ in rates of insulin secretion, threshold sensitivity to stimuli and possibly rates of hormone synthesis. This diversity of cellular responsiveness and geographic degranulation gradient support the hypothesis that cellular recruitment is the basis for the glucose dose response characteristics exhibited by islets.

According to this theory increasing levels of glucose would stimulate greater numbers of B-cells so that at a maximal stimulatory dose all the cells capable of responding to the particular stimulus have been recruited. In addition there may be a temporal component involved in the recruitability of the different cell populations which implies that the applied stimulus is not the only determinant for initiation of secretion (glucose potentiation). This B-cell heterogeneity may also be responsible for the compartmental properties of insulin storage and the kinetic pattern of insulin release observed in islets.

Consistent with this hypothesis, when islets were chronically hyperstimulated (tolbutamide treatment) they secreted newly synthesized and stored insulin in a homogeneous manner; islets from tolbutamide treated rats secreted insulin with the same specific activity as their intra-islet insulin. This indicated that treated islets were no longer compartmental-

ized. This can be interpreted as showing that following *in vivo* tolbutamide "priming," individual B-cells have become more similar in their response characteristics. With this same logic, insulin secreting tumors, which are believed to originate from a single progenitor B-cell, should secrete insulin in a homogeneous manner and indeed that is what was observed. This further supports the proposal that islets compartmentalization of the hormone reservoir and the dynamic kinetics of insulin release are the consequence of the heterogeneous responsiveness of individual normal B-cells.

In addition, because of their decreased content, tolbutamide treated islets secreted a greater fractional amount of both immunoreactive and radioactive insulin than that observed in normal islets. Similar patterns of homogeneous insulin release were also observed in rat insulin secreting tumors. These tumors, which are unresponsive to many external stimuli and reportedly contain little stored hormone, secreted immunoreactive and radioactive insulin in similar fractional rates as tolbutamide treated islets. These data demonstrate that heterogeneous hormone release is a property of regulated secretion that depends on the continued presence of an intracellular hormone reservoir and a graded stimulus responsiveness.

Initial reports describing the effect of prolonged glucose stimulation on insulin secretion suggested that the observed decrease of insulin release may result from the exhaustion of a labile secretory compartment [Matchinsky 1986] or a desensitization of the secretory apparatus to the glucose

induced signal [Bolaffi 1986b]. Based on the B-cell heterogeneity described, a mechanism can be envisaged in which glucose stimulates a set of B-cells having diverse insulin secretory, storage and synthetic characteristics. If secretion in these cells exceeded the rate of insulin synthesis stimulation would deplete their insulin reservoir at different rates. Continued exposure would result in a gradual decrease in the size of the "labile" compartment and amount of insulin secreted, with the insulin secretory rate becoming progressively more dependent on the rate of insulin synthesis. Because stimulation with higher glucose concentrations or other secretogouges would recruit greater numbers of heterogeneous cells there would be expected, as was observed, a greater amount of insulin released without a significant alteration in kinetics [Bolaffi personal communication]. Furthermore, this model predicts that preferential release of newly synthesized hormone can only occur in secretory systems that retain their heterogeneous cellular response characteristics and maintain a less responsive pool of secretory material. Therefore, islets isolated from tolbutamide treated rats and insulin secreting tumors should not exhibit the same phasic pattern of stimulated insulin secretion observed in normal islets (in particular the decreased secretion following prolonged exposure to stimuli). Rather, the maximal rate of stimulated secretion would be limited to and have a greater dependence on the rate of hormone synthesis. Although the possibility that a signal desensitization phenomena may contribute to the attenuation of insulin secretion cannot be dismissed, the experimental find-

ings of altered compartmental characteristics coincident with phasic insulin secretion strongly support the cell heterogeneity/recruitment hypothesis.

We can not exclude the possible participation of intracellular secretory granule heterogeneity in this phenomenon, however, at present no evidence exists in support of this hypothesis in islets. If a physical segregation of secretory granules within the B-cell were responsible for the heterogeneous release, disruption of the microtubule network should have randomized this organization, this was not observed (figure 18). Islets quantitatively exhibited the same preferential release of newly synthesized insulin before and after acute hypothermic exposure, which should have temporarily disrupted the existing microtubule filaments. Furthermore, findings in other secretory cell systems which proposed that secretory granule heterogeneity was responsible for the observed differential secretory properties (heterogeneous release and decreased hormone externalization in response to continuous stimulation) can also be explained on the basis of heterogeneous cellular characteristics.

In one such system, as described in the introduction, dispersed parathyroid cells secreted both new and old PTH when media calcium is lowered, but when exposed to c-AMP only old PTH was released [Morrissey 1979]. Those investigators suggested that this response was a result of intra-cellular granular sinecence, however, this can also be explained by heterogeneity of cellular responsiveness to the two stimuli (cellular recruitment). One can envisage a mechanism whereby

decreased calcium - the physiologic stimulus - recruits all parathyroid cells while c-AMP stimulates a subpopulation of cells that synthesize hormone at a slower rate. Alternatively extracellular calcium may continuously stimulate an inhibitor of secretion in all cells and c-AMP may deinhibit only a subset of those cells. In either case, exposure to c-AMP would result in the preferential release of older hormone while cells stimulated with calcium would secrete both new and old PTH. Thus it appears that cell heterogeneity/recruitment may be a common secretory cell characteristic responsible for stimulus dose responsivness, compartmental secretory properties and stimulus "desensitization".

Through our investigations we also defined proinsulin synthesis and processing at various times and conditions. Other laboratories have demonstrated that glucose stimulates insulin synthesis at both the transcriptional [Permutt 1972a, 1974, Itoh 1980] and translational level [Ashcroft 1978, Permutt 1972b, Welsh 1986]. In isolated islets the translational effect was observed within minutes of glucose exposure while an increase in insulin gene transcription was observed following at least an hour of exposure [Itho 1980, Giddings 1982].

Our studies demonstrated that (pro)insulin synthesis reached a maximal rate by 45 min of exposure and remained at that level for at least 180 min thereafter. Others in this laboratory reported that islets exposed to 11 mM glucose synthesized hormone at a constant rate during 24 hrs of culture [Nagamatsu 1987]. Although insulin mRNA levels were not determined, based on data from other laboratories, culture with 11

mM glucose maintains insulin mRNA levels relatively constant for the 24 hr period studied [Brunstedt 1982, Welsh 1986] (the mechanisms responsible for this observation remain controvertial). These data imply that under conditions of continuous glucose stimulation, where mRNA levels are sustained the translational process appears to have reached a maximal level (saturated) after only 45 min of stimulation.

Further investigation of the apparent translational limitaion phenomenon would be critical in our understanding of the regulation of protein specific expression. Glucose has long been known to stimulate insulin gene transcription, however, its effect on insuiln mRNA stability or degradation remains unclear [Welsh 1985, Nielsen 1985]. It is important to appreciate that in addition to stimulating insulin synthesis, glucose stimulates the synthesis of many other proteins (including the putative converting enzyme [Nagamatsu, personal communication]). This apparent glucose enhanced expression of many islet proteins may result in the saturation of the cells translational mechanism. Although no evidence is presently available in support of this proposal one could postulate that any number of factors involved in translation (ribosome number, ribosome active state, amount of SRP or number of SRP receptors, etc.) could be responsible for limiting this process.

Glucose was also shown to stimulate synthesis of both murine insulins (I,II) to the same extent. Whether exposure to 20 mM glucose was 15, 60 or 240 min, insulin I and II were synthesized in proportion to their relative content within rat

islets (approximately 60:40% insulin I:II, as previously reported [Clark 1969, Rall 1979]. This data is consistent with reports of the effect of glucose on insulin I,II synthesis in normal islets cultured overnight [Rhodes 1979] and is further supported by studies showing that chronic in vivo hyperglycemia stimulates the synthesis of both insulin mRNA's to the same extent [Giddings 1986].

The two insulins in mouse islets were also found to be present in similar amounts. Note that in mice the two insulin genes are located on separate chromosomes [Lalley 1984]. Because of this segregation is was unexpected to find equal glucose regulation of synthesis and presumably mRNA levels of insulin I and II. Thus, genomic separation of the two insulin genes does not differentially affect expression or glucose regulated synthesis of insulin I and II.

These studies contradict previous reports that glucose preferentially stimulates synthesis of insulin I vs. II [Kakita 1982]. This discrepancy may result from a methodological artifact present in the original reports which interfered with the quantitative recovery, separation or detection of the hormones. Insulin I,II recovery and separation are very sensitive to ambient pH [Trump 1984]. Furthermore, specific anti-insulin antisera used for detection may exhibit a preference for one or the other insulins; as demonstrated by our by our immunoassay studies of individual insulin I and II from mouse islets (figure 31, this was corrected for all other studies reported here). As described in methods our quantitative analysis using HPLC and consistent recovery yields precluded

these complications.

Although our studies and those of other laboratories [Clark 1969, Rall 1981] show insulin I:II to be present in approximately 60:40% distribution, we cannot exclude the possibility that islets isolated from rats and mice of different strains contain different proportions of the two insulins. Our studies with mice demonstrating a similar insulin I:II distribution (in mice the two non-allelic genes are located on different chromosomes) suggest this is improbable but does not conclusively eliminate this possibility.

Interestingly, disproportionate elevated expression of insulin I vs. II has consistently been reported in rat B-cell tumors [Cordell 1982, Giddings 1986]. In all cases investigated, the elevated rate of insulin I synthesis resulted from both differential translational or pre-translational processes. Although this preferential expression of rat insulin I in tumors was assumed to result from alterations in cellular mechanisms caused by tumor induction, it may be that individual B-cells do not express the two insulins equally. Because of the similarity in their 5'-flanking regions and relative close genomic proximity (100 kb apart) it was supposed that individual B-cells within normal rat islets would synthesize both insulins in similar amounts. As studies with B-cell tumors demonstrate this may be a false assumption. It remains to be determined if similar amounts of insulin I and II are present within individual B-cells of normal islets. In angler fish islets, which synthesize two SRIF's that are products of non-allelic genes, the individual cells reportedly

contain either SRIF I or II exclusively [McDonald]. It is conceivable that different B-cells may exhibit selectivity in hormone expression, so that the relative proportions of insulin I and II observed in islets are a consequence of the number of cells synthesizing each hormone. Our demonstration that the relative distribution of insulin I and II is similar in islets of different primordial origin and cellular composition within the same pancreas of a particular strain of rats indicate that if this determination exists it remains constant for all islets studied from a particular animal. Furthermore, our studies demonstrate that specific retention or secretion of insulin I vs. II does not affect the character of the hormone pool observed in normal islets.

Because our analysis only evaluated the insulin present in the hormone pool, it was necessary to define proinsulin to insulin conversion for each set of conditions.

In a series of experiments by another laboratory [Logothetopolus 1980], it was observed that islets from rats maintained at chronic hyperglycemic states apparently convert proinsulin to insulin faster than islets from normal rats. This implied that the prohormone conversion rate does not remain constant and may be regulated by the environmental milieu *in vivo*.

In these studies we demonstrated that hyperstimulation of islets *in vivo* (tolbutamide treatment) significantly accelerates the rate of proinsulin to insulin conversion. Islets isolated from rats treated with tolbutamide (3 days) apparently processed proinsulin twice as fast as islets from

untreated rats ($t_{1/2}$ = 20 min, 50 min respectfully). Tolbutamide administration did not cause elevated plasma glucose levels, suggesting that chronic hyperglycemia is not a unique inducer of accelerated processing *in vivo*.

The accelerated prohormone conversion phenomena was also induced *in vitro*. Pulse-labelling experiments showed that post-translational incubation conditions had no effect on the proinsulin processing mechanism. However, preincubating islets in 20 mM glucose for 225 min vs. 45 min resulted in a doubling of the proinsulin to insulin conversion rate. The kinetics of this accelerated proinsulin modification were similar to that observed in islets from tolbutamide treated rats, indicating that a 20 min conversion half life is probably the maximal processing rate. Others in this laboratory have demonstrated that this *in vitro* acceleration of proinsulin processing depends on protein synthesis and can be induced by agents other than glucose [Nagamatsu]. Thus, accelerated proinsulin processing is not a glucose specific phenomena, but rather may result from a general activation of the islets metabolic state.

Analysis of proinsulin to insulin processing in these studies measured the end result of two events: 1) transport from the ER to the conversion site, and 2) enzymatic cleavage. Histochemical [Orci 1974] and biochemical [Clark 1969, Kemmler 1971] studies in other laboratories have reported that in freshly isolated islets the transport rate of newly synthesized prohormone is much faster than the apparent rate of proinsulin cleavage (approximately $t_{1/2}$= 10-20 min vs. 50 min

respectively). Based on these observations computerized mathematical calculations were performed which integrated both rates and computed a resultant overall rate of proinsulin processing. As expected, this analysis demonstrated that alterations in the transport rate had relatively little effect on the computed processing rate while changes in the rate of cleavage directly correlated with the observed alteration in processing.

Consistent with the mathematical computations, inhibition of ER to Golgi transport (using Antimycin A to uncouple ATP synthesis) at various times following the pulse demonstrated that, under non accelerated conversion conditions (only 45 min preincubation before pulse), 75% of the prohormone had apparently been transported into the conversion compartment within 20 min of pulse initiation (figure 34). Others in our laboratory have shown that accelerated proinsulin transport could not solely account for the increased processing rate observed [Nagamatsu personal communication]. Note, antimycin A had previously been shown to be a very effective inhibitor of intracellular transport, however, because the onset of action had not been determined the calculated transport time is not a definitive number and may indeed be longer than that assumed. Further investigation has determined that glucose induced accelerated conversion is dependent on protein synthesis [Nagamatsu 1987], exhibits a glucose dose response curve similar to that observed for insulin synthesis and is associated with an apparent increased islet concentration of procathepsin-B (the putative proinsulin converting enzyme [Nagamatsu,

personal communication]. These observations support the hypothesis that the amount of converting enzyme available before granule formation may control the rate of processing activity.

Evaluation of processing of the individual insulins I,II revealed that under non-accelerated and accelerated conversion conditions, the kinetics of insulin I maturation were faster than insulin II (figure 33). These differences in processing kinetics for the two insulin s appear to result from differential rates of transport of the two prohormones to the conversion compartment. Because proinsulin I and II could not be quantitatively separated by the methods employed, the rates for the individual prohormone conversion could not be determined. As previously discussed it is not clear whether the two insulins are exclusively expressed in individual B-cells or both present in all B-cells. Therefore, the observed differences in processing kinetics may represent different rates of transport in cells that selectively synthesize insulin I vs. II (in this case intra-cellular transport would be faster in cells synthesizing insulin I). Alternatively, differences in the amino acid structure of the proinsulins may confer selectivity for their intra-cellular transport. Differences in rates of intra-celluar secretory and membrane protein transport have been reported for several cell systems [Lodish 1983, Fries 1984]. However, the rat B-cell may be the first cell in which differential transport of such similar proteins was shown to occur within the regulated secretory pathway.

In summary, the hormone reservoir in islets exhibits a

dynamic compartmental character which is associated with alterations in the phasic pattern of insulin secretion. Furthermore, environmental conditions which effect the state of the reservoir profoundly alter the islets hormone synthetic and processing characteristics. The augmentation of these parameters and the heterogeneous compartmentalization are not restricted to a subset of pancreatic islets or selective for a specific hormone product (insulin I,II). Therefore the basis for the hormone reservoir compartmentalization and the kinetics of the secretory response result from either intracellular compartmentalization of secretory granules or heterogeneity of B-cell responsiveness within islets. Although we cannot exclude the possible contribution of secretory granule segregation, our investigations, and those of other laboratories, support the hypothesis that the dynamic hormone storage and secretory properties observed in islets are a consequence of complex inter-cellular interactions involving recruitment of heterogeneous B-cell populations within the islet.

METHODS

ISLET PREPARATION

Islets were isolated from ab. lib fed, 280-350 g male Long-Evans rats (Simmonsen Company, Gilroy, CA) following the method of Lacy and Kostianovsky [Lacy 1967]. Islets from different regions of the pancreas (head vs. tail) were obtained by excising, in situ, comparable amount of tissue from division of each pancreatic primordia (ventral and dorsal respectively). Because of the extended time period over which these experiments were conducted two different, yet similar , procedures for pancreatic tissue digestion were utilized. The difference was due to the use of a different collagenase which required altering the concentration and duration of exposure for each of the digestion steps, however, both procedures yielded similar number of islets with comparable activity. Minced pancreatic tissue (1 cm) was digested in 4.0 ml Hank's balanced salt solution, minus magnesium, at 37 C. Collagenase (1; CLS IV, Worthington Biochemical Corp., Freehold, NJ, 2; grade V, Sigma Chemical Co., St. Louis, MO: the following procedure was used with collagenase 2, procedure for collagenase 1 is described in [Figlewicz 1980]) was used in a three step procedure utilizing vortex induced agitation and a high intensity lamp as a heat source - temperature being monitored by a probe immersed in the digestion media. The first period was with 20 mg for 12 min, the second with 10 mg for 5 min, the third 5 mg for 5 min; all calculated on the basis of tissue from one pancreas in 4 ml Hank's buffer. Each digestion was followed by two buffer washes and centrifugation to

sediment the tissue. The final washed digest was centrifuged for 15 min at 1500 g on an ice cold discontinuous density gradient consisting of 9 ml 60% and 9 ml 40% (vol/vol) Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) to Hank's. Islets present at the interface between the 60:40% Percoll layers were collected and manually selected under a dissecting microscope with a drawn glass pipette.

INCUBATION AND LABELING OF ISLETS

In each experiment islets were put into 15 X 45-mm glass vials, which, like all glass- and plasticware, were siliconized with Prosil-28 (PCR Research Chemicals, Inc., Gainesville, FL). Buffer was removed, and 0.5 ml of Kregs-Ringer Bicarbonate (KRB) + 25mM N-2-hydroxyethyl-piperazine-N-2-ethane sulfonic acid, pH 7.3, was added. Incubation were at 37° C in an atmosphere of 95:5% O_2:CO_2. Islets were preincubated and chased in KRB containing different glucose concentrations for various time periods, however for the pulse fresh KRB + 20mM glucose + 400 uCi/ml ^{3}H-leucine (TRK-170, Amersham Corp., Arlington Heights, IL) was introduced; for reference this time was called 0 min. Pulse was terminated by removing the radioactivity buffer and then washing and incubating in KRB + 0.2 mM leucine +/- glucose. All subsequent buffers also contained 0.2 mM leucine. Samples of secreted hormone were collected after incubation buffer was removed and islets washed to eliminate all previously secreted hormones. Fresh KRB at 37 C was next introduced, and the islets were returned to incubation. A sample

of hormones secreted during intervals specified in Results was removed and filtered through a plug of tightly packed siliconized glass wool. Secreted hormone and hormone remaining stored within the islets at the end of the sampling interval were either frozen immediately or extracted and purified separately. Purification was done at 4 C without the addition of carrier insulin as described below.

ANALYSIS

Samples were analyzed using reverse phase-High Performance Liquid Chromatography (HPLC) and/or affinity and bio-gel chromatography. Samples were specifically prepared for each of these analysis procedures.

AFFINITY, BIO-GEL CHROMATOGRAPHY

Chemical extraction: All samples were adjusted to 1.0 ml with fresh KRB and mixed with 4.28 ml of 356-ml ethanol: 64.1 ml water: 7.5 ml concentrated hydrochloric acid [Davoren 1962]. The next morning 50 ul of 2 M ammonium acetate was added, and the pH was adjusted to 8.3 with ammonium hydroxide. After centrifugation, the resulting supernates were decanted, adjusted to pH 5.3 with 1.0 M hydrochloric acid, and 10 ml of ethanol and 20 ml of ether were added sequentially with mixing. The precipitate that formed after 2-4 hr, was pelleted by centrifugation and air dried. The samples were dissolved

and stored frozen in 4.0 ml of affinity column buffer (40 mM sodium phosphate, pH 8.0, + 140 mM sodium chloride + 0.2% bovine serum albumin + 0.025% thimerosal). A 70 % yield after the entire chemical extraction procedure was estimated by addition of a trace of porcine 125I-insulin to a sample of intact islets + 1.0 ml of incubation buffer.

Affinity Chromatography : Antiinsulin-Sepharose was made from guinea pig antiinsulin serum (Miles Yeda, Israel) and Sepharose 4B (Pharmacia Fine Chemicals). A crude globulin fraction was prepared by dissolving lyophilized antiserum in 8 vol of water, with stirring, adding saturated ammonium sulfate to a final concentration of 40% saturated. Precipitated protein was pelleted by centrifugation, redissolved and then reprecipitated with the same procedure. The final precipitate was dissolved with and extensively dialyzed against water. Dialyzed, crude globulin from 3.0 ml of antiserum was covalently coupled to 1.5 cm (packed volume) of freshly prepared cyanogen bromide-activated Sepharose at pH 8.0 by stirring for 15 hr at 4 C; excess reactive sites were blocked by stirring the beads for an additional 4 hr in 1.0 M methylamine. Beads were extensively washed with acidic and basic buffers as described [Berne 1975], and then packed into columns that bound in excess of 15 ug porcine insulin/1.0 cm (packed volume).

Thawed experimental samples were applied to 1.0-cm (packed volume) columns that were equilibrated with column buffer. 50 ml of column buffer was used to wash out unbound proteins, and elution of insulin and proinsulin was started with 50 ml of

1.0 N acetic acid. Insulin and proinsulin eluted with the first 10 ml of acetic acid and were precipitated from this sample with 10% (final) trichloroacetic acid. The precipitate was collected by centrifugation, washed three times with ether, then dissolved and frozen in 10 mM hydrochloric acid + 0.05% bovine serum albumin (Pentex Albumin fraction V, Miles Laboratories Inc., Elkhart, IN). A yield of 85% was estimated with a trace of porcine I-insulin applied to antiinsulin-Sepharose columns.

Bio-gel Chromatography: Insulin and proinsulin were separated from each other on 1 X 110-cm columns of Bio-gel P-30 (Bio-Rad Laboratories, Richmond, CA) that were equilibrated and eluted with 3.0 N acetic acid + 0.05% bovine serum albumin at room temperature. Insulin [Lundquist 1976] from the insulin peak was measured by radioimmunoassay with rat insulin (Novo Research Laboratories, Bagsvaerd, Denmark) as a standard, and radioactivity in all peaks was measured in Aquasol (New England Nuclear, Boston, MA) with a Packard Model 2425 Tri-Carb Liquid Scintilation Counter (Packard Instrument Co., Downers Grove, IL).

Figure 38 is an example of an elution profile of a column of Biogel P-30 loaded with a sample of islets that were incubated for 95 min at 37 C, then extracted and the hormones purified as described above. There were only two peaks of radioactivity: the first was coincident with a small immunoreactive peak that marked the elution position of proinsulin; the second was coincident with the large peak of immunoreac-

tive insulin. Insulin eluted with nearly constant specific activity (counts per minute/nanograms immunoreactive insulin) throughout its peak, indicating relative homogeneity. 100% of a trace porcine 125I-insulin, but only 40% of total immunoreactive insulin, was recovered in the insulin peak after day 2 of elution in 3.0 N acetic acid. For this reason the calculation of specific activity of insulin was done as follows: counts per minute eluted in the insulin region per nanogram immunoreactive insulin in sample before chromatography. Measurements of specific activity, therefore, included a small error (<10%) due to proinsulin and processing intermediates in the original sample. Fractional secretion rates were calculated with 100% equal to the sum of the hormone secreted during the interval plus the hormone remaining stored in the islets at the end of the interval.

When required the insulin and glucagon [Gerich 1974] (Unger antiserum 04A) contents of islets were measured by radioimmunoassy of buffered dilutions of extracts that were obtained by mixing the islets for 2 hr at 4 C in acid-ethanol + 50 mM benzamidine [Ensink 1972]. All numbers are reported as the mean + SE. Differences were assessed by Student's unpaired, two-tailed t-test.

Calculations: Fractional rates of secretion were calculated by assigning the value of 100% to the sum of all islet ans secreted hormone. Measurements of radioactivity were corrected for the loss of C-peptide during the purification procedures by multiplying radioactivity eluting in the insulin peak by 11/6. Half-lives for the conversion of proinsulin to

insulin were calculated by the least-squares method (logarithm of percent intact proinsulin versus time) for all points in the linear portion of the conversion curve.

Computer modeling of insulin biosynthesis, compartmental storage, fractional secretion and proinsulin processing were done on an IBM PC incorporating a flow pattern for labeled and unlabeled proinsulin and insulin (illustrated in figure 14 [Landhal 1982]. This flow pattern features two cellular insulin storage compartments: one compartment (labile) represents a preferentially recruitable hormone pool (high fractional secretory rate). The second compartment (stable) contains the remainder of the total stored islet insulin. The same rates for transport and conversion from proinsulin were used for labeled and unlabeled insulin. In fitting this pattern, rate of biosynthesis of labeled proinsulin, transport rate to secretory granules, fractional secretion of both labeled and unlabeled insulins, rate of proinsulin to insulin conversion and islet content of labeled and unlabeled insulin were fixed by experimentally determined values taken from the results presented in this discourse.

HPLC-Analysis

Samples : Following incubation secreted sample media and islets (100 ul KRB) were transferred to 1.5 ml microcentrifuge tubes (Eppindorf) and immediately frozen in a solid CO_2 /ethanol bath. Samples were lyophilized, resuspended in 100-250 ul of 1M acetic acid and maintained on ice till media samples were redissolved. All samples were then frozen,

thawed, vortexed, sonicated in ice bath, vortexed and refrozen for analysis.

Separation : The two rat insulins, processing intermediates and proinsulin were separated by reverse phase-HPLC ( ) using gradient elution with acetonitrile and water, both containing 0.1% morpholine and 0.1% trifluro acetic acid vol/vol, using a Dupont pep ER-8 column ( ).

Optimal separation of rat insulin I and II (7 min peak to peak ; figure 39) was achieved using acetonitrile gradient 32-34% over 35 min. In samples where separation of both insulins and proinsulin were required the following four segment gradient was used: percentages correspond to the relative % acetonitrile in solution, 32-35% 30 min, 35-39% 7 min, 39-44% 30 min and a flow rate of 1.5 ml/min. In the latter protocol, insulin I and II eluted in approximately the same positions depicted in figure 39. Under these conditions the two proinsulins were inseparable and eluted as one peak between 50 - 60 min. Difficulty in segregating rat proinsulins has consistently been reported (Halban 1987). Note that ambient temperature in the vicinity of the column appeared to have a profound effect on column retention time (i.e. retention time increased and peak definition declined with increasing temperature). Therefore, HPLC separation was performed only when temperature could be maintained below 24° C. To eliminate residual protein retention and "ghost peaking" elution after each sample, columns were subjected to two 25-75% acetonitrile gradient washes of 10 min each.

One min elution fraction were collected directly into scintilation vials or into tubes containing an equal volume of twice concentrated phosphate immunoassay buffer (maintained in ice bath). Samples eluted into assay buffer were immediately vortexed upon collection and volumes split for scintilation counting and radioimmunoassay. To each fraction for scitilation counting 5 ml of Hydrofluor was added and counted using a Packard scintilation counter.

Identification: Identity of insulin I and II elution fractions were confirmed using immunopurified ^{3}H-leucine and ^{35}S-methionene labeled insulin. Only insulin II contains methionene, which eluted in the peak contained in minutes 20 - 25 (figure 39). Purity of the fractions in each peak were determined using SDS polyacrilamide gel electrophoresis flourography [Skinner 1983]. The only radioactive substance present in the fractions exhibited a molecular weight identical to that of immunopurified insulin.

Recovery: Immunoreactive insulin content of samples was determined prior to HPLC. Samples were then subjected to gradient elution on HPLC and minute elution fractions, throughout the gradient run, were collected into tubes containing twice concentrated phosphate immunoassay buffer and immediately vortexed so as to insure mixing of solutions. These samples were then split and analyzed for immunoreactive insulin content and radioactivity. Comparison of the cumulative IRI following HPLC separation with that prior to this procedure, indicated that 76% of total IRI was recovered.

IRI and radioactivity recovery of individual insulins I

and II were also determined. 1 ml HPLC elution fractions, corresponding to insulin I and II peaks, were collected separately into albumin coated tubes containing 50 ul of 5% bovine serum albumin in water (weight/vol). The collected fractions were then lyophilyzed (4 hrs) and redissolved in 250 ul of 1 M acetic acid. Aliquots of 25 ul each were taken for IRI and scintilation counting for each insulin I and II sample. 200 ul of the remaining samples were subjected to reverse phase HPLC and then eluted minute fractions analyzed for IRI and radioactivity as described above. Comparison of IRI and radioactivity measurements of individual insulins prior to and following HPLC revealed greater then 95% recovery of both radioactive insulins and an average 85% recovery for both insulin I and II.

Data represented in figure 31b were compiled from experiments in which eluted fractions were not vortexed immediately upon collection; rather the two solutions, which remain separate, were mixed only at the completion of the HPLC run. The disparity in the individual insulin I and II IRI suggest that following this procedure the amount of murine insulin I was preferentially reduced. In all subsequent analysis eluted fractions were vortexed immediately following collection. Adoption of this procedure resulted in the IRI contents of insulin I in rat or mouse samples to always be greater than that of insulin II.

REFERENCES

Aizawa T, Hinkle PM (1985) Endocrinology 116:909

Allan D, Billah MM, Fineau JB, Michell RH (1976) Nature 261:58

Ashcroft SJH, Bunce J, Lowry M, Hansen SE, Hedskov CJ (1978) Biochem J 174:517

Ashcroft SJH (1980) Diabetologia 18:5

Ashcroft SJH, Hammonds P, Harrison DE (1986) Diabetologia 29:727

Atwater I, Beigelman PM (1976) J Physiol Paris 72:769

Baetens D, Malaisse-Lague F, Perrelet A, Orci L (1979) Science 206:1323

Bendayan M (1984) Histochem J 16:85

Bergmann JE, Singer SJ (1983) J Cell Biol 97:1777

Berne C (1975) Endocrinology 97:1241

Best L, Dunlop M, Malaisse WJ (1984) Experientia 40:1085

Blobel G, Dobberstein B (1975) J Cell Biol 67:835

Blobel G (1980) Proc Natl Acad Sci USA 77:1496

Bolafi JL, Heldt A, Grodsky GM (1986a) 68 th Annual Meeting of The Endocrine Society, Anaheim CA (abstract)

Bolafi JL, Heldt AH, Grodsky GM (1986b) Diabetes 35:370

Bonner-Weir S, Orci L (1982) Diabetes 31:883

Bonner-Weir S (1984) Diabetes 33:81A

Brisson GR, Malaisse-Lagae F, Malaisse WJ (1972) J Clin Invest 51:232

Brown WJ, Farquhar ME (1984a) CEll 36:295

Brown WJ, Lonstatinescu E, Farquhar ME (1984b) J Cell Biol 99:320

Burgess T, Kelly RB (1984) J Cell Biol 99:2223

Burgess T, Craik C, Kelly RB (1985) J Cell Biol 101:639

Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y

(1982) J Biol Chem 257:7847

Cate RL, Chick W, Gilbert W (1983) J Biol Chem 258:6645

Cerasi E (1975) Acta Endocr 79:483

Chafouleas JG, Dedman JR, Munjaal RP, Means AR (1979) J Biol Chem 254:10262

Chan SJ, Keim P, Steiner DF, (1976) Proc Natl Acad Sci USA 73:1964

Charles MA, Lamecki I, Pictet R, Grodsky GM (1975) J Biol Chem 250:6134

Chick, WL, Warren S, Chute RN, Like AA, Luaris V, Kitchen KC (1977) Proc Natl Acad Sci USA 74:628

Clark JL, Steiner DF (1969) Proc Natl Acad Sci USA 62:278

Cohn DV, Elting J (1983) Recent Prog Horm Res 39:181

Conn PM, Ganong BR, Ebeling J, Staley D, Neidel JE, Bell RM (1985) Biochem Biophys Res Commun 126:532

Cordell B, Bell G, Tischer E, DeNoto FM, Ullrich A, Pictet R, Rutter WJ, Goodman HM (1979) Cell 18:533

Cordell B (1982) Cell 31:531

Creutzfeldt in The Pancreas. Fitzgerald PJ, Morrison AB eds., Williams and Wilkens, Baltimore :208

Davoren PR (1962) Biochim Biophys Acta 63:150

Delears M, Catagna M, Malaisse WJ (1981) Cancer Lett 14:109

Docherty K, Carroll R, Steiner DF (1982a) Proc Natl Acad Sci USA 79:4613

Docherty K, Steiner DF (1982b) Ann Rev Physiol 44:625

Docherty K, Hutton JC (1983) FEBS Lett 162:137

Dunphy WG, Rothman JE (1985a) Cell 42:13

Dunphy WG, Brands R, Rothman JE (1985b) Cell 40:463

Edlund T, Walker MD, Barr PJ, Rutter WJ (1985) Science 230:912

Eipper BA, Mains RE (1980) Endocinol Rev 1:1

Engelman DM, Steitz TA (1981) Cell 23:411

Ensinck JW, Shepard C, Dudl RJ, Williams RH (1972) J Clin Endocrinol Metab 35:463

Farquhar MG, Palade GE (1981) J Cell Biol 91:77s

Farquhar MG (1985) Ann Rev Cell Biol 1:447

Felsenfed G, McGhee J (1982) Nature (Lond) 296:602

Fries E, Rothman JE (1980) Proc Natl Acad Sci USA 77:3870

Fries E, Gustofsson L, Peterson PA (1984) EMBO J 3:147

Gabel CA, Goldberg DE, Kornfeld S (1983) Proxc Natl Acad Sci USA 80:775

Gabel CA, Kornfeld S (1984) J Cell Biol 99:296

Gerich JE, Charles MA, Grodsky GM (1974) J Clin Invest 54:833

Geuze HJ, Slot JW, Strous GJAM Hasilik A, von Figura K (1984a) J Cell Biol 98:2047

Geuze HJ (1984b) Cell 37:195

Giddings SJ, Chirgwin J, Permutt MA (1981) J Clin Invest 67:952

Giddings SJ, Chirgwin J, Permutt MA (1982) Diabetes 31:624

Giddings S, Swyers J, Carnaghi L (1986) Diabetes 35 suppl 1:44A

Gold G, Karoly K, Freeman C, Adelman A (1976) Biochem Biophys Res Commun 73:1003

Gold G, Reaven GM, Reaven EP (1981) Diabetes 30:77

Gold G, Pou J, Nowlain RE, Grodsky GM (1984) Diabetes 33:1019

Goldberg DE, Kornfeld S (1983) J Biol Chem 258:3159

Gonzales-Noriega A, Grubb JH, Talkad V, Sly WS (1980) J Cell Biol 85:839

Goodman RH, Aron DC, Roos BA (1983) J Biol Chem 258:5570

Green J, Griffiths G, Louvard P, Quinn G, Warren G (1981) J Mol Biol 152:663

Green R, Shields D (1984) J Cell Biol 99:97

Grill V, Adamson U, Cerasi E (1978) J Clin Invest 61:1034

Grill V (1981) Am J Physiol 240:E24

Griffiths G, Quinn P, Warren G (1983) J Cell Biol 96:835

Griffiths G, Pfeifer S, Simons K, Matlink K (1985) J Cell Biol 101:949

Grodsky GM, Batts AA, Bennett LL Veclla C, McWilliams NB, Smith DF (1963) Am J Physiol 205:638

Grodsky GM, Bennett LL (1966) Diabetes 15:910

Grodsky GM, Curry D, Landahl H, Bennett LL (1969) Acta Diabet Lat 6:554

Gumbiner B, Kelly RB (1982) Cell 28:51

Halban PA, Wollheim CB (1980a) J Biol Chem 255:6003

Halban PA, Wollheim CB, Blondel B, Renold AE (1980b) Biochem Pharmacol 29:2625

Halban PA (1982) J Biol Chem 257:13177

Halban PA, Mutkoski R, Dodson G, Orci L (1987) Diabetologia 30:348

Hammends P, Schofield PN, Ashcroft SJH (1987) FEBS Lett 213:149

Hawthorne JN (1982) Nature 295:281

Hellerman (1984) Proc Natl Acad Sci USA 81:5340

Hobart P, Crawford R, Shen LP, Pictet R, Rutter WJ (1980) Nature (Lond) 288:137

Hoenig M, MacGregor LC, Matschinsky FM (1986) Endocrinol Metab 13:E502

Hoff JD, Lasley BL, Wang CF, Yen SSC (1977) J Clin Endocrinol

Metab 44:302

Howell SL, Parry DG, Taylor KW (1965) Nature (Lond) 208:487

Howell SL, Kastanovski M, Lacy PE (1969) J Cell Biol 42:695

Hubbard SC, Ivatt RJ (1981) Ann Rev Biochem 50:555

Hubinont C, Best L, Sener A, Malaisse WJ (1984) FEBS Lett 170:247

Jamieson JD, Palade GE (1968) J Cell Biol 39:580

Itoh N, Okamoto H (1980) Nature (Lond) 283:100

Johnson LM, Bankaitis VA, Emr SD (1987) Cell 48:875

Kaelin D, Renold AE, Sharp GWG (1979) Diabetologia 14:329

Kakita K, Giddings S, Permutt MA (1982a) Proc Natl Acad Sci USA 79:2803

Kakita K, O'Connell K, Permutt MA (1982b) Diabetes 31:841

Kakita K, O'Connell K, Permutt MA (1985) in Methods in Diabetes Research Vol. 1, Laboratory Methods, part C (Lorner J and Pohl SL eds.) :419

Kaiser CA, Preuss D, Grisafi P, Batstein D (1987) Science 235:312

Kelly RB (1985) Science 230:25

Kemmler W, Peterson JD, Rubenstein AH, Steiner DF (1971) Diabetes 21:572

Kishimoto A, Takai Y, Mori T, Kikkawa U, Nishizuka Y (1980) J Biol Chem 255:2273

Kornfeld R, Kornfeld S (1985) Ann Rev Biochem

Kraft AS, Anderson WB (1983) Nature (Lond) 301:621

Kuo JF, Anderson RGG, Wise BC, Mackerlova L, Salomonson I, Brackett NL, Katoh N, Shoji M, Wrenn RW (1980) Proc Natl Acad Sci USA 77:7039

Laimins L, Holmgren-Konig M, Khoury G (1986) Proc Natl Acad Sci USA 83:3151

Lalley PA, Chirgwin JM (1984) Cytogenet Cell Genet 37:515

Landahl HD, Grodsky GM (1982) Bull Math Biol 44:399

Laub O, Rutter WJ (1983) J Biol Chem 258:6043

Lacy PE, Kostianovsky M (1967) Diabetes 16:35

Lee JC, Grodsky GM, Bennett LL, Smith-kyle DF, Craw L (1970) Diabetologia 6:542

Ledford BE, Davis DF (1983) J Biol Chem 258:3304

Levy J, Malaisse WJ (1975) Biochem Pharmacol 24:235

Lin BJ, Haist RE (1973) Endocrinology 92:735

Lodish HF, Kong N, Snider M, Strous GJAM (1983) Nature (Lond) 304:80

Lodish HF, Kong N, Hirami S, Rasmussen J (1987) J Cell Biol 104:221

Logothetopoulos J, Jain K (1980) Diabetes 29:801

Loh YP (1986) Annals New York Acad Sci 495:292

Lomedico P, Rosenthal N, Efstratiadis A, Gilbert N Kolodner R, Tizard R (1979) Cell 18:545

Lomedico PT (1982) Proc Natl Acad Sci USA 79:5798

Lundquist I, Fanska R, Grodsky GM (1976) Endocrinology 99:1304

MacGregor RR, Hamilton JW, Cohn DV (1975) Endocrinology 97:178

Machamer J (1985) Mol Cell Biol 25:135

Mackin RB, Noe BD (1987a) J Biol Chem 262:6453

Mackin RB, Noe DB (1987b) Endocrinology 120:457

Malaisse-Lagae F, Amherdt M, Ravazzola M, Sener A, Hutton JC, Orci L, Malaisse WJ (1979) J Clin Invest 63:1284

Malaisse WJ, Lebrun P, Herchuelz A, Sener A, Malaisse-Lagae F

(1983) Endocrinology 113:1870

Maldonato A, Renold AE, Sharp GWG, Cerasi E (1977) Diabetes 26:538

Markussen J (1971) Int J Peptide Protein Res 3:149

McDonald JK, Greiner F, Bauer GG, Elde RP, Noe BD (1987) J Histochem Cytochem 35:155

McGhee JD, Felsenfeld G (1980) Ann Rev Biochem 49:1115

McLean WG, Keen P (1973) Exp Cell Res 80:345

Meda P, Denef JF, Perrelet A, Orci L (1980) Am J Physiol 238:C114

Meda P, Perrelet A, Orci L (1984) in Modern Cell Biology, Satir BH ed., Alan Liss New York 3:131

Meglasson MD, Burch PT, Berner DK, Najafi H, Vogin AP, Matchinsky FM (1983) Proc Natl Acad Sci USA 80:85

Michell RH (1982) Cell Calcium 3:285

Montminy MR, Goodman RH, Horovitch SJ, Habner JF (1984) Proc Natl Acad Sci USA 81:3337

Moore HP, Walker MD, Lee F, Kelly RB (1983a) Cell 35:531

Moore HP, Gumbiner B, Kelly RB (1983b) J Cell Biol 97:810

Moor HP, Gumbiner B, Kelly RB (1983c) Nature (Lond) 302:434

Moore HP, Kelly RB (1986) J Cell Biol

Morel A, Gluschankof P, Gomez S, Fafeur V, Cohen P (1984) Proc Natl Acad Sci USA 81:7003

Morris GE, Korner A (1970) Biochim Biophys Acta 208:404

Morrissey JJ, Cohn DV (1979) J Cell Biol 82:93

Munro S, Pelham HRB (1987) Cell 48:899

Nagamatsu S, Bolafi JL, Grodsky GM (1987) Endocrinology 120:1225

Niedel JE, Kuhn LJ, Vandenbark GR (1983) Proc Natl Acad Sci USA 80:36

Nielsen DA, Welsh M, Casadaban MJ, Steiner DF (1985) J Biol Chem 260:13585

Nishizuka Y (1984) Science 225:1365

Noe BD (1981) J Bio Chem 256:9397

Noe BD, Spiess J (1983) J Bio Chem 258:1121

Noe BD, Debo G, Spiess J (1984) J Cell Biol 99:578

Noe BD, Andrews PC, Dixon JE, Spiess J (1986) J Cell Biol 103:1205

Novick P, Schekman R (1979) Proc Natl Acad Sci USA 76:1858

Novick P, Field C, Schekman R (1981) Cell 21:205

O'Connor MDL, Landahl HD, Grodsky GM (1980) Am J Physiol 238:R378

Ohlssom H, Edlund T (1986) Cell 45:35

Orci L, Montesano R, Meda P, Malaisee-Lagae F, Brown D, Perrelet A, Vassalli JD (1981) Proc Natl Acad Sci USA 78:293

Orci L (1974) Diabetologia 10:163

Orci L (1982) Diabetes 31:538

Orci L, Halban P, Amherdt M, Ravazzola M, Vassalli JD, Perrelet A (1984a) Cell 39:39

Orci L, Ravazzola M, Louvard D, Perrelet A (1984b) C.R. Acad Sci 299:697

Orci L, Ravazzola M, Amherdt M, Yanaihara C, Ynaihara N, Halban P, Renold AE, Perrelet A (1984c) J Cell Biol 98:222

Orci L, Halban P, Amherdt M, Ravazzola M, Vassalli, JD, Perrelet A (1984d) J Cell Biol 99:2187

Orci L (1984e) Proc Natl Acad Sci USA 81:6743

Orci L, Ravazzola M. Amherdt M, Louvard D, Perrelet A (1985) Proc Natl Acad Sci USA 82:5385

Orci L, Glick BS, Rothman JE (1986a) Cell 46:171

Orci L, Ravazzola M, Amherdt M, Madsen O, Perrelet A, Vassalli JD, Anderson RG (1986b) J Cell Biol 103:2273

Osborne R, Tashjian AH (1981) Endocrinology 108:1164

Owada M, Neufeld EF (1982) Biochem Biophys Res Commun 105:814

Pace CS, Goldsmith KT (1985) Am J Physiol 248:C527

Palmitter R, Gagnon DJ, Ericsson LH, Walsh KA (1977) J Biol Chem 252:6386

Patzelt C, Labrecque AD, Duguid JR, Carroll RJ, Kein P, Heinrikson RL, Steiner DF (1978) Proc Natl Acad Sci USA 75:1260

Pearse BMF, Bretscher MS (1981) Ann Rev Biochem 50:85

Permutt MA, Kipnis DM (1972a) Proc Natl Acad Sci USA 69:505

Permutt MA, Kipnis DM (1972b) J Biol Chem 247:1194

Permutt MA (1974) J Biol Chem 249:2738

Pipeleers DG, in't Veld PA, Van DE Winkel M, Maes E, Schuit FC, Gepts W (1985) Endocrinology 117:806

Pipeleers D (1987) Diabetologia 30:277

Patzelt C, Labrecque AD, Daguid JR, Carroll RJ, Keim PS, Heinriksom RL, Steiner DF (1978) Proc Natl Acad Sci USA 75:1260

Praz GA, Halban PA, Wollheim CB, Blondel B, Stauss AJ, Renold AE (1983) Biochem J 210:345

Prentki M, Wollheim CB (1984) Experientia 40:1052

Pruss RM, Moskal JR, Eiden LE, Beinfeld MC (1985) Endocrinology 117:1020

Rall LB, Pictet RL, Rutter WJ (1979) Endocrinology 105:835

Rebois RV, Patel Jitendra (1985) J Biol Chem 260:8026

Reed R, Maniatis T (1985) Cell 41:95

Reitman ML, Kornfeld S (1981) J Biol Chem 256:4275

Reuchlin S (1983) N Engl J Med 309:1495

Rhodes CJ, Lucas CA, Halban PA (1987a) FEBS Lett 215:179

Rhodes CJ, Halban PA (1987b) J Biol Chem 262:10712

Rhor G, Keim V (1984) Dig Dis Sci 29:965

Rose JK, Bergmann P (1982) Cell 30:753

Roth J, Berger EG (1982) J Cell Biol 93:223

Rothman JE, Fine RE (1980) Proc Natl Acad Sci USA 77:780

Rothman JE (1981) Science 213:1212

Sabatini DD, Kreibich G, Morimoto T, Adesnik M (1982) J Cell Biol 92:1

Sachs H, Faucett P, Takabataki Y, Prtanova R (1969) Recent Prog Hormone Res 25:447

Salomon D, Meda P (1986) Exp Cell Res 162:507

Sando H, Borg J Steiner DF (1972) J Clin Invest 51:1476

Sando H, Grodsky GM (1973) Diabetes 22:354

Sano K, Takai Y, Yamanishi J, Nishizuka Y (1983) J Biol Chem 258:2010

Schatz H, Laube H, Sieradzki J, Kamenisch W, Pfeifler EF (1978) Horm Metab Res 26:9

Scheele G, Tartakoff A (1985) J Biol Chem 260:926

Schuit FC, in't Veld PA, Gepts W, Pipeleers DG (1987) J Cell Sci in press

Schwaiger H, Hasilik A, von Figura K, Wiemken A, Tanner W (1984) Biochem Biophys Res Commun 104:950

Selden RF, Skoskiewicz MJ, Russell PS, Goodman HM (1987) N Engl J Med 317:1067

Shen LP, Rutter WJ (1984) Science 224:168

Shields D, Blobel G (1977) Proc Natl Acad Sci USA 74:2059

Slaby F, Bryan J (1976) J Biol Chem 251:5078

Slot JW, Geuze HJ (1983) J Histochem Cytochem 31:1049

Sly WS, Fischer HD (1981) J Cell Biochem 18:2047

Smith LF (1966) Am J Med 40:662

Soares MB, Schon E, Henderson A, Karathanasis SK, Cate R, Zeitlin S, Chirgwin J, Efstatiadis A (1985) Mol Cell Biol 5:2090

Sodoyez JC, Sodoyez-Goffaux F, Dunbar JC, Foa PP, (1970) Diabetes 19:603

Stachura ME, Tyler JM, Farmer PK (1985) Endocrinology 116:698

Steiner DF, Kemmler W, Tager HS, Peterson JD (1974) Fed Proc 33:2105

Steiner DF (1983) Harvey Lecture Series 78:191

Steiner DF, Docherty K, Carroll R (1984) J Cell Biochem 24:121

Steiner DF, Chan SJ, Kwok SCM, Welsh JM (1985) Ann Rev Genetics 19:463

Steiner DF, Chan SJ, Welsh JM, Nielsen D, Michael J, Tager HS, Rubenstein AH (1986) Clin Invest Med 9:328

Steinman RM, Mellman IS,Muller WA, Cohn ZA (1983) J Cell Biol 96:1

Strous GJAM, Lodish HF (1980) Cell 22:709

Sugden MC, Christie MR, Aschroft SJH (1979) FEBS Lett 105:95

Swearigen KC (1971) Endocrinology 85:1037

Tabas I, Kornfeld S (1978) J Biol Chem 253:7779

Tabas I, Kornfeld S (1980)

Takai Y, Kaibuchi K, Sano K, Nishizuka Y (1982) J Biochem 91:403

Tanese T, Lazarus NR, Devrin S, Recant L (1970) J Clin Invest 49:1394

Tanigawa K, Kuzuya H, Imura H, Taniguchi H, Baba S, Takai Y, Nishizuka Y (1982) FEBS Lett 138:183

Tavianini MA, Hayes TE, Magazin MD, Minth CD, Dixon JE (1984) J Biol Chem 259:11798

Tomlinson S, Walker SW, Brown BL (1982) Diabetologia 22:1

Trimble ER, Renold AE (1981) Am J Physiol 240:E422

Trimble ER, Halban PA, Wollheim CB, Renold AE (1982) J Clin Invest 69:405

Trump G, Hildemann WH, Tebow GB (1984) Analytical Biochem 138:298

Ullrich SJ, Hawkes SP (1983) Exp Cell Res 148:377

Valverde I, Vandermeers A, Anjanylu R, Malaisse WJ (1979) Science 206:225

Valverde I, Malaisse WJ (1984) Experimentia 40:1061

Villa-Komaroff L, Estratiadis A, Broome S, Lomedico P, Tizard R, Naber SP, Chick WL, Gilbert N (1978) Proc Natl Acad Sci USA 75:3727

Virji MA, Steffes MW, Estensen RD (1978) Endocrinology 102:706

von Heijne G (1984) J Mol Biol 184:99

Waheed AR, Pohlmann R, Hasilik A, von Figura K (1981a) J Biol Chem 256:4150

Waheed AR, Hasilik A, von Figura K (1981b) J Biol Chem 256:5717

Waheed A (1982) Biochem Biophys Res Commun 105:1052

Walker MD, Elund T, Boulet AM, Rutter WJ (1983) Nature (Lond) 306:557

Walker MD, Edlund T, Boulet AM, Rutter WJ (1984) Mol Biol of

Dev 3:481

Walter P, Jackson RC, Marcus MM, Lingappa VR, Blobel G (1979) Proc Natl Acad Sci USA 76:1795

Walter P, Blobel G (1981) J Cell Biol 91:557

Walter P, Gilmore R, Blobel G (1984) Cell 38:5

Welsh M, Nielsen DA, Mackrell AJ, Steiner DF (1985) J Biol Chem 260:13590

Welsh M, Scherberg N, Gilmore R, Steiner DF (1986) Biochem J 235:459

Welsh M, Hammer RE, Brinster RL, Steiner DF (1986) J Biol Chem 261:12915

Welsh M, Brunstedt J, Hellerstrom C (1986) Diabetes 35:228

Wentworth BM, Schaefer IM, Villa-Komaroff L, Chirgwin JM (1986) J Mol Evol 23:305

Whitman M, Kaplan D, Cantley L, Roberts TM, Schaffhausen B (1986) Fed Proc 45:2647

Wickner WT, Lodish HF (1985) Science 230:400

Williams DB, Swiedler SJ, Hart GW (1985) J Cell Biology 101:725

Witters LA, Vater CA, Lienhard GE (1985) Nature (Lond) 315:777

Wolff DJ, Brostrom CO (1979) Adv Cyclic Nucl Res 11:27

Wollheim GB, Sharp GWG (1981) Physiol Res 61:914

Wu C, Gilbert W (1981) Proc Natl Acad Sci USA 78:1577

Zawalich W, Brown C, Rasmussen H (1983) Biochem Biophys Res Commun 117:448

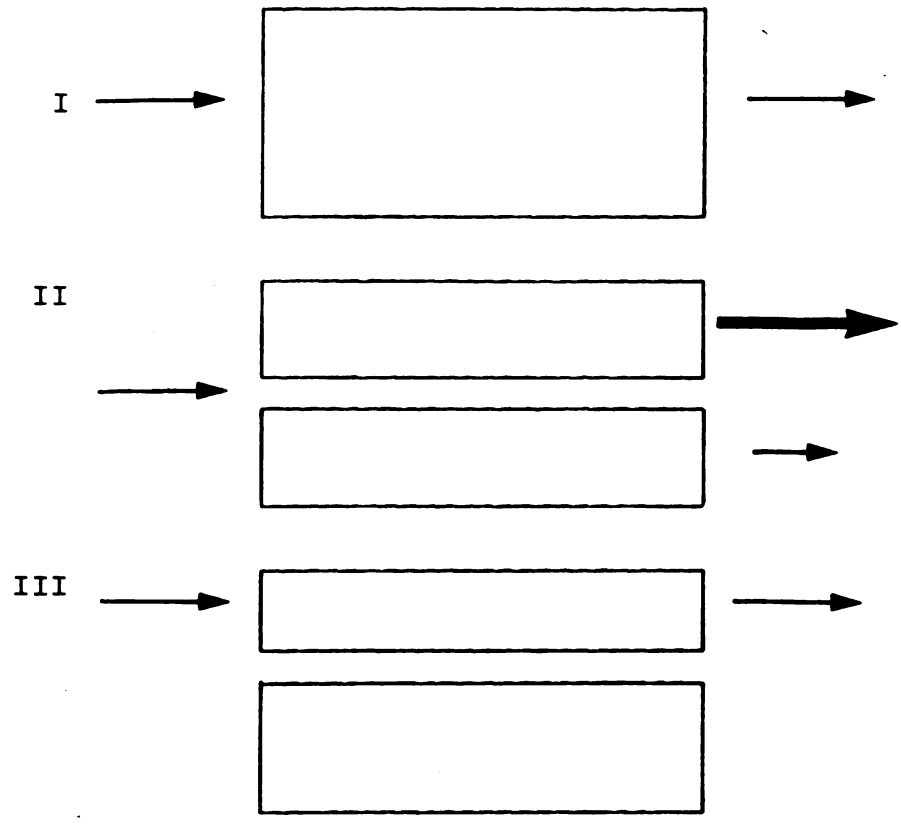


Figure 1: These schematic illustrations depict 3 conceptual models for the organization of the hormone reservoir to be discussed; (I) a homogeneous pool, (II) heterogeneous compartments (labile and stable) that differ in their response characteristics (synthetic and secretory) and (III) an extension of model II, a compartmental arrangement one of which contains hormone but remains dormant, thus synthesis and release involve only one compartment (active vs. inert).

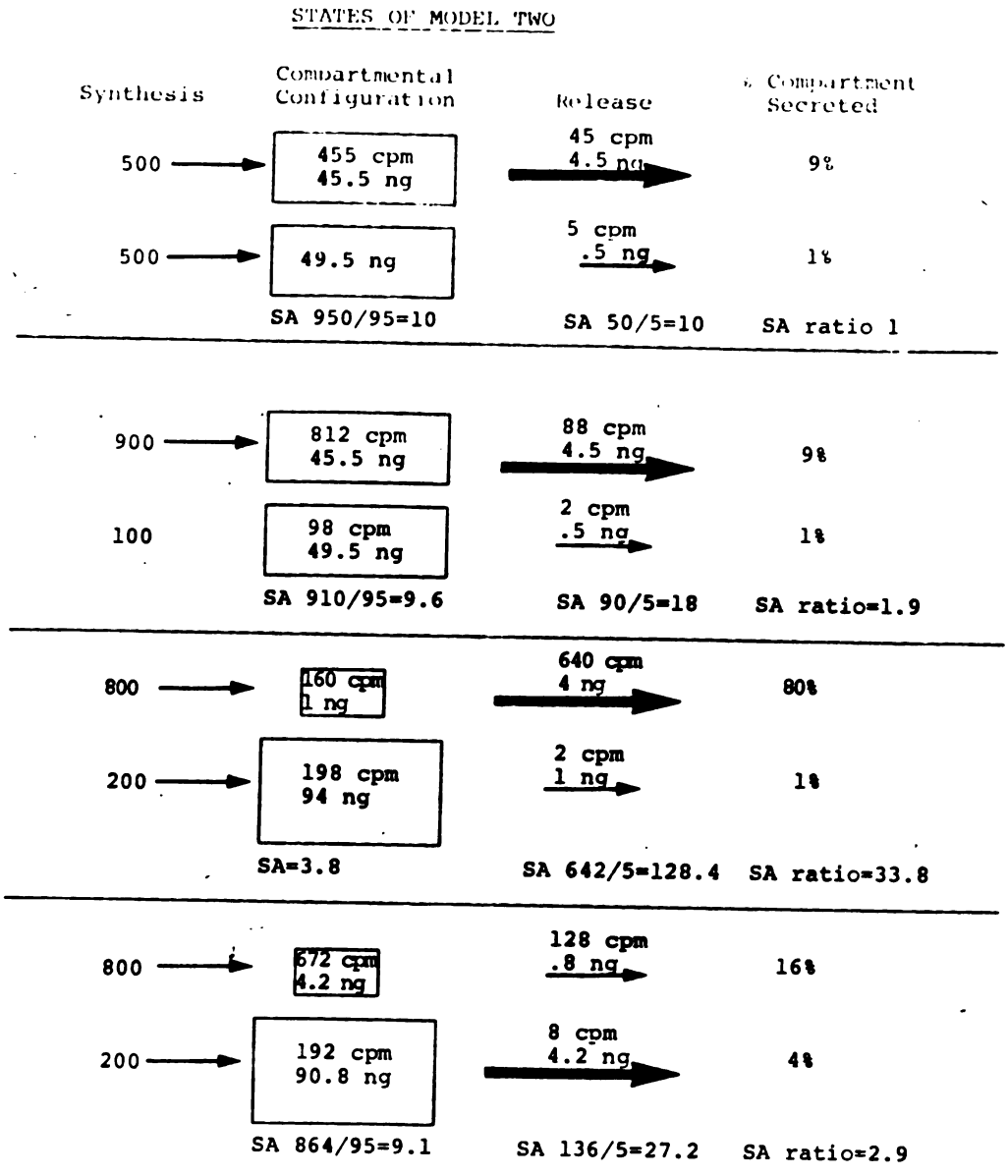


Figure 2: The effect of alterations in the individual compartment size and distribution of newly synthesized hormone on the storage and secretory state of the hormone reservoir, based on model II in figure 1. In each state the following were held constant: initial amount of total radioactive hormone and the total amount of immunoreactive hormone released. The specific activity (SA) of the released hormone and that remaining in the compartments represents the radioactive hormone divided by the immunoreactive hormone (cpm/ng). The SA ratio represents a relative measure of the hormone specific activity of secreted vs. that retained in the reservoir. SA ratio >1 indicates a greater fractional amount of radioactive vs. immunoreactive hormone was released. These states are not meant to correlate with specific experimental results to be discussed later, nor do they represent absolutes; rather they illustrate the effect of alterations in the parameters determining compartmental configuration.

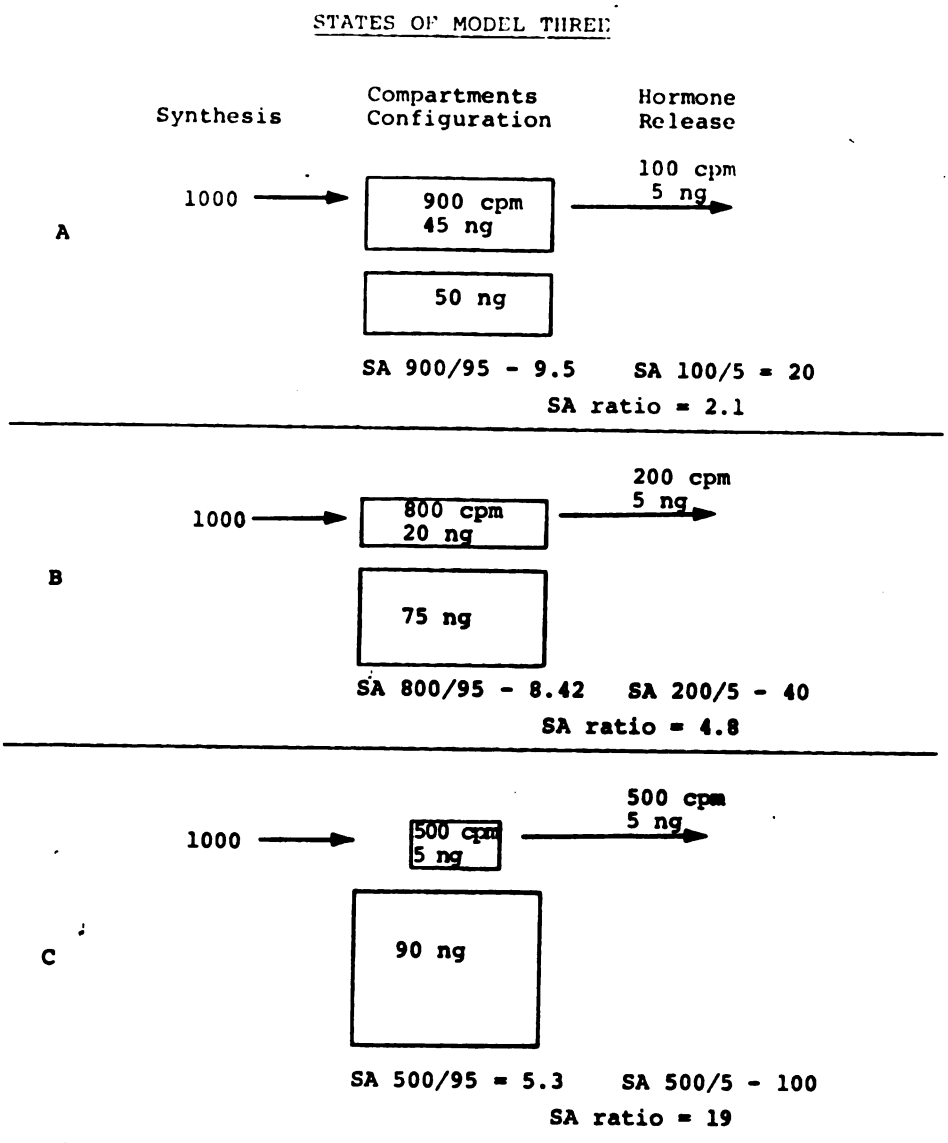


Figure 3: The effect of changes in size of compartments on the hormone reservoirs secretory and storage character based on model III in figure 1. As the size of the active compartment decreases radioactive hormone is secreted at a greater fractional rate; evidenced by higher specific activity of the secreted insulin and increased SA ratio. The same calculations were performed as described in figure 2.

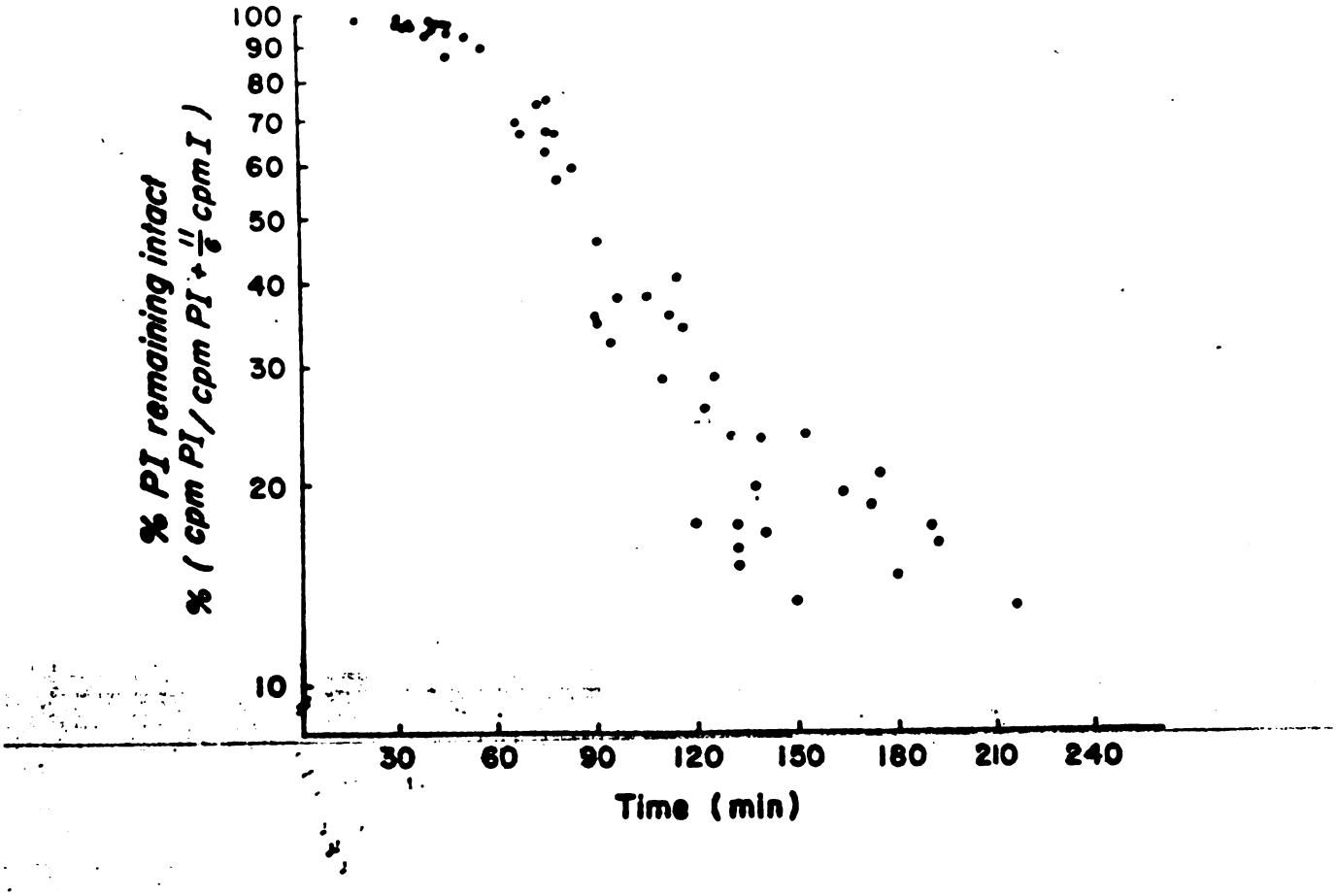


Figure 4: Effect of time on the conversion of newly synthesized proinsulin to insulin. Islets were incubated continuously in KRB + 20 mM glucose beginning at -45 min and exposed to 3H-leucine between 0 - 15 min. Samples were obtained by extracting the islets and the accumulated incubation buffer together. The radioactivity eluting from columns of Biogel P-30 in the insulin peak was multiplied by 11/6 to correct for the difference in leucine content between insulin and proinsulin.

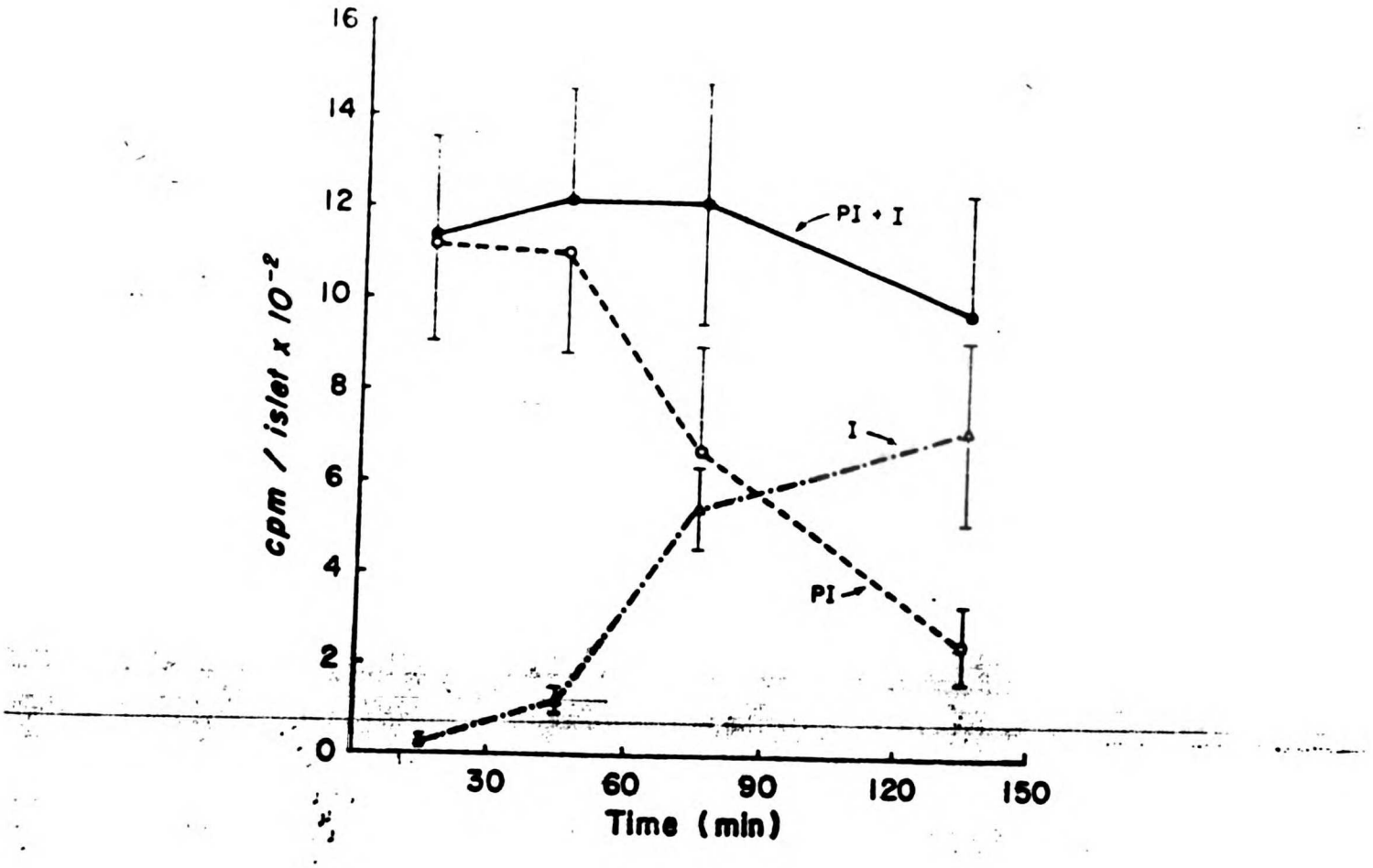


Figure 5: Effect of time on the recovery of proinsulin and insulin in the islets plus incubation buffer. Samples were obtained and calculation performed as described in figure 4. Points represent the mean ± SE from six experiments.

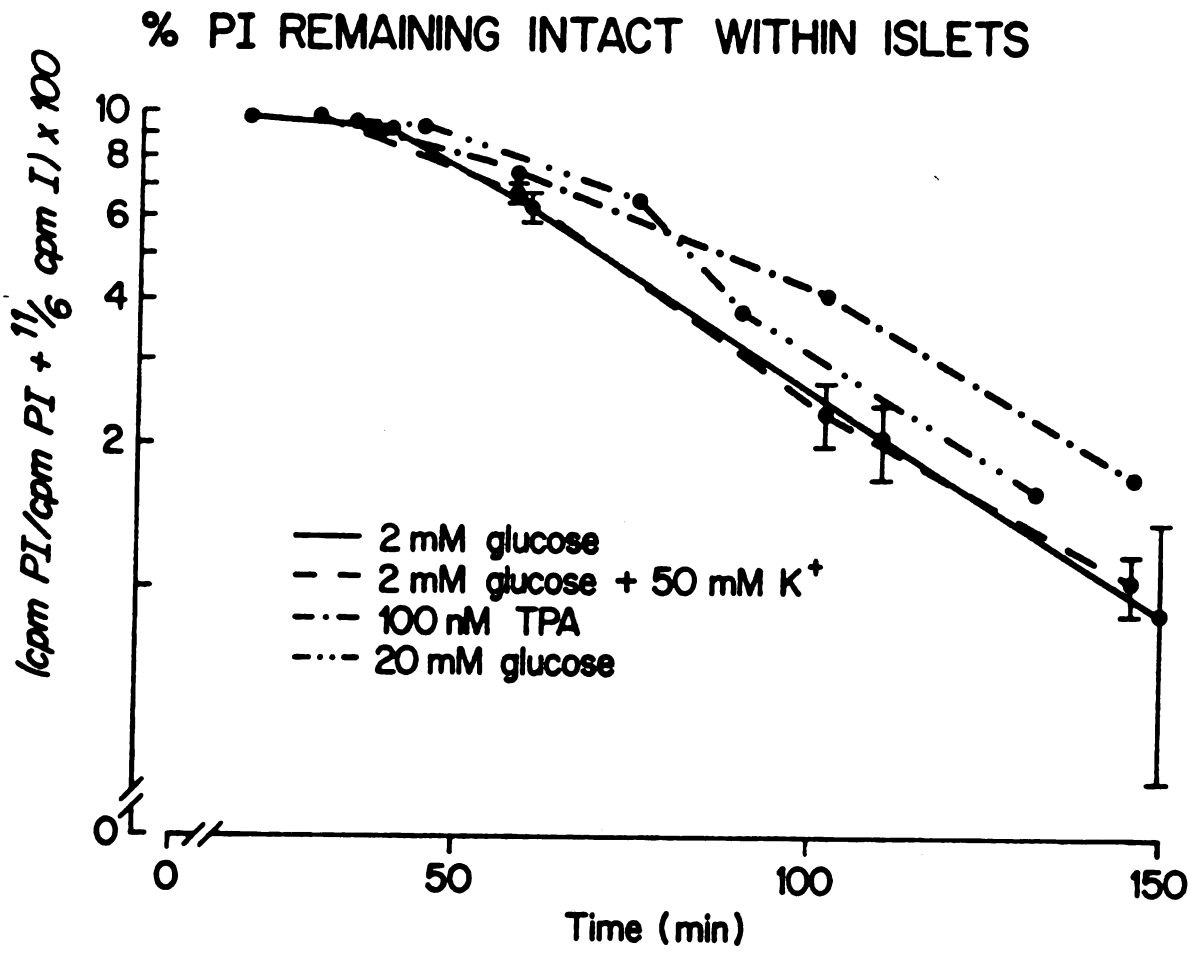


Figure 6: Effect of various post pulse culture conditions on proinsulin to insulin processing kinetics. Islets were incubated in KRB + 20 mM glucose beginning at -45 min including the pulse exposure to ^{3}H-leucine between 0 - 15 min. Immediately following the pulse islets were extensively washed and cultured for up to min 150 in KRB containing these agents: 2 mM glucose, 2 mM glucose + 50 uM K+, 0 mM glucose + 100 nM TPA or 20 mM glucose. Samples were obtained by extracting the islets and media collections separately. The radioactivity eluting from columns of Biogel P-30 in the insulin peak was multiplied by 11/6 to correct for the difference in leucine content between insulin and proinsulin. Graph depicts percent of proinsulin remaining intact within islets; following the 40 min time point the percent proinsulin remaining intact in the secreted media paralleled that observed in islet samples (data not shown). Points represent 3 to 9 experiments.

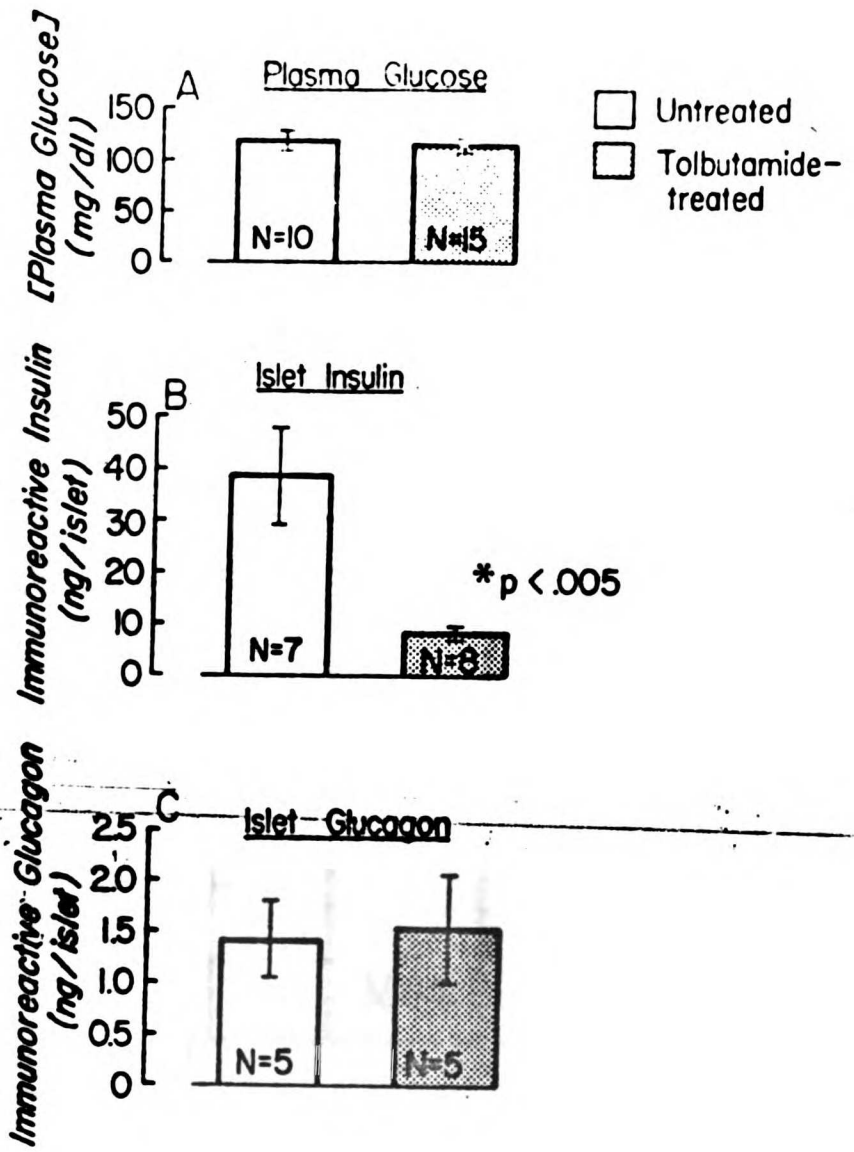


Figure 7: (A) Effect of tolbutamide pretreatment on plasma glucose levels. Tolbutamide-treated rats were administered tolbutamide orally (500 mg/kg body weight) twice daily for three consecutive days. Plasma glucose concentrations were measured on tail blood, which was drawn at 9 a.m. from anesthetized animals (approximately 16 hrs after the last tolbutamide administration). (B) Effect of tolbutamide pretreatment on the insulin content of isolated islets. Isolated islets were extracted at 4 C with acid-ethanol containing bezamidine. Insulin was measured by radioimmunoassay. Statistical differences were assessed with Student's two tailed, unpaired t-test. (C) Effect of tolbutamide pretreatment on the glucagon content of isolated islets. Isolated islets were extracted as above in the presence of benzamidine. Glucagon was measured by radioimmunoassay.

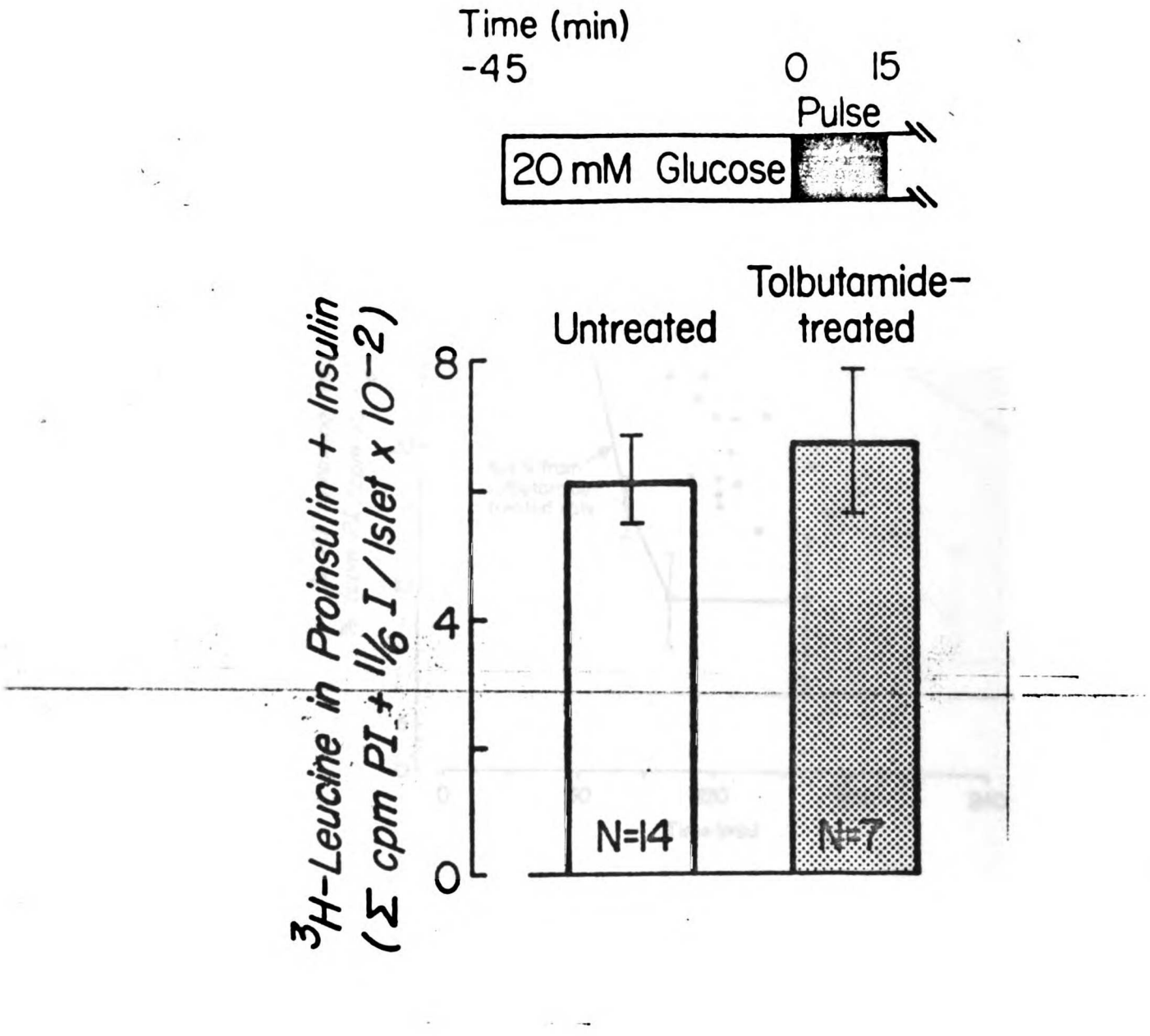


Figure 8: Effect of tolbutamide pretreatment on the incorporation of 3H-leucine into proinsulin + insulin in isolated islets. Values represent islets collected at later times (typically 131 min) plus all labeled proinsulin and insulin secreted from the time of the pulse to the time of islet collection. The radioactivity eluted from columns of Biogel P-30 in the insulin peak was multiplied by 11/6 to correct for the difference in leucine content between insulin and proinsulin.

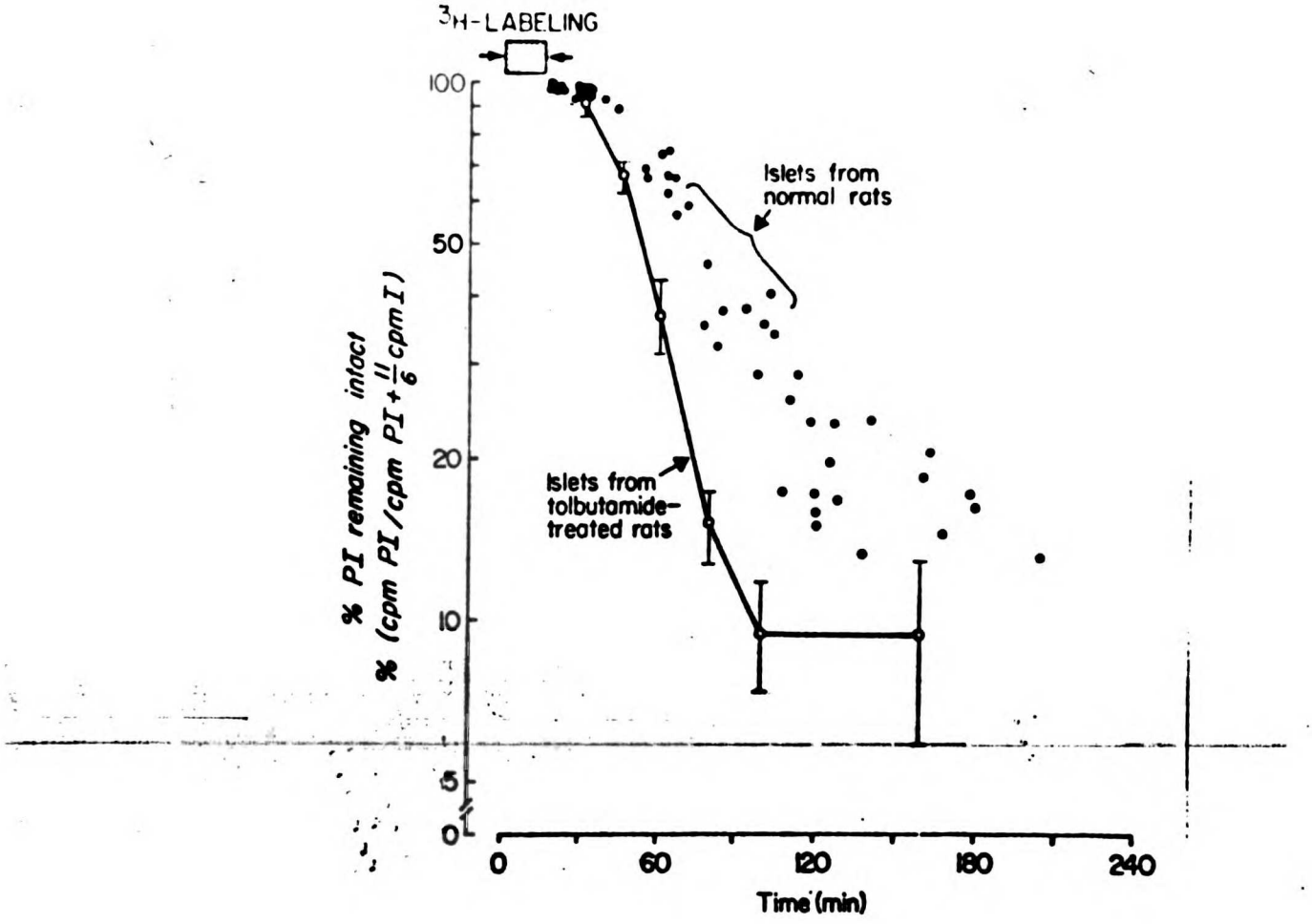


Figure 9: Effect of time on the conversion of labeled proinsulin to insulin in islets isolated from control and tolbutamide-treated rats. Radioactivity eluting from columns of Biogel P-30 in the insulin peak was multiplied by 11/6 to correct for the different leucine content between rat insulin and proinsulin. Closed circles represent experiments with islets from control rats (as illustrated in figure 4) and open circles represent the mean ± SEM of three experiments done on separate days with freshly isolated islets from tolbutamide treated rats. Linear regression lines were calculated for each set of measurements using all values between 10% and 80% intact proinsulin: slopes of the two lines were significantly different ($P<0.001$).

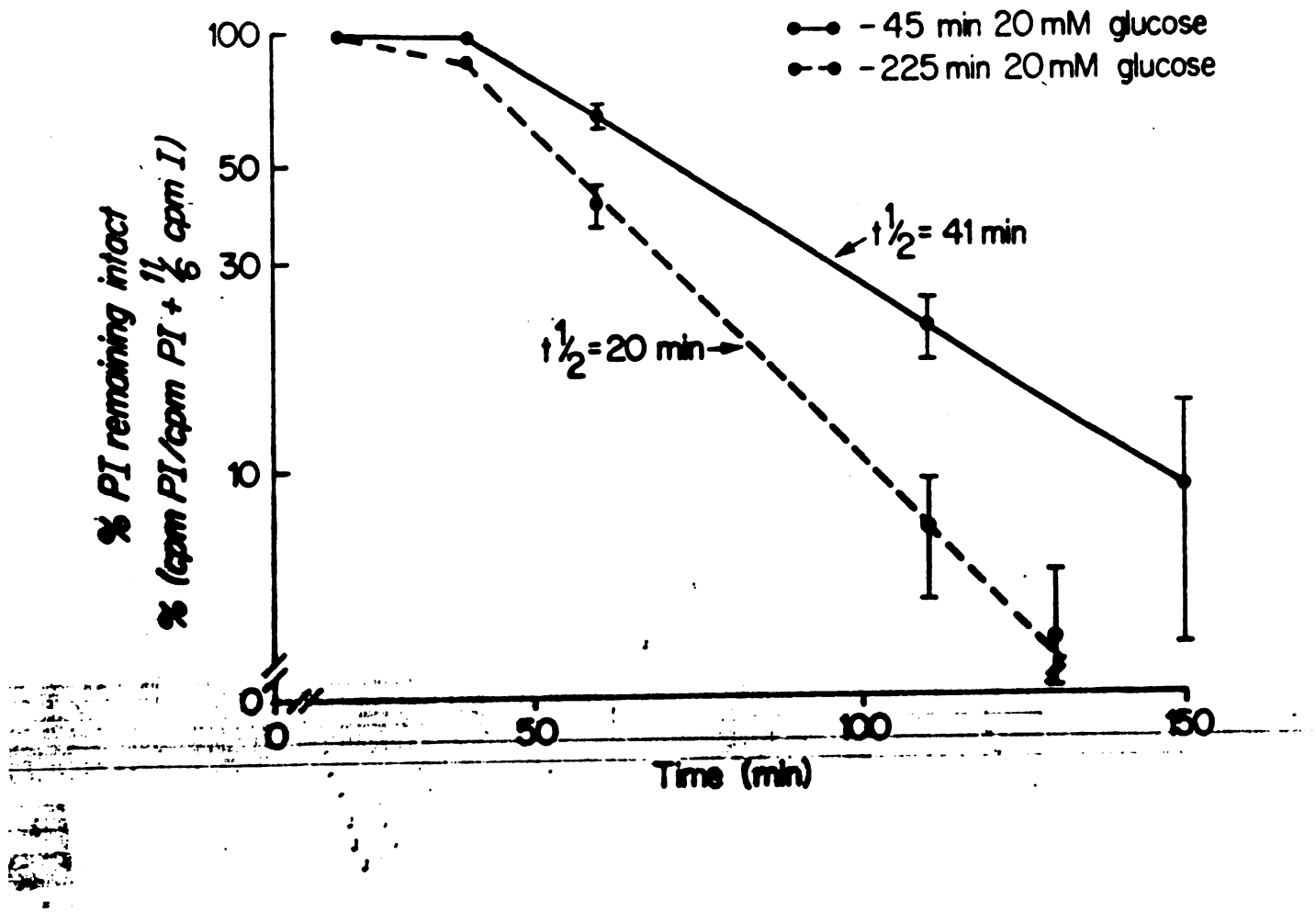


Figure 10 : Effect of prolonged exposure to 20 mM glucose on proinsulin to insulin processing. Islets were preincubated for either 45 min or 225 min in KRB containing 20 mM glucose, then pulsed with ^{3}H-leucine between 0 - 15 min, also in the presence of 20 mM glucose. After the pulse, islets were washed with 2 mM glucose KRB and maintained in this buffer until the termination of the experiment. Combined islet and accumulated incubation buffer samples were frozen immediately; then proinsulin and insulin I,II were separated and analyzed using reverse-phase HPLC. Radioactivity eluting in the insulin I,II peaks was multiplied by 11/6 to correct for the different leucine content between rat insulins and proinsulins. Total radioactivity (proinsulins + 11/6 insulins) remained constant; processing intermediates were not included in these calculations. Linear regression lines were calculated for each set of measurements. Data points represent mean ± SE of 3 - 5 experiments.

MATHEMATICAL MODEL PROJECTIONS

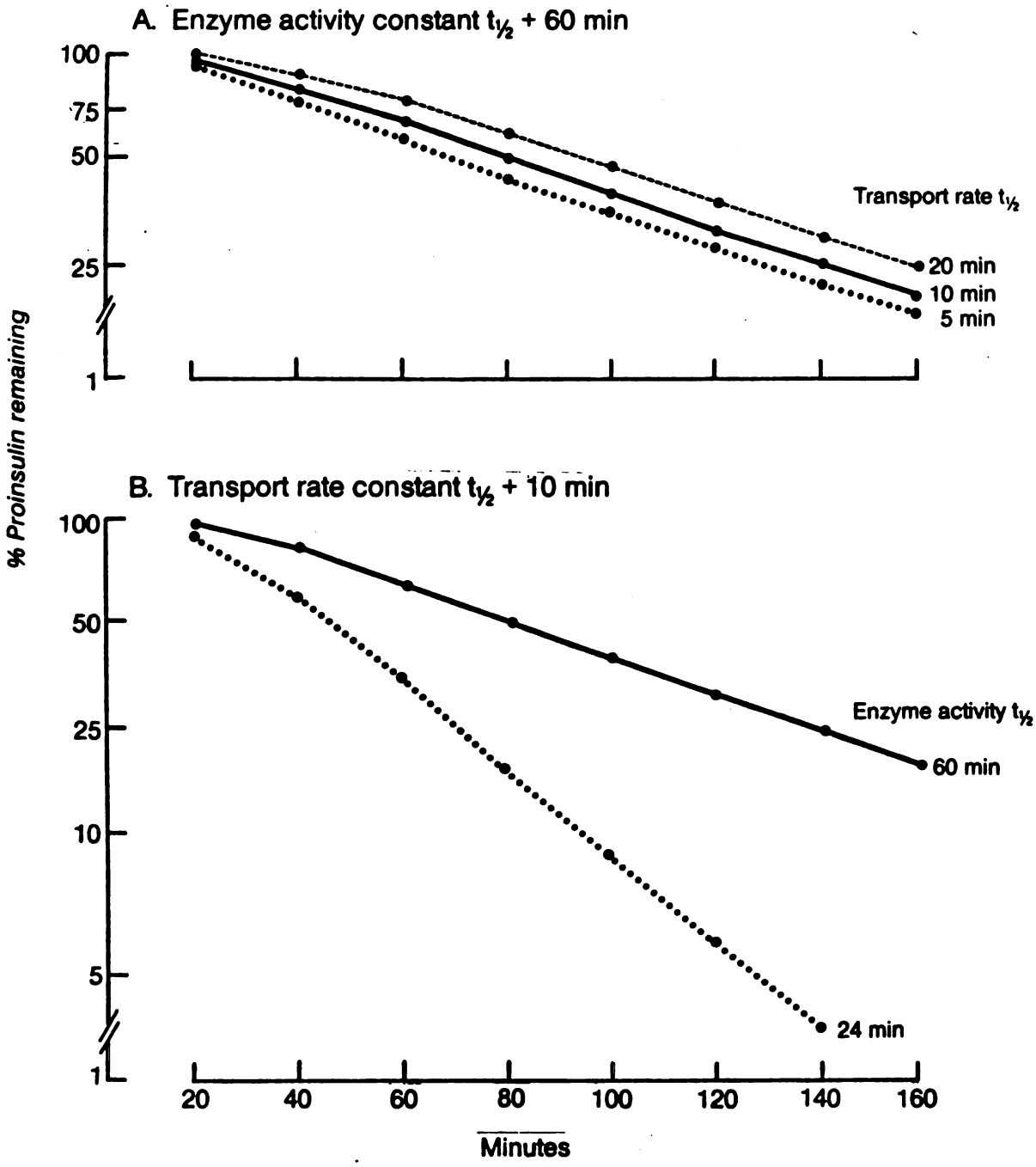


Figure 11 : Mathematical model for proinsulin to insulin processing was derived which allowed for individual variations in 1) transport activity and 2) rate of enzyme cleavage. Computer analysis provided calculated minute integrations for each set of parameters. (A) Effect of alterations in transport rate on overall proinsulin to insulin processing; enzyme activity remained constant, $t_{1/2}$ = 60 min. (B) Effect of alterations in enzyme cleavage activity on overall proinsulin to insulin processing; transport rate to the conversion compartment remained constant, $t_{1/2}$ = 10 min.

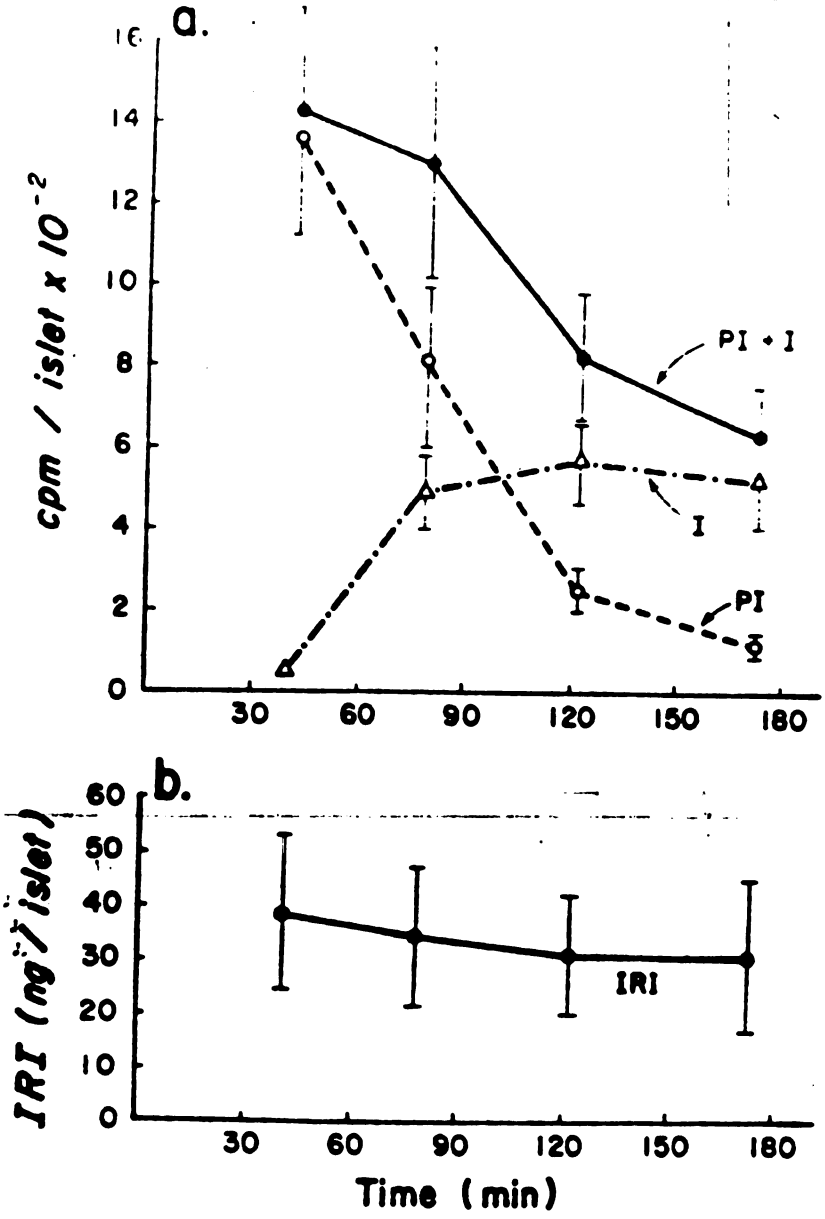


Figure 12 : Effect of time on the recovery of proinsulin and insulin in islets. Samples were obtained from islets, which were incubated continuously in KRB + 20 mM glucose beginning at -45 min and exposed to ^{3}H-leucine between 0 - 15 min. In fig a, the radioactivity eluting from columns of Biogel P-30 in the insulin peak was multiplied by 11/6 to correct for the difference in leucine content between insulin and proinsulin. Points represent the mean $\pm$ SE from eight experiments. In fig b, the actual amount of immunoreactive insulin recovered from islets after chemical extraction and affinity chromatography was plotted. Points represent the mean $\pm$ SE from eight experiments.

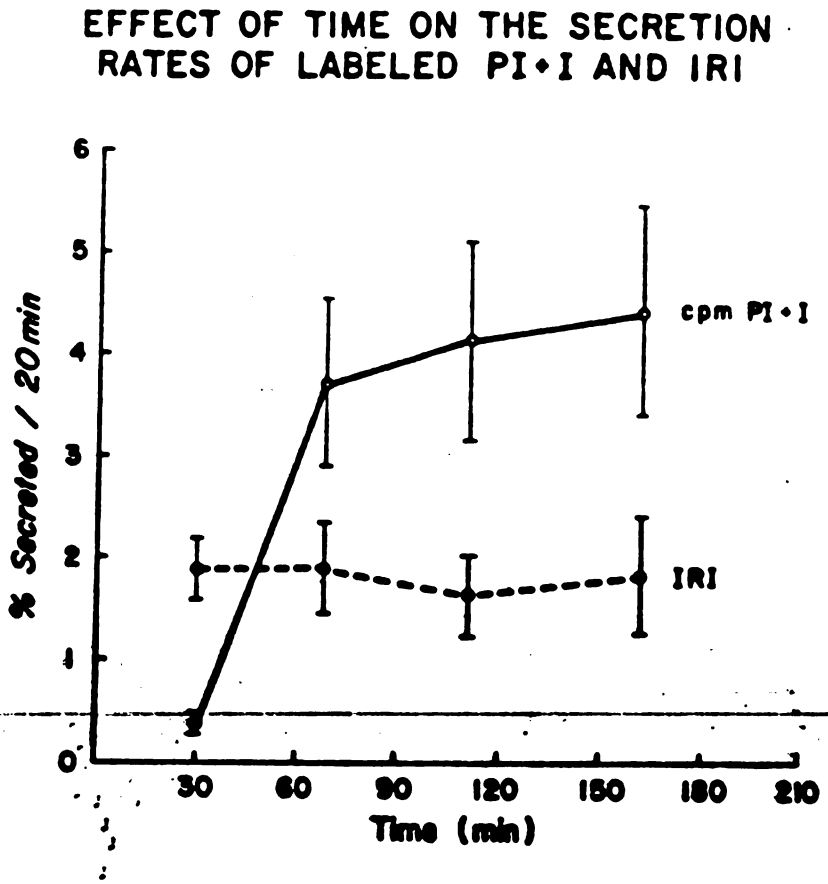


Figure 13 : Effect of time on both the fractional secretory rate of radioactive proinsulin plus insulin and the fractional secretory rate of immunoreactive insulin. Samples were obtained from islets which were incubated continuously in KRB + 20 mM glucose beginning at -45 min and exposed to ^{3}H-leucine between 0-15 min. Radioactivity eluting from columns of Biogel P-30 in the insuiln peak was multiplied by 11/6 to correct for the difference in leucine content between insulin and proinsulin. Points were drawn at the center of the 20 min interval, and represent the mean + SE from eight experiments.

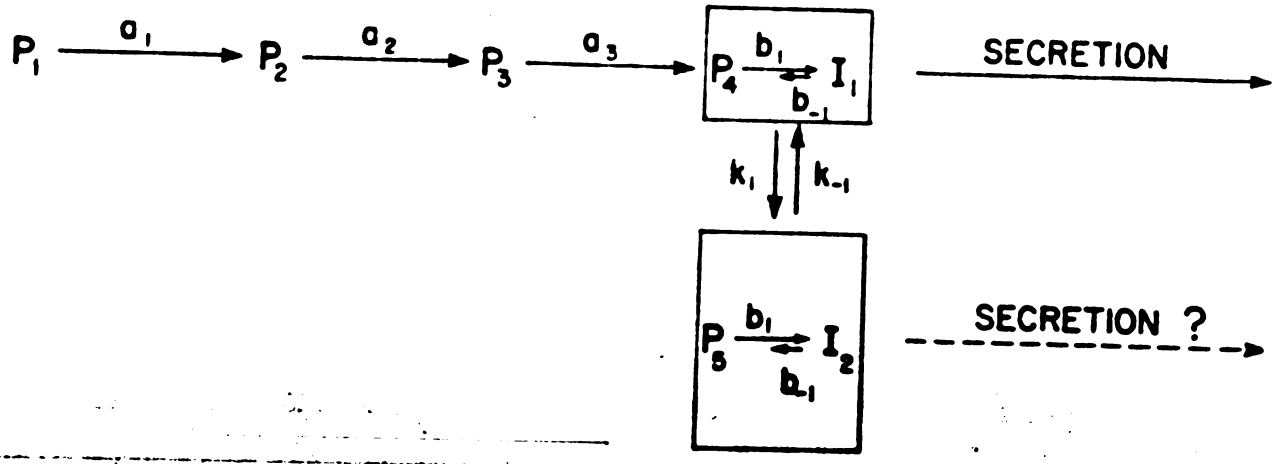


Figure 14 : Schematic diagram of a storage-limited representation of B-cell function depicting proinsulin and other insulin precursors; I represents insulin, P represents insulin precursors; lower case letters are rate constants.

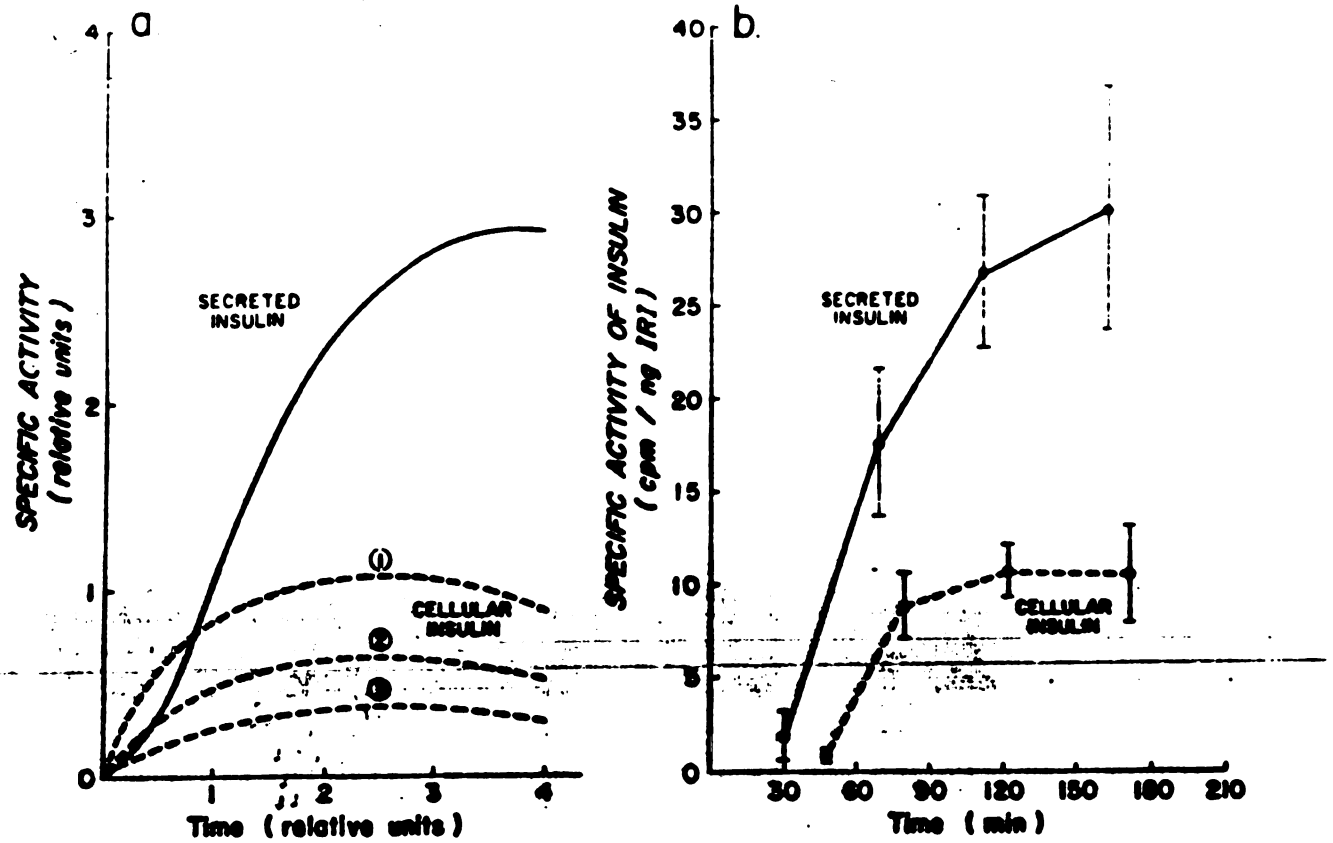


Figure 15: Effect of time on the predicted and experimentally determined specific activity of secreted and cellular insulin. In fig (a) steady state equations were used to predict the relationships between the specific activity of secreted and cellular insulin if the percentage of total islet insulin contained in the glucose-labile compartment equals 33% in No.1, 20% in No.2 or 11% in No.3. In fig (b) experimental data were obtained from islets continuously incubated in KRB + 20 mM glucose as previously described. Points representing secreted hormone are drawn at the center of the 20 min interval and all points represent the mean ± SE from eight experiments.

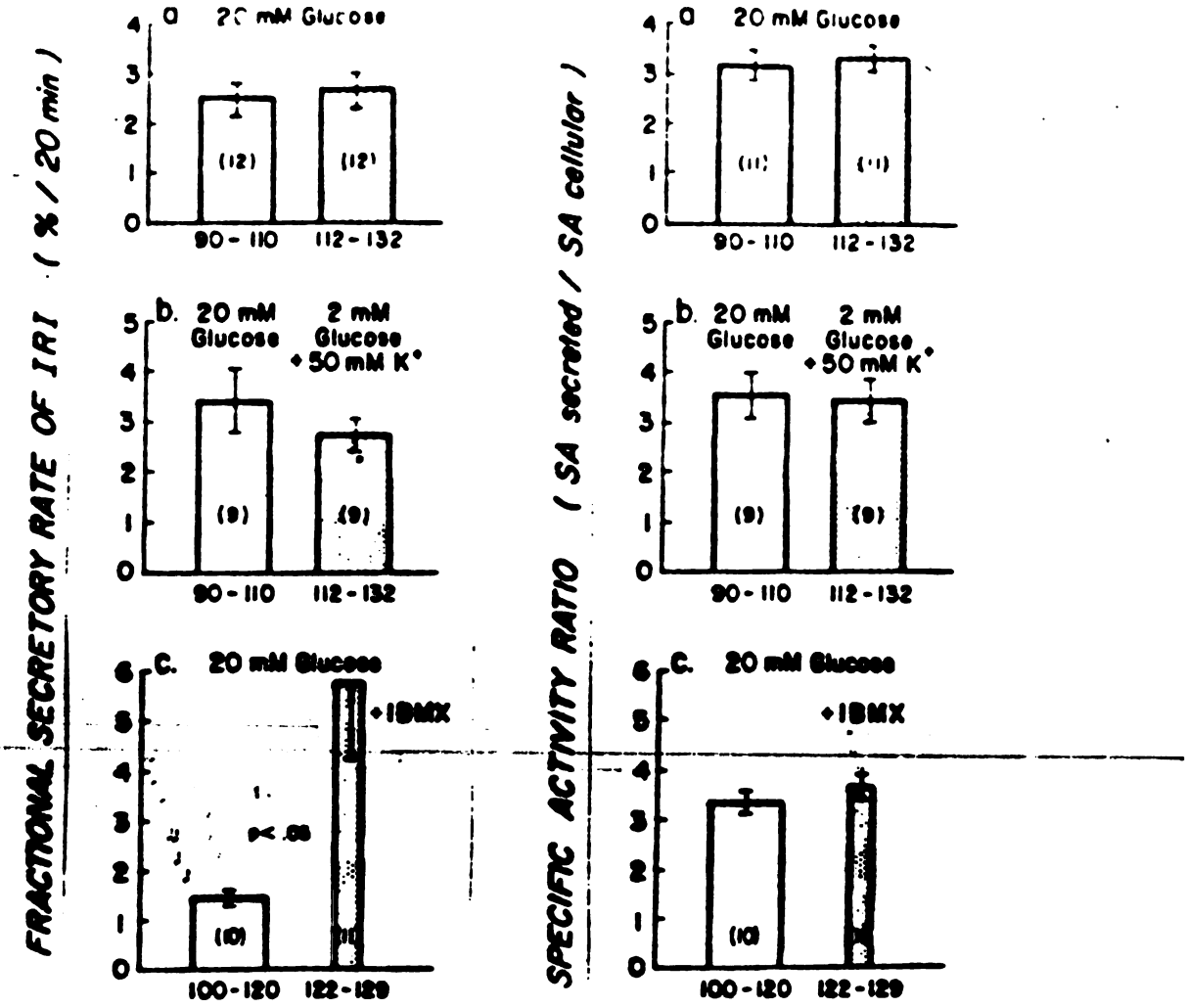


Figure 16 : Effect of different stimuli on the rate of insulin secretion and specific activity ratio between secreted and cellular insulin. Islets were continuously incubated in KRB + 20 mM glucose beginning at -45 min and exposed to ^{3}H-leucine between 0 - 15 min. The radioactivity eluting from columns of Biogel P-30 in the insulin peak was used for the calculation of the specific activity of insulin. In fig a, islets were washed twice with KRB + 20 mM glucose between 87 and 90 min and once between 110 and 112 min. Bars represent the mean ± SE from 11 or 12 experiments. In fig b, islets were washed twice with KRB + 20 mM glucose between 87 and 90 min and once with KRB + 2 mM glucose between 110 and 112 min. Bars represent mean ± SE from nine experiments. In fig c, islets were washed twice with KRB + 20 mM glucose between 97 and 100 min and once between 120 and 122 min. A 7 min, rather than a 20 min sample of secreted hormone was collected in KRB + 1 mM IBMX in order to analyze equivalent amounts of secreted insulin in both intervals. Bars represent the mean ± SE from 10 or 11 experiments.

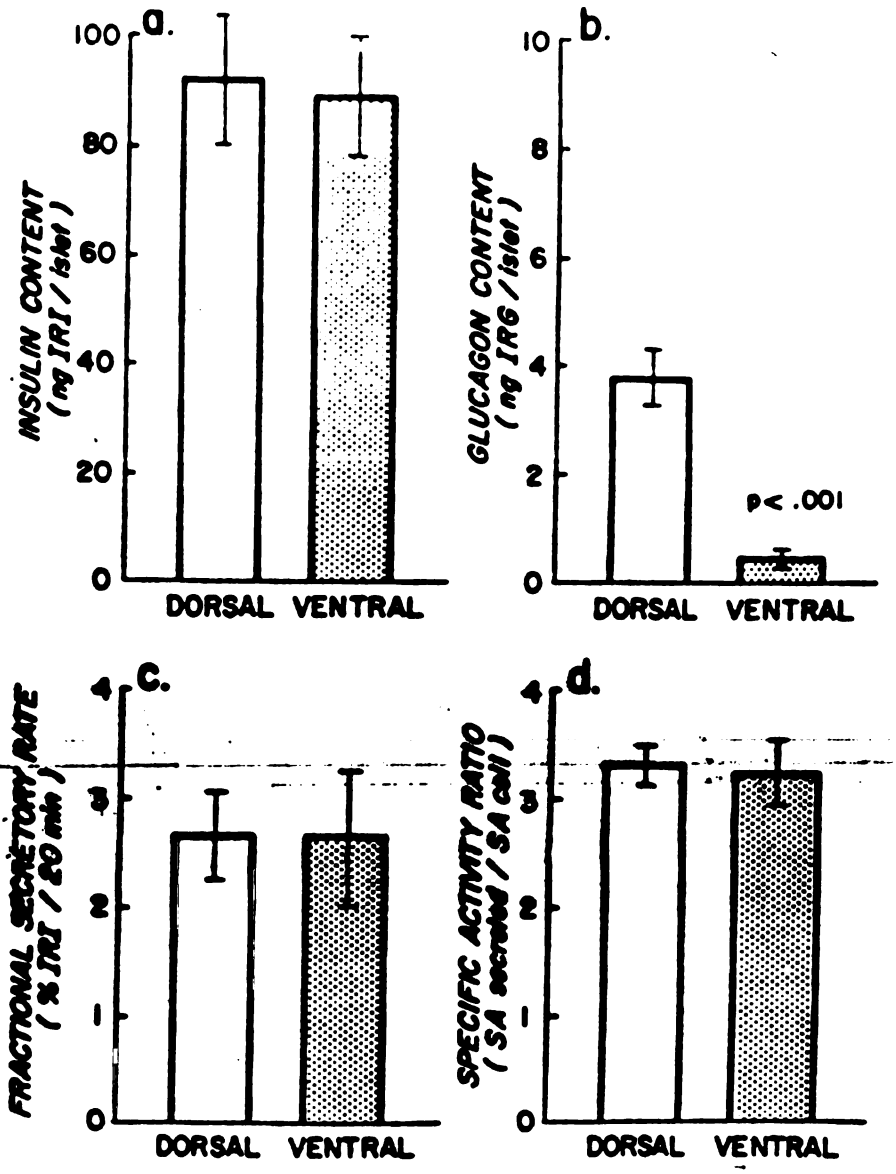


Figure 17: Effect of pancreatic region of origin on the insulin content, glucagon content and insulin secretory rates of isolated islets. Experiments and analysis were performed as previously described (continuous 20 mM glucose exposure). Bars represent the mean ± SE from six experiments.

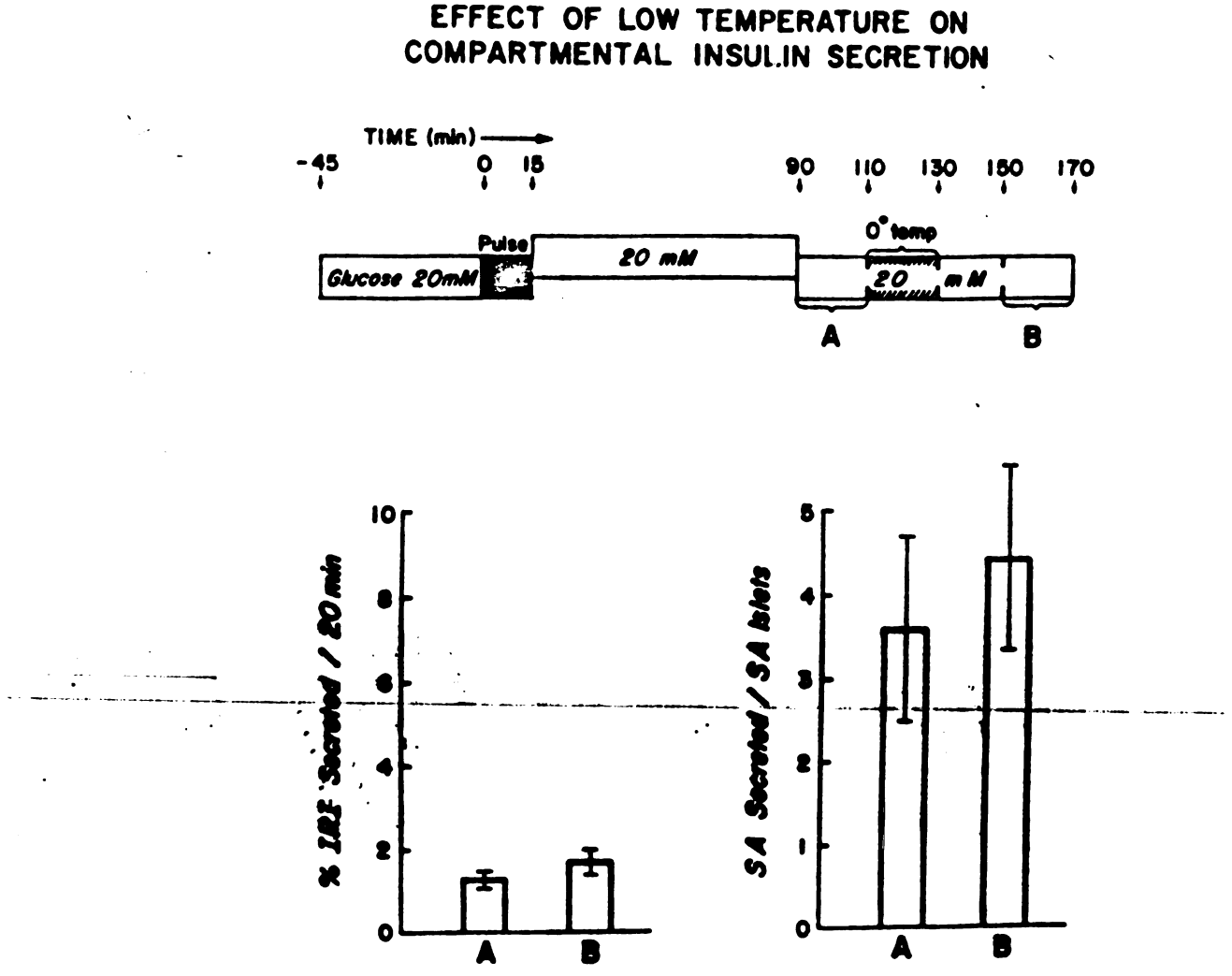


Figure 18: Effect of 20 min period at 0o C on the secreted/islet insulin specific activity ratio. Islets were incubated continuously in 20 mM glucose beginning at -45 min and exposed to ^{3}H-leucine between 0 and 15 min. After labeling, islets were incubated at 37° C in 20 mM glucose until 110 min, chilled to 0° from 110 to 130 min, then returned to incubation at 37° for the duration of the experiment. Noncumulative samples of secreted insulin were collected at 90-110 min and 150-170 min. All buffers were preequilebrated to their desired temperature prior to exposure to islets. Insulin from secreted and islet samples were purified by acid-ethanol extraction, affinity chromatography and chromatography of columns of Biogel P-30. Bars represent the mean ± SE from four experiments.

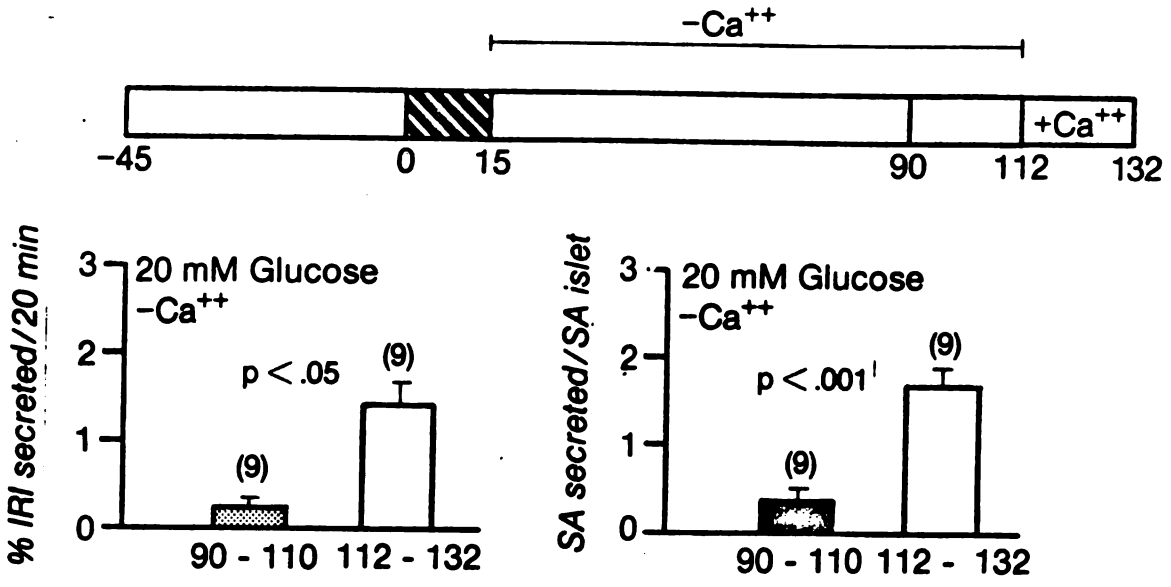


Figure 19: Effect of calcium omission on the rate of insulin secretion and the specific activity ratio between the secreted and islet insulin. Following pulse, islets were washed twice each time with KRB + 20 mM glucose without added calcium, first at 15 - 18 min and again at 87 - 90 min. Islets were washed once with KRB + 20 mM glucose with calcium between 110 and 112 min. Bars represent the mean + SE from nine experiments.

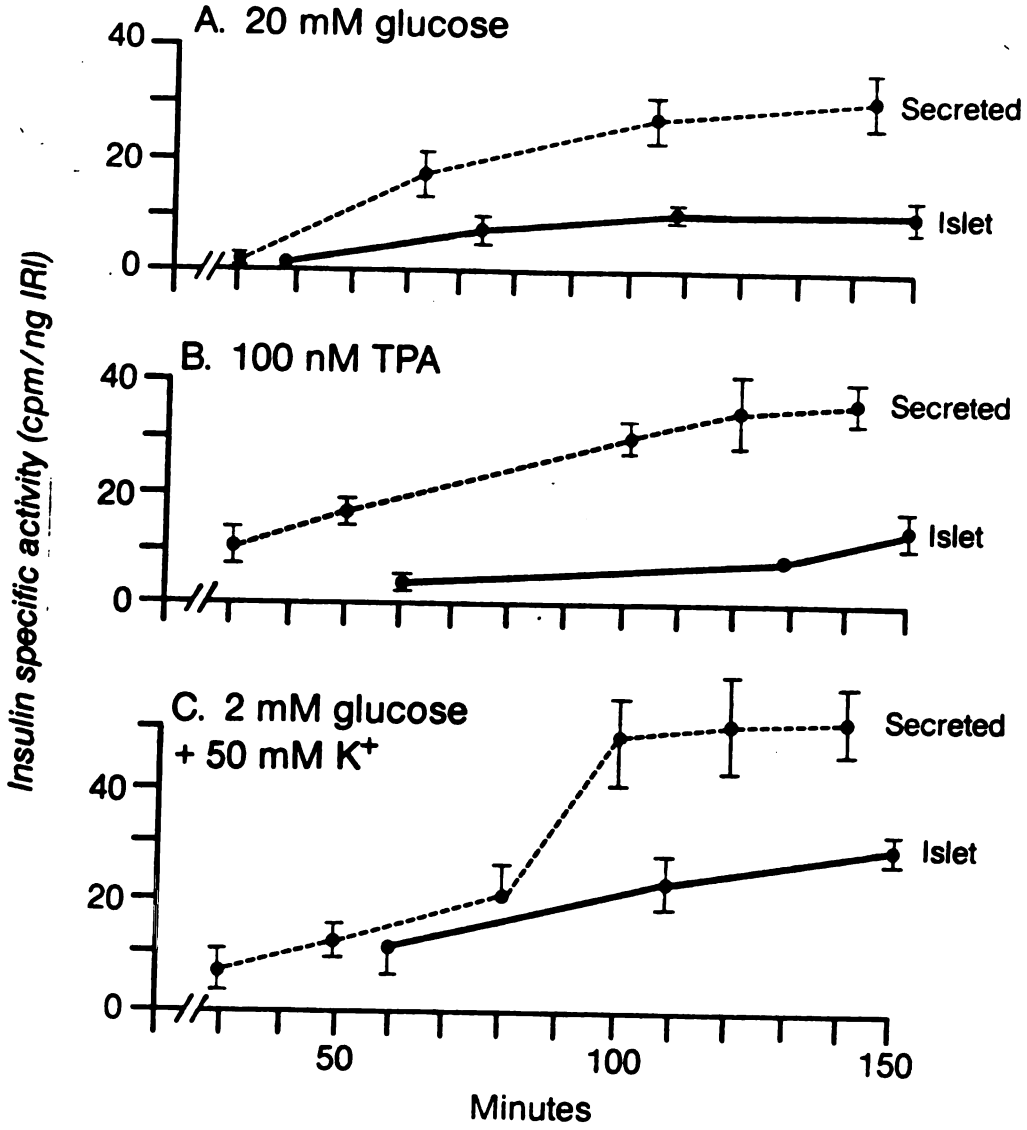


Figure 20: Effect of time and post pulse culture conditions on the specific activity of secreted and islet insulin. In all experiments islets were exposed to 20 mM glucose beginning at -45 min and through the ^{3}H-leucine pulse period, 0-15 min. After the pulse islets were washed and maintained in KRB containing the following: (a) 20 mM glucose, same as described in figure 4, (b) 100 nM TPA with no glucose present, and (c) 2 mM glucose + 50 mM K^{+}. Data represent mean + SE of 3 - 9 experiments.

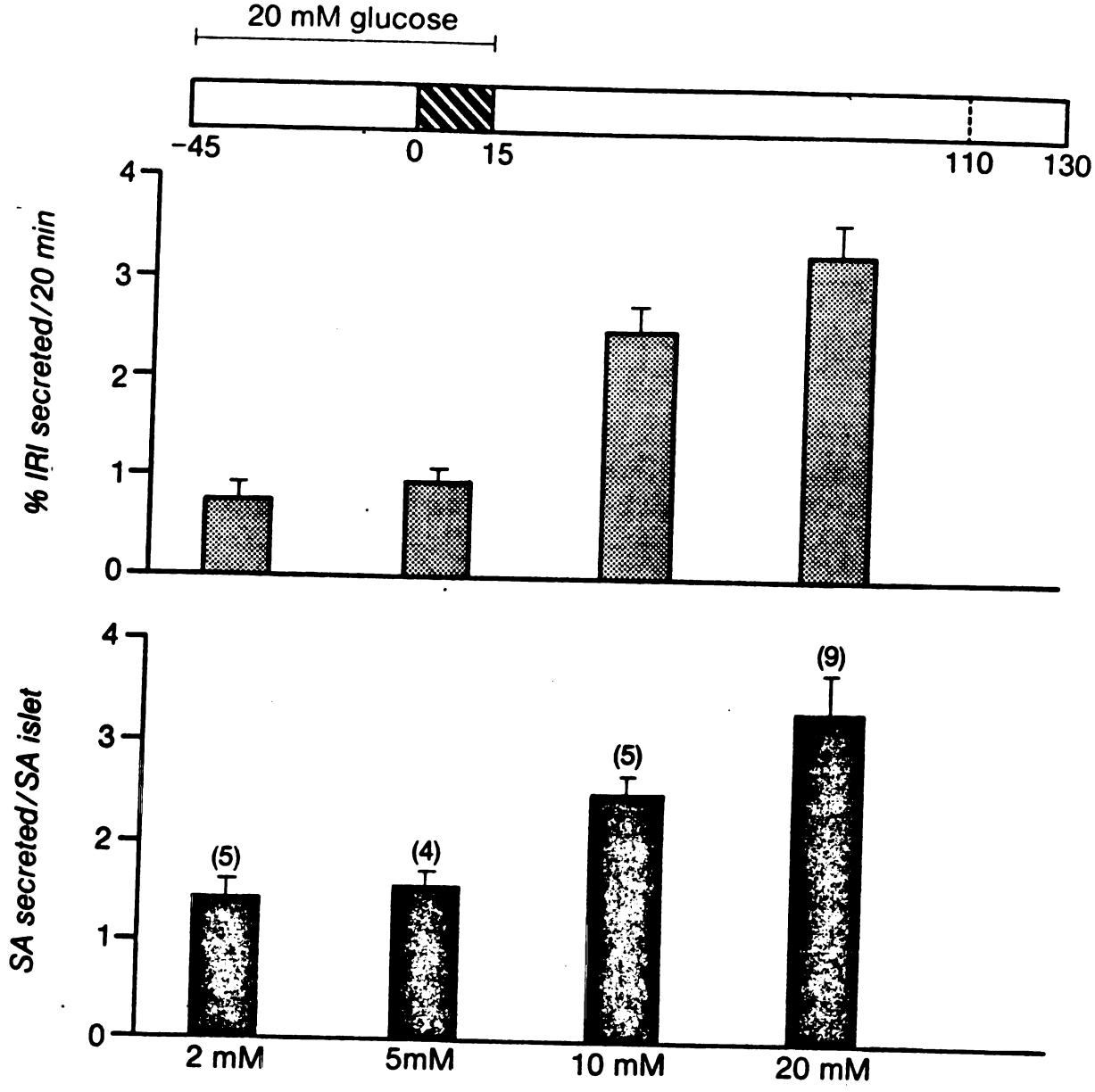


Figure 1: Effect of post pulse glucose concentration on secretory rate and secreted/islet specific activity ratio. The data represented in these graphs were compiled from studies conducted at different times over a two year period in which similar experimental paradigms were followed. In all experiments islets were exposed to 20 mM glucose beginning at -45 min and through the ^{3}H-leucine pulse period, 0-15 min. After the pulse islets were washed with KRB containing the appropriate glucose concentration and maintained under those conditions. At 110-130 min a 20 min media sample was collected. With the exception of islets maintained at 2 mM glucose, secreted samples were collected in glucose concentrations in which they were cultured. Because islets cultured in 2 mM glucose secrete only basal amounts of hormone (below the levels necessary to make specific activity measurements) this group of islets was exposed to 20 mM for the 20 min secretion collection period (110-130).

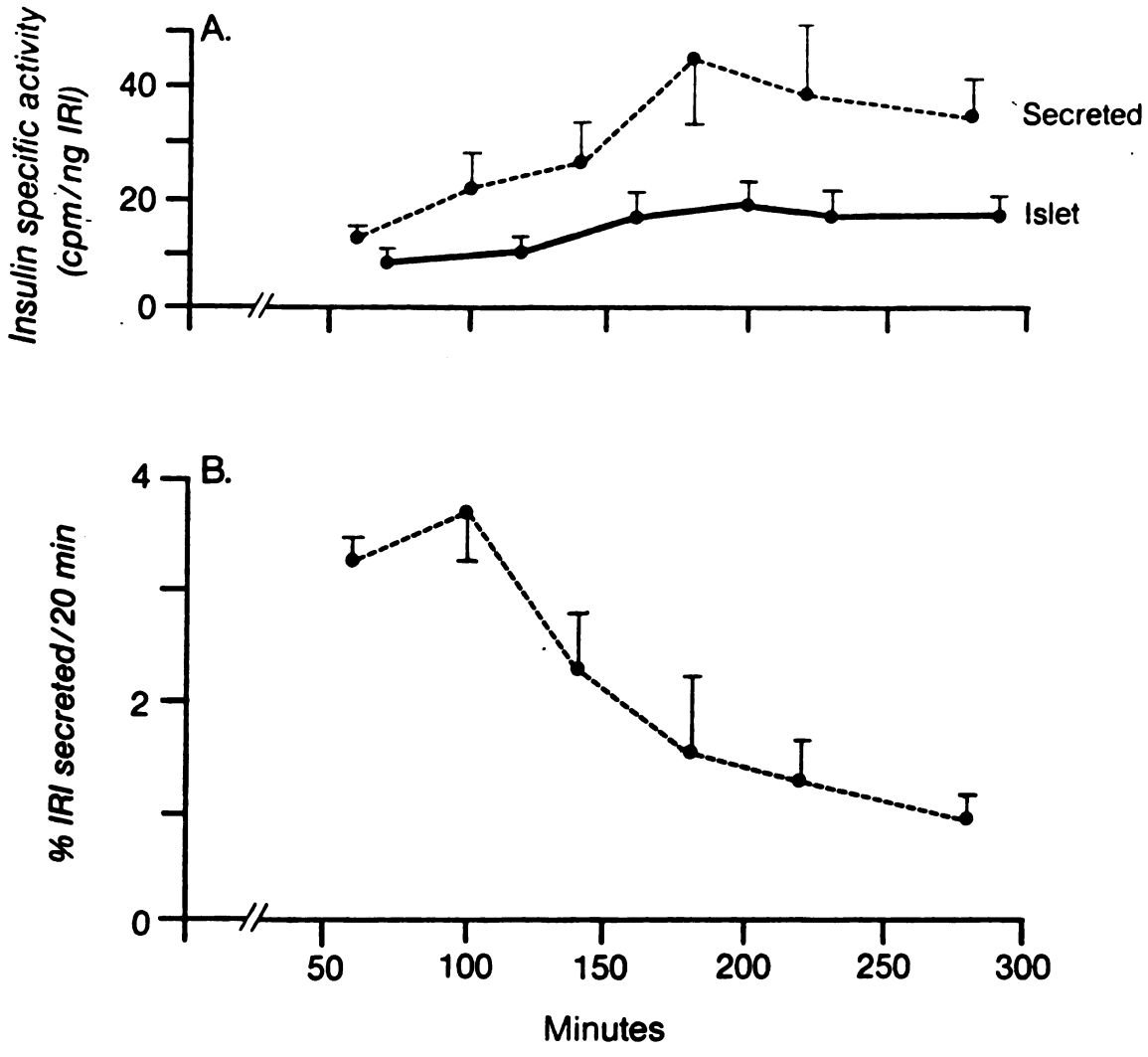


Figure22 : (A) Effect of time on the specific activity of secreted and stored insulin remaining in islets cultured with 2 mM glucose. Islets were exposed to 20 mM glucose beginning at -45 min and including the ^{3}H-leucine pulse, 0-15 min. Following the pulse, islets were washed three times with KRB containing 2 mM glucose and maintained under these conditions until reexposed to 20 mM glucose for 20 min. Secreted samples were collected during 20 min exposure to 20 mM glucose following which individual islet and media samples were immediately frozen and subsequently analyzed using reverse-phase HPLC.
(B) Represents the percent of immunoreactive insulin secreted in response to 20 mM glucose stimulation following culture in 2 mM glucose for various durations. These points correspond to secretion periods depicted in fig A. The total amount of immunoreactive insulin (islet + media) was similar at all times measured. Data points represent mean + SE of 3 - 5 experiments.

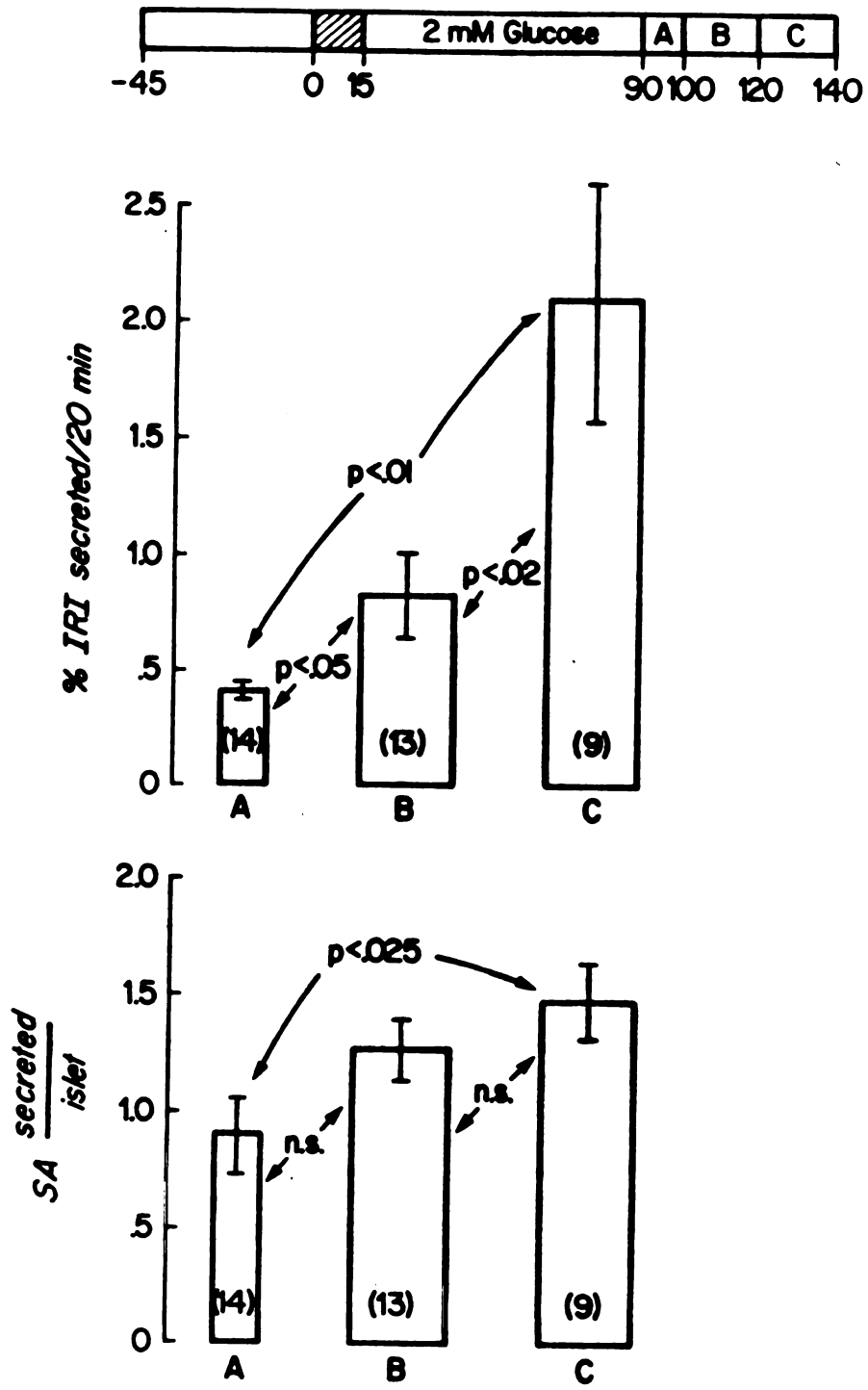


Figure 3: Secreted/islet insulin specific activity ratio during first and second phase insulin release. Islets were exposed to glucose beginning at -45 min and through the ^{3}H-leucine pulse, 0-15 min. After the pulse islets were washed with KRB containing 2 mM glucose and maintained in this buffer until 90 min. At min 90 islets were exposed to 20 mM glucose for three sequential periods of 10, 20 and 20 min during which secretion was measured. This temporal collection of media conformed to the observed kinetics of phasic insulin secretion [ref]; first phase occurring within the first 10 min of exposure. All samples were acid-ethanol extracted and individually analyzed.

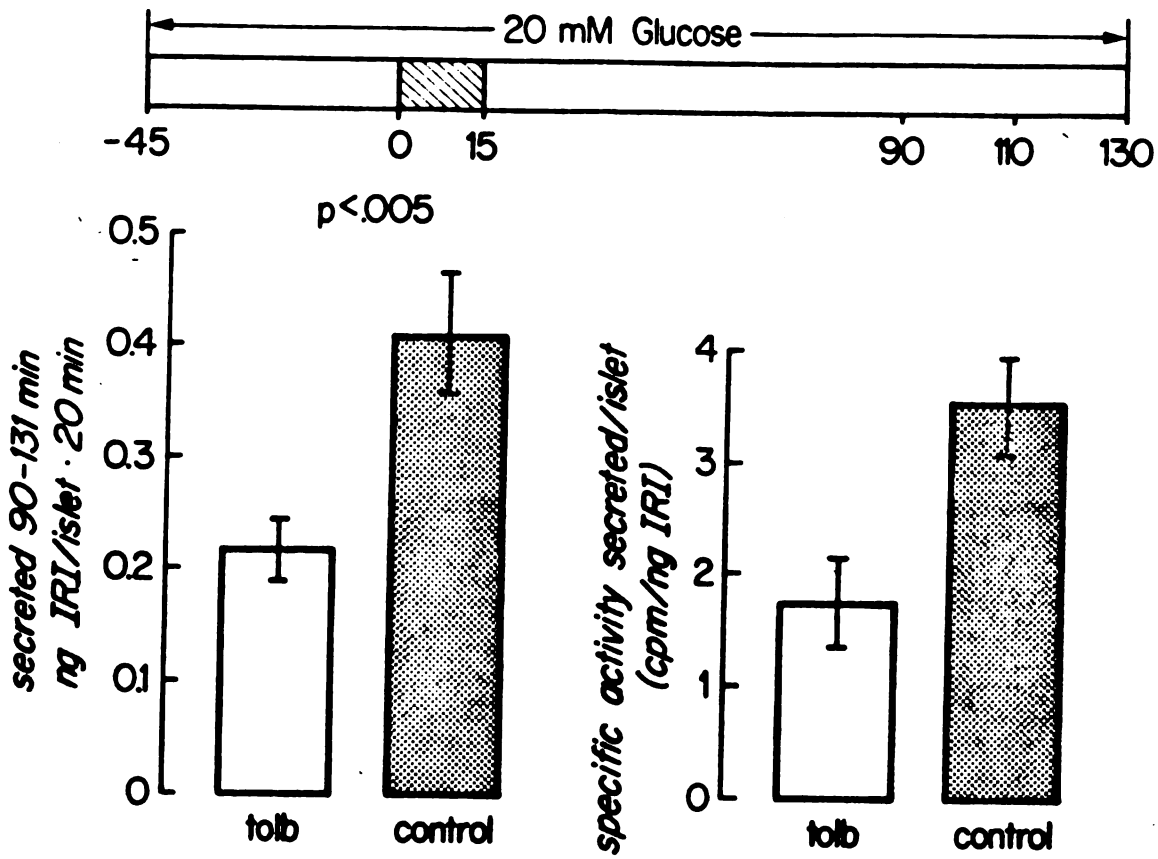


Figure 24: Comparison of secretory rate and secreted/islet insulin specific activity ratio in islets from tolbutamide treated vs. normal rats. Islets were continuously maintained in KRB containing 20 mM glucose and exposed to ^{3}H-leucine between 0 - 15 min. Islet and media samples were extracted in acid-ethanol and chromatographed using Biogel P-30 columns. Secretion was collected for 2 sequential 20 min periods, 90-110 min and 110-130 min. Because data from these periods was very similar, measurements were analysed as one collection period. Therefore, bars represent the + SEM from seven experiments, or 14 data measurements each.

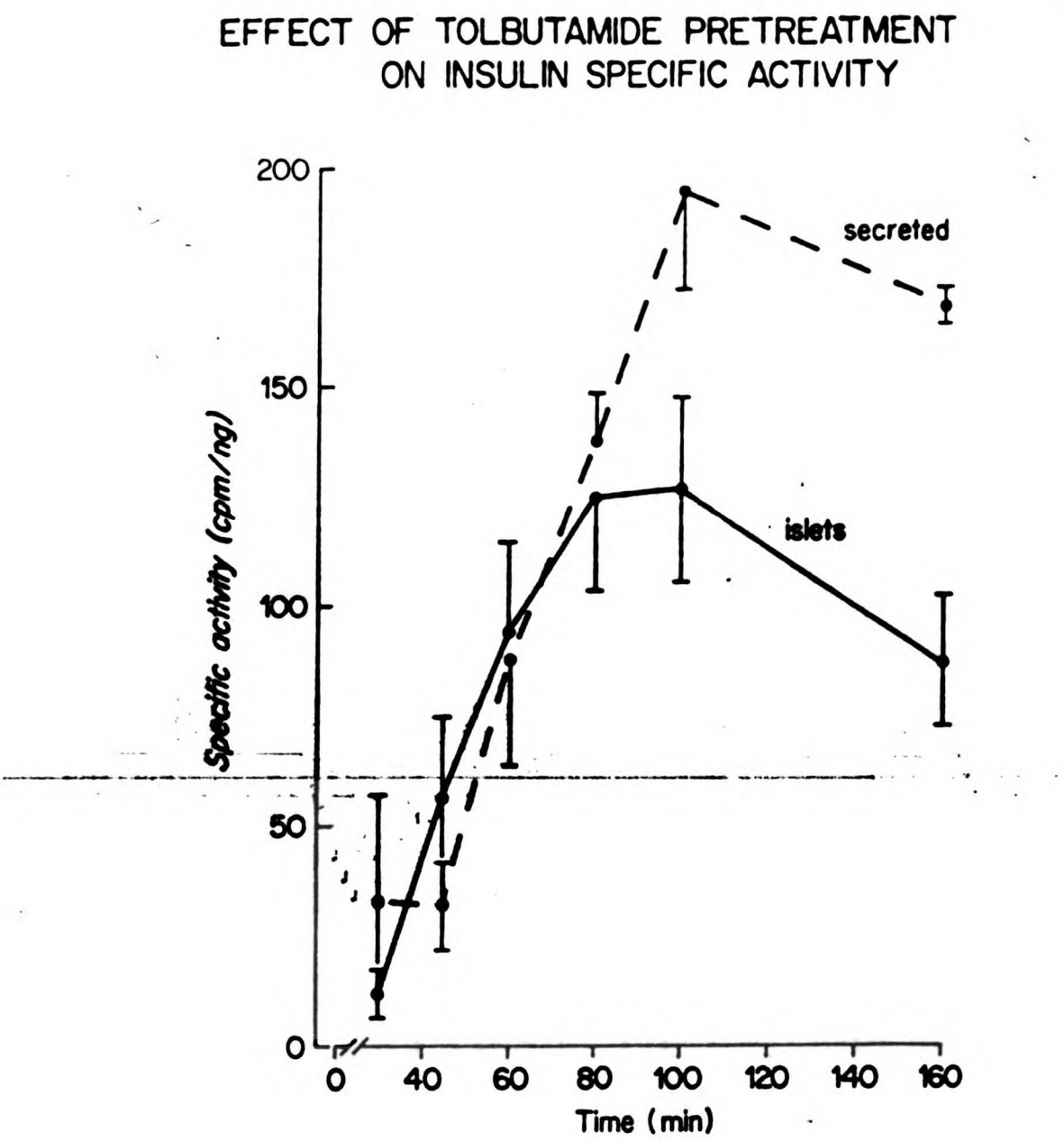


Figure 25 Effect of time on the specific activity of secreted and stored insulin in islets isolated from tolbutamide treated rats. Islets were continuously maintained in 20 mM glucose beginning at -45 min and exposed to 3H-leucine between 0-15 min. Points represent mean ± three or more experiments. Data points are plotted at the center of the intervals, i.e., 15-45, in which incubation buffers were removed and replaced with fresh KRB. All samples were acid-ethanol extracted and chromatographed on Biogel P-30 columns.

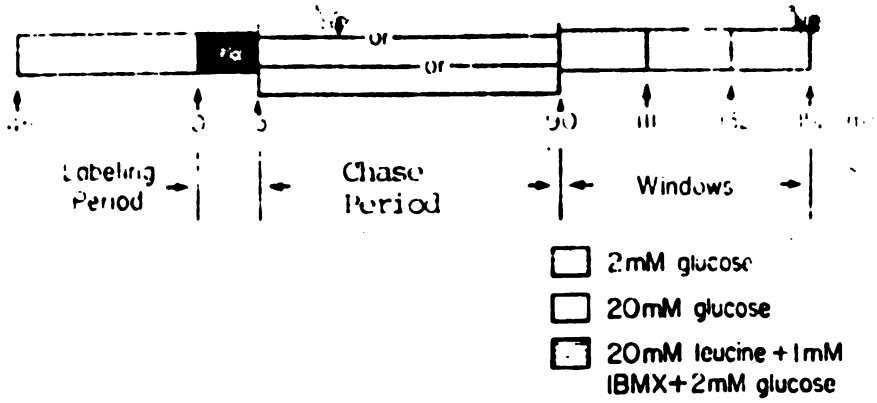


Figure 26 : Schematic representation of the pulse-labeling experiments done with freshly isolated tumor cells. Tumor cells were washed with fresh buffer during each transition period, then resuspended and incubated in fresh buffer. Thus, noncumulative samples of secreted hormone were obtained during each period.

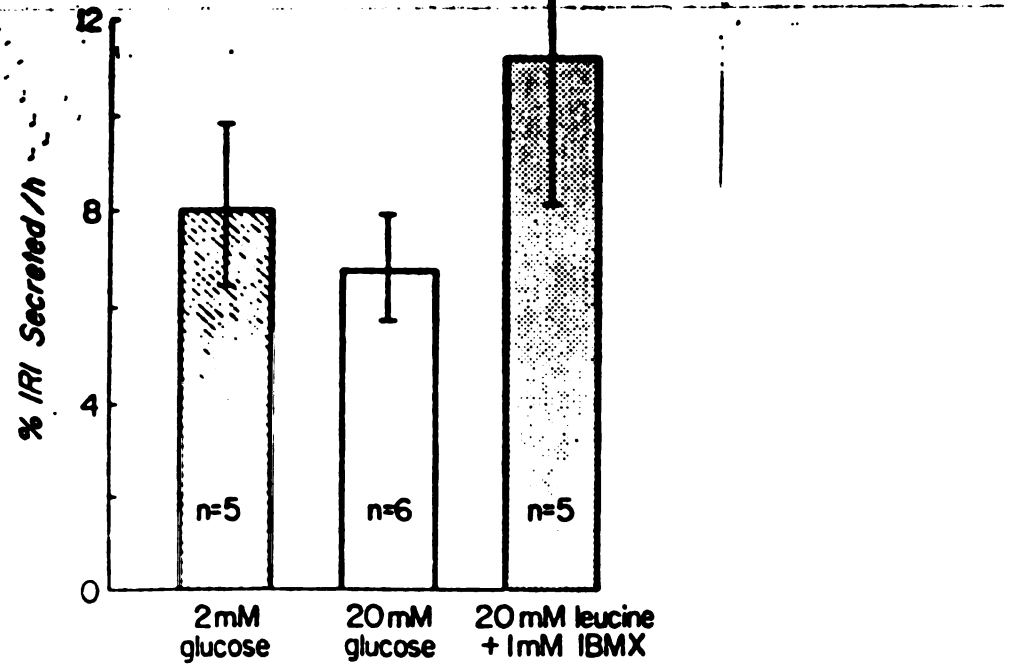


Figure 27 : Effect of various secretory stimuli on the fractional secretion rate of immunoreactive insulin from rat tumor cells during the chase period (between 15-90 min). Data from 5 to 6 experiments are represented as mean + SEM.

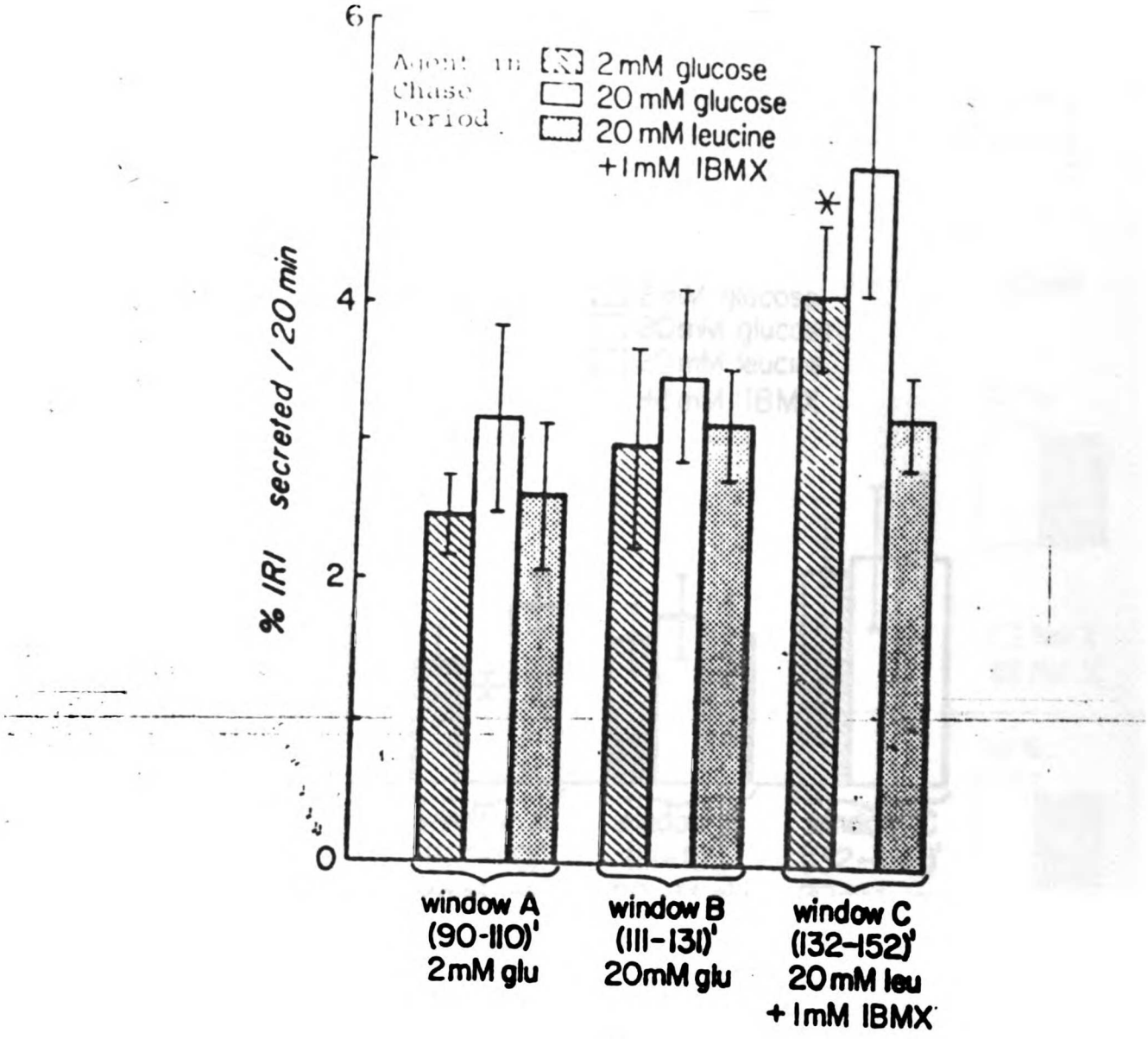


Figure28: Effect of secretory stimuli during the windows on the fractional secretory rate of immunoreactive insulin from rat tumor cells. Three bars are ploted during each window to represent data from experiments with cells incubated in 2 mM glucose, 20 mM glucose, or 20 mM leucine plus 1 mM IBMX during the marking period.

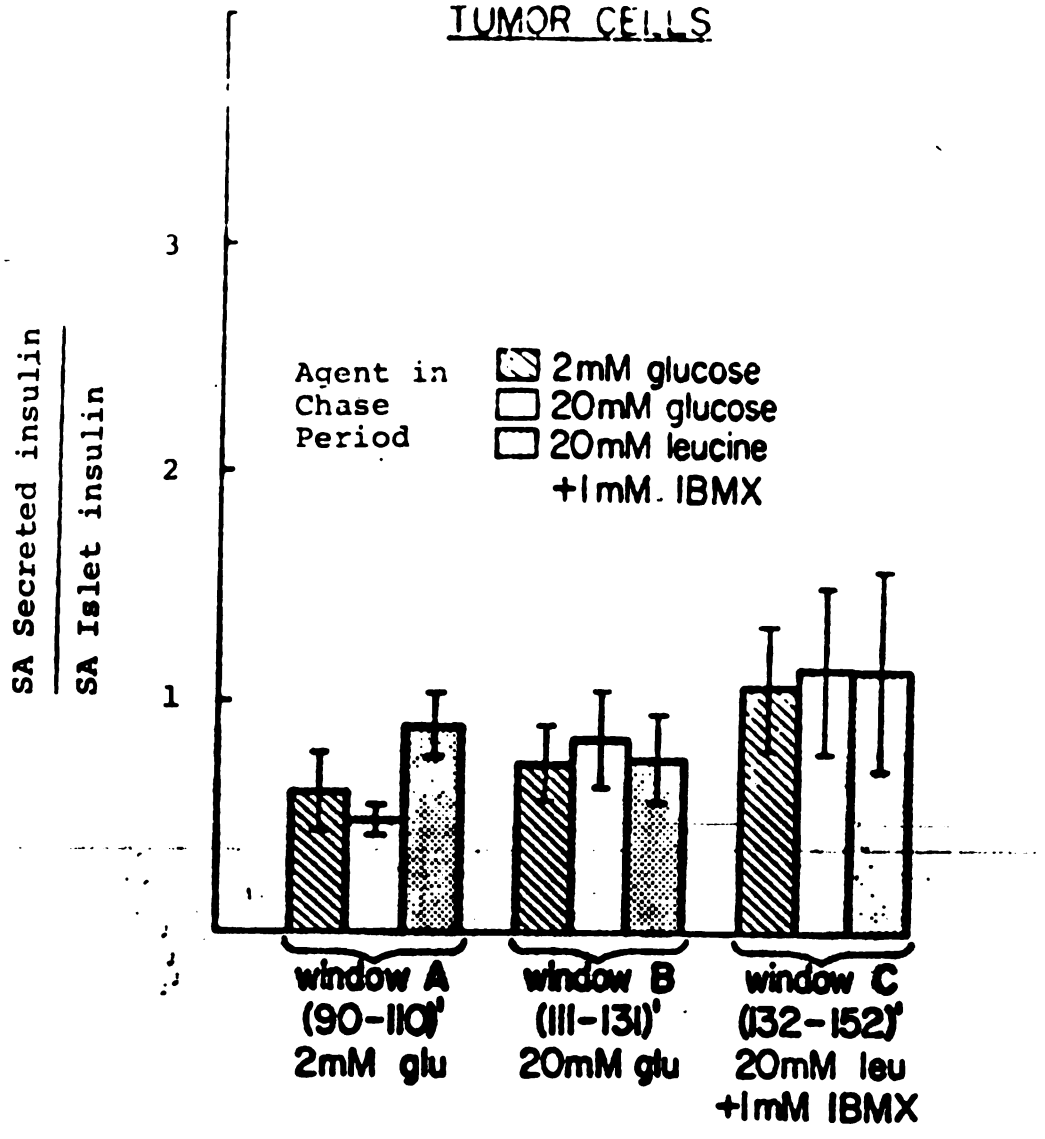


Figure 29 : Effect of chase conditions (15-90 min) and secretory stimuli on the specific activity of secreted and cellular insulin.

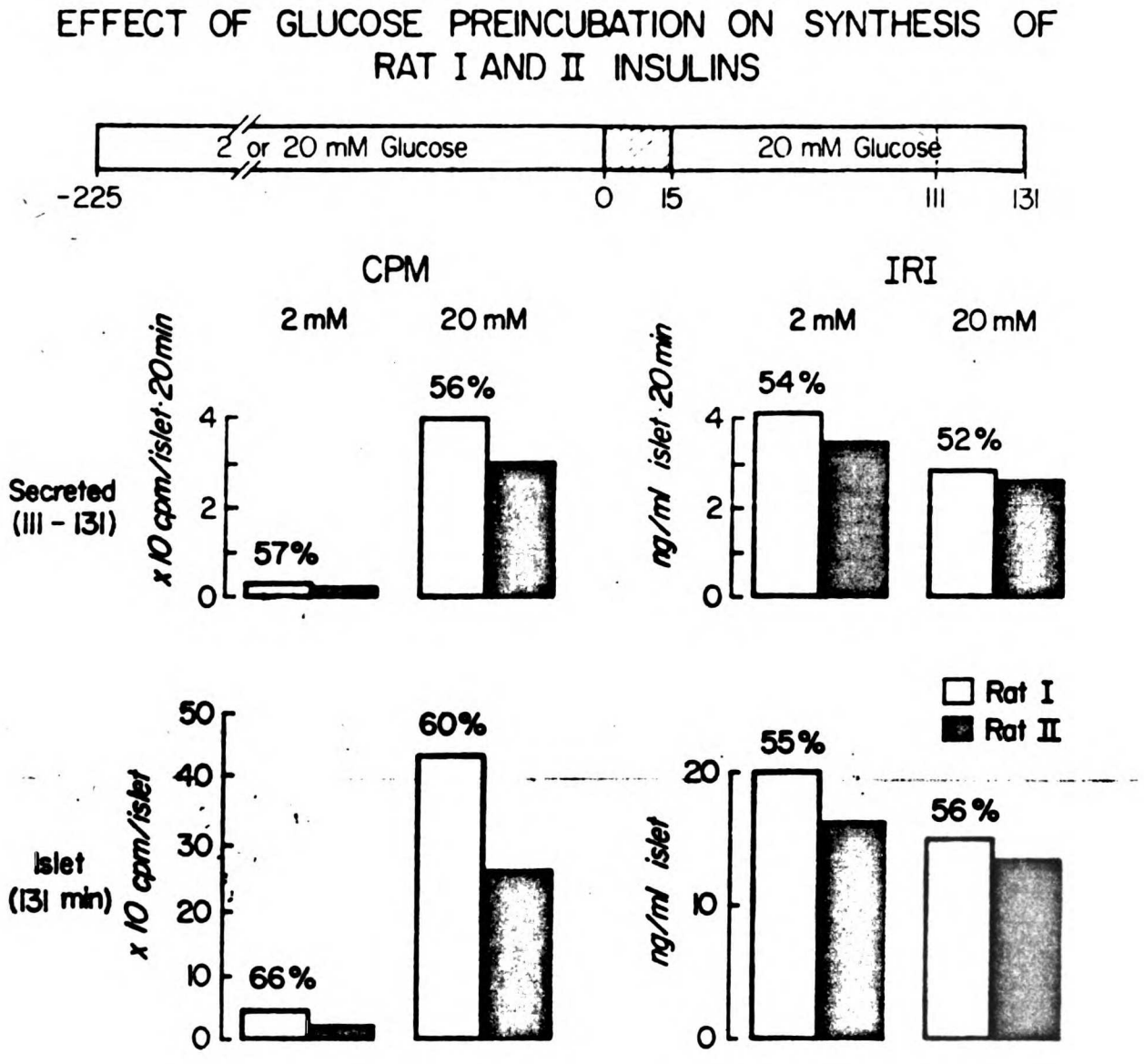


Figure 30: Effect of glucose preincubation on synthesis of rat I and II insulins. Islets were cultured for 225 min in KRB containing either 2 or 20 mM glucose, then exposed to ^{3}H-leucine, in the presence of 20 mM glucose between 0-15 min. Following the pulse islets were washed and maintained in KRB with 20 mM glucose for the remainer of the experiment. A 20 min secretion sample was collected between 111-131 min. All samples were immediately frozen, then lyophilyzed and the two rat insulins seperated by reverse-phase HPLC. Individual rat insulin I and II measurements were performed HPLC eluted samples. Bars represent the mean of 4 to 7 experiments. Percentages above the the bar corresponding to rat insulin I, represent the mean relative percentage of radioactive or immunoreactive insulin I.

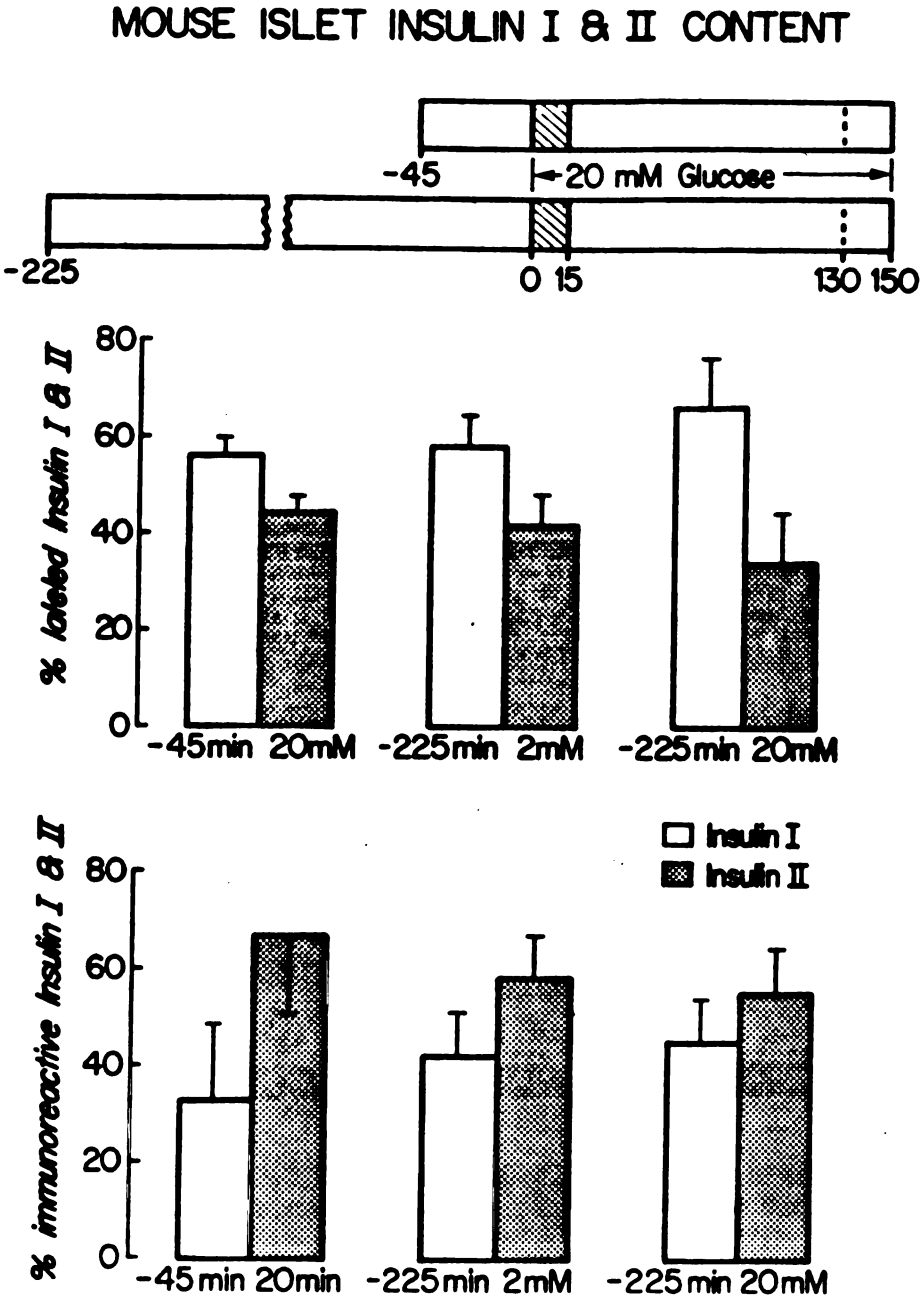


Figure31: Effect of duration of glucose exposure on insulin I and II synthesis in mouse islets. Prior to pulse, mouse islets were cultured under the following conditions: -45 min incubation in 20 mM glucose, -225 min incubation in 2 mM glucose or -225 min incubation in 20 mM glucose. All islets were then exposed to ^{3}H-leucine, in the presence of 20 mM glucose, between 0-15 min. Following the pulse islets were washed and maintained in 20 mM glucose for the remainder of the experiment. Islet samples, excluding culture media, were immediately frozen and subsequently analyzed using reverse-phase HPLC. The top panel represents the relative percentage of radioactive insulin I and II present in mouse islets at min 150. The bottom panel the corresponding immunoreactive measurements. Bars represent mean + SE of 3 to 6 experiments.

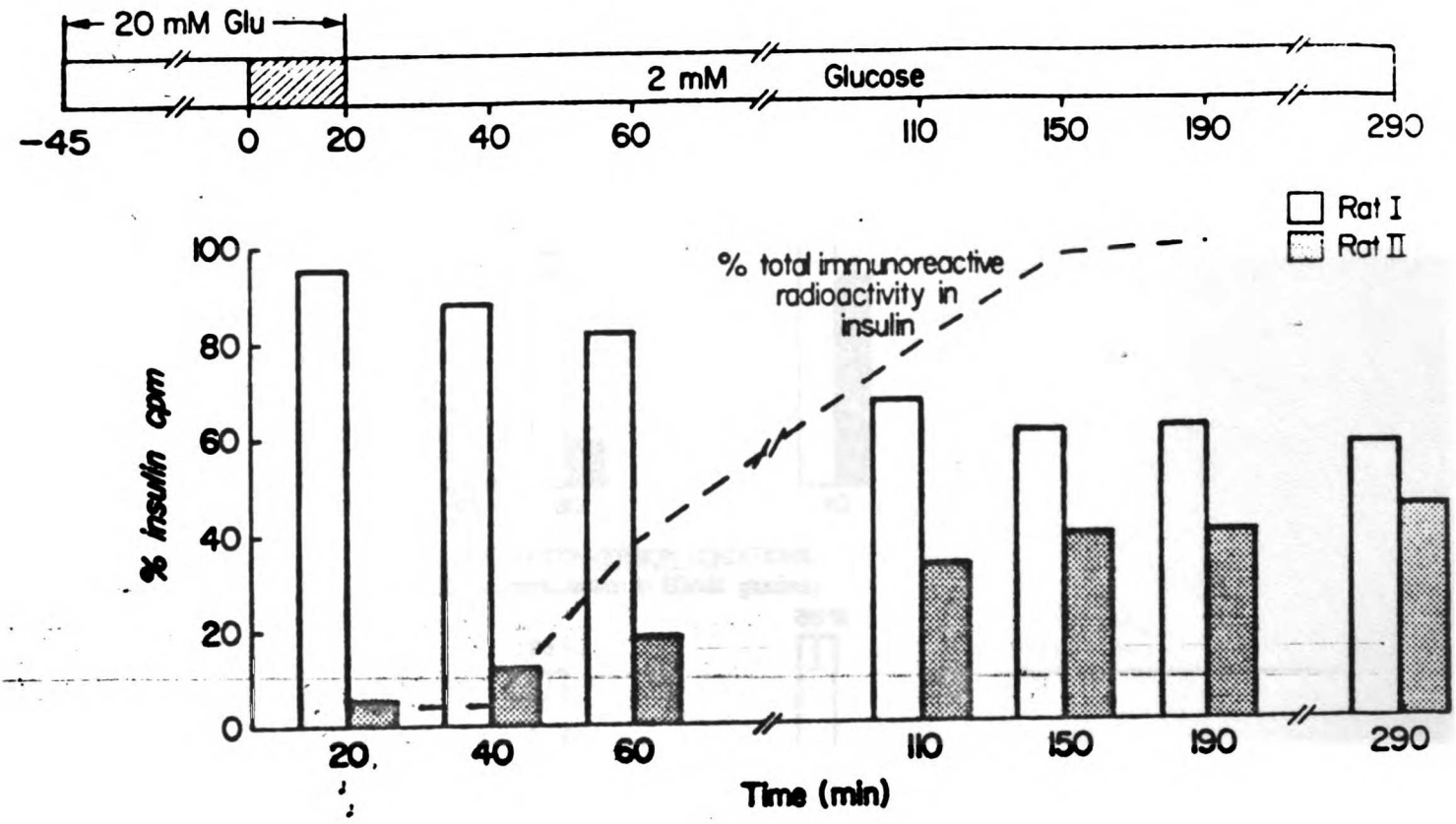


Figure 32: Effect of time on the relative appearance of radioactive insulins I and II in rat islets. Islets were exposed to 20 mM glucose beginning at -45 min and including the ^{3}H-leucine pulse, 0-20 min. After the pulse islets were washed three times with KRB containing 2 mM glucose and maintained under these conditions for up to 250 min. Samples were collected at times designated; media and islets were not segregated, rather, frozen together and rat insulin I, II and proinsulins analyzed using reverse-phase HPLC. Bars illustrate the mean relative percentage of labeled rat insulin I and II, the dashed line depicts the % total immunoreactive radioactivity in insulin at each time, normalized to the total amount of radioactive proinsulins present at 20 min. All radioactive insulin I and II measurements were multiplied by 11/6, to correct for loss of C-peptide. Each time point represent 3 to 7 experiments.

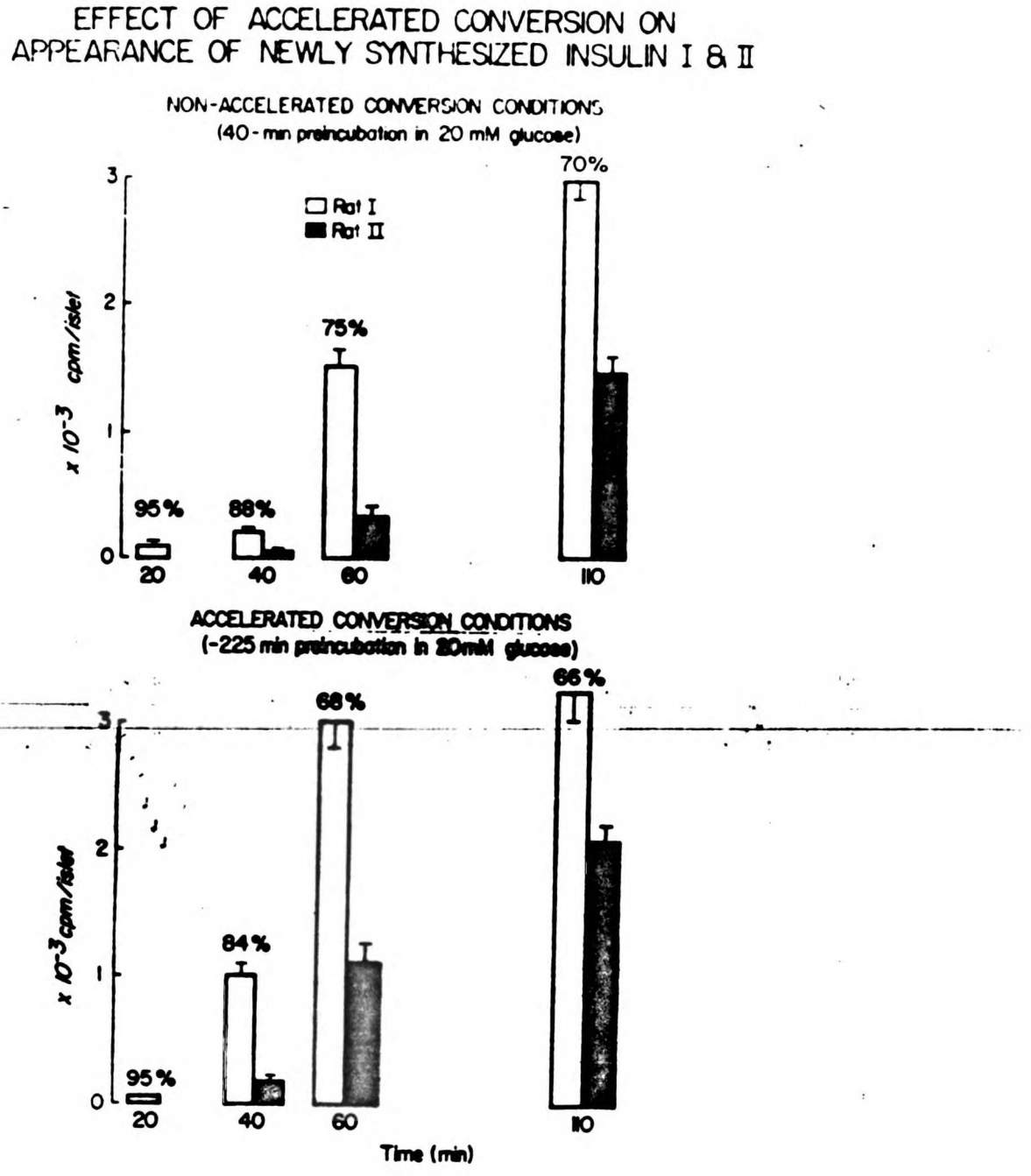


Figure33: Effect of accelerated conversion on appearance of newly synthesized insulin I and II in rat islets. Islets were preincubated in 20 mM glucose for either 40 min or 225 min, then exposed to ^{3}H-leucine between 0 - 20 min, also in the presence of 20 mM glucose. Following the pulse, islets were washed with KRB containing 2 mM glucose and maintained at that glucose concentration for the duration of the experiment. At times designated, islets were collected and immediately frozen for subsequent analysis using HPLC. The bars depict the mean number of counts + SE incorporated into insulin I and II per islet at each time. The percentage above each set of bars represents the relative percentage of insulin I present. Data are from three to five experiments.

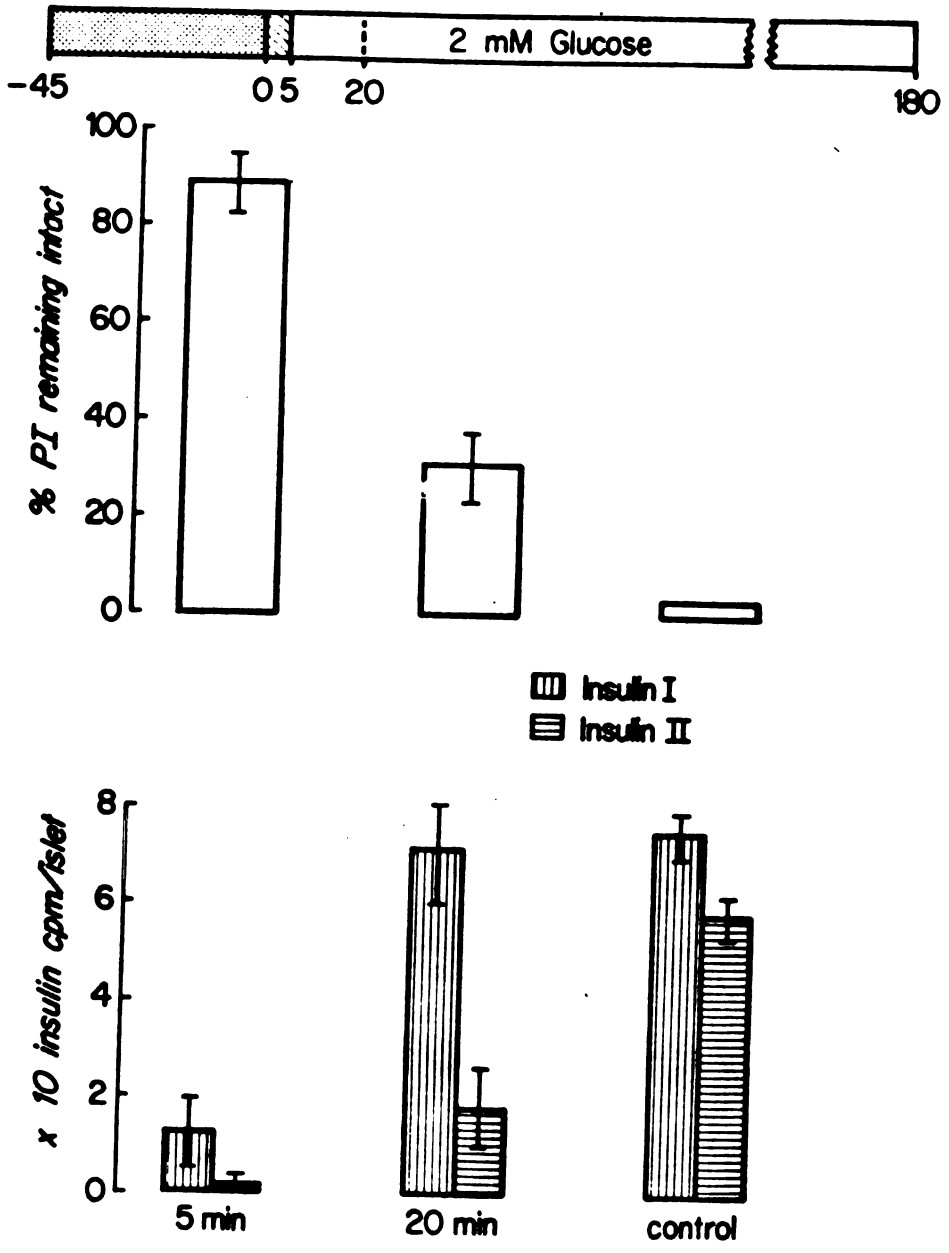
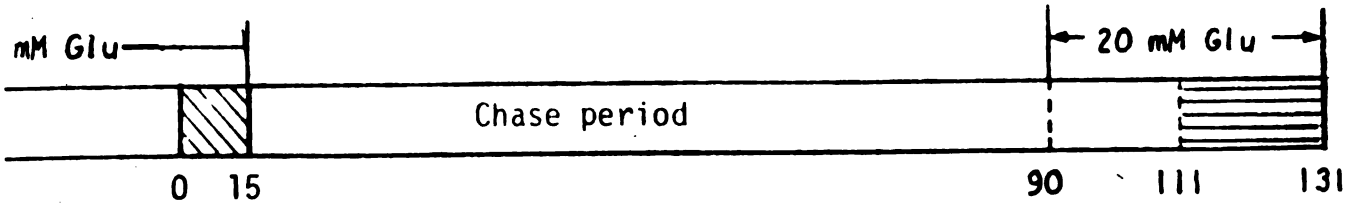
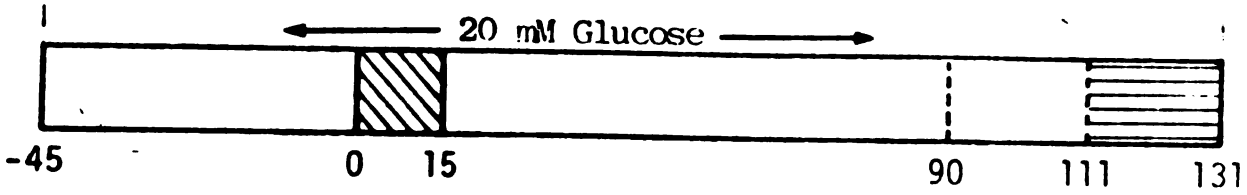


Figure 34: Effect of ATP synthesis inhibition on appearance of insulin I and II in islets. Islets were exposed to 20 mM glucose beginning at -45 min including the ^{3}H-leucine pulse, 0-5 min. After the pulse, islets were washed three times with KRB containing 2 mM glucose and cultured with 2 mM glucose KRB in the presence or absence of 50 uM antimycin A, between 5 and 180 min. Another group of islets were similarly washed and cultured in 2 mM glucose KRB without antimycin A between 5 and 20 min. At 20 min media was replaced with 2 mM glucose KRB containing 50 uM antimycin A and maintained in this buffer for the remainder of the experiment. Bars represent mean + SE of three experiments.



Chase Conditions	% IRI secreted (15-90 min)	% Newly synthesized insulin Rat I & II	
		Secreted (111-131 min)	Islet (131 min)
1) 0 mM glucose	1.9	56:44	62:38
2) 0 mM glucose + 25 uM forskolin	6.8	58:42	65:35
3) 0 mM glucose + 100 nM TPA	21.5	59:41	65:35
4) 20 mM glucose	15.0	53:47	62:38

Figure 35: Effect of chase conditions on the relative distribution of secreted and islet rat insulin I and II. Islets were exposed to 20 mM glucose beginning at -45 min including the ^{3}H-leucine pulse, between 0 - 15 min. After the pulse, islets were washed three times with KRB containing the agents listed under chase conditions and maintained in media containing these agents until 90 min. At 90 min islets were washed with KRB containing 20 mM glucose and exposed to these conditions for the remainder of the experiment. A 20 min secreted sample was collected between 111-131 min after which both media and islet samples were immediately frozen and subsequently analyzed using reverse-phase HPLC. Percent of immunoreactive insulin secreted during the chase period (15 - 90 min) was calculated based on the amount of islet insulin present at the end of the experiment, 131 min, for each set of chase conditions. Data represent mean of 4 to 7 experiments.



	SPECIFIC ACTIVITY (cpm/ng insulin/ml)	
	Secreted	Islet
Rat I	89.9	42.1
Rat II	68.9	30.9
$\frac{\text{SA Rat I}}{\text{SA Rat II}}$	1.3	1.4

Figure 36: Effect of continuous glucose exposure on the specific activity of individual rat insulin I and II in secreted and islet samples. Islets were maintained in KRB containing 20 mM glucose throughout the experiment and exposed to ^{3}H-leucine between 0 - 15 min. Secreted samples were collected between 111 - 131 min following which both media and islet samples were immediately frozen. The two insulins were separated using reverse-phase HPLC and the individual amount of radioactive and immunoreactive insulin I and II determined. Data represent the mean of 5 experiments.

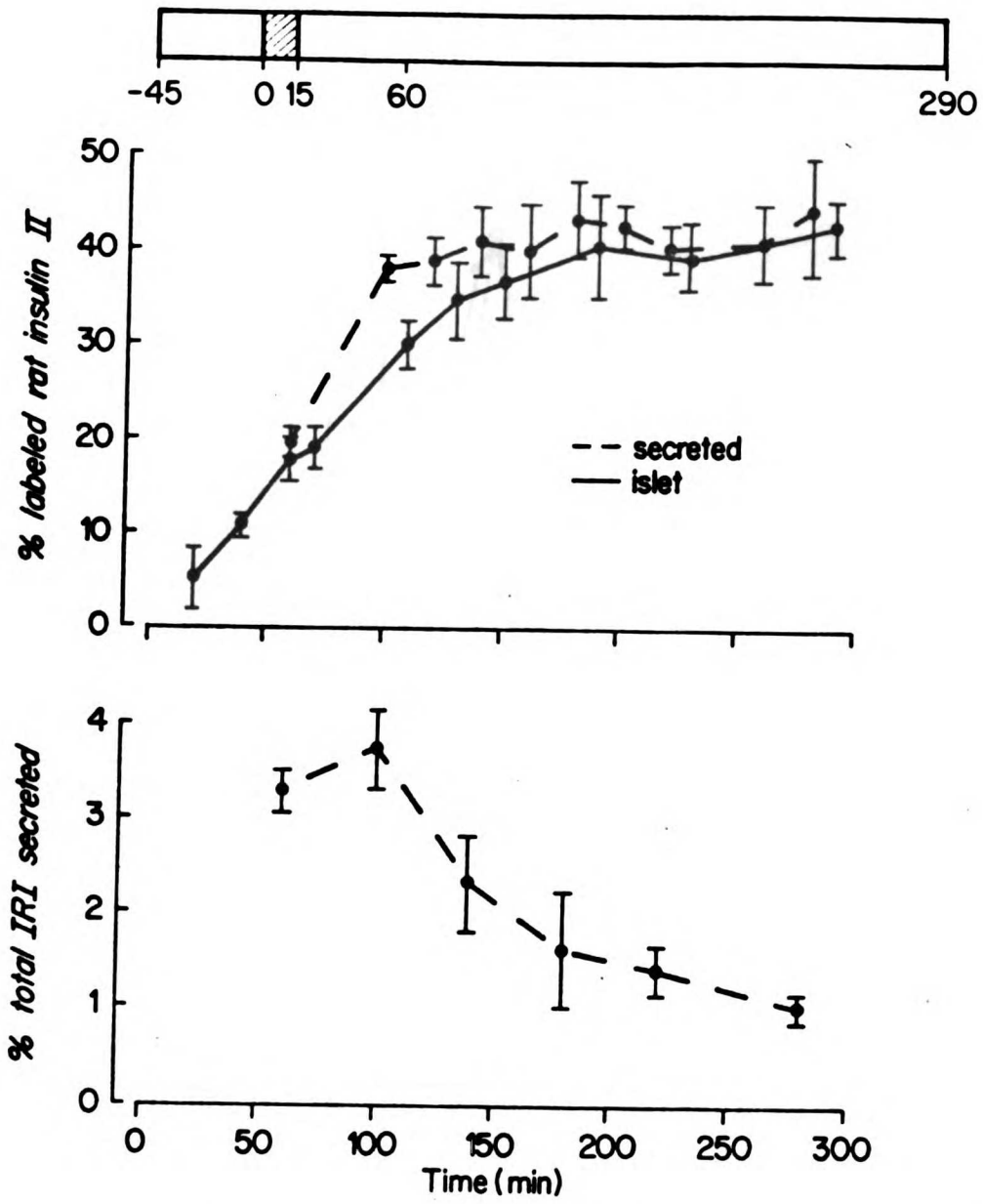


Figure37: Effect of time on the relative percentage of radioactive insulin II present in secreted and rat islet samples. Islets were exposed to 20 mM glucose beginning at -45 min and including the ^{3}H-leucine pulse, 0 - 15 min. After the pulse, islets were washed and cultured for various times in KRB containing 2 mM glucose. Secreted samples were collected during 20 min exposure of islets to 20 mM glucose (i.e., for data point at 60 min, islets were cultured from 15 - 50 min in 2 mM glucose then exposed to 20 mM glucose between 50 - 70 min; for data point at 290 min islets were cultured until 250 min in 2 mM glucose, then exposed for 2 sequential 20 min periods to 20 mM glucose). The lower panel represents the percent of immunoreactive insulin secreted, corresponding to the stimulation periods. Immunoreactivity was based on measurement of IRI prior to HPLC analysis. These data are complied from experiments carried out over a two year period. Each point represents the mean + SE of 4 to 10 experiments.

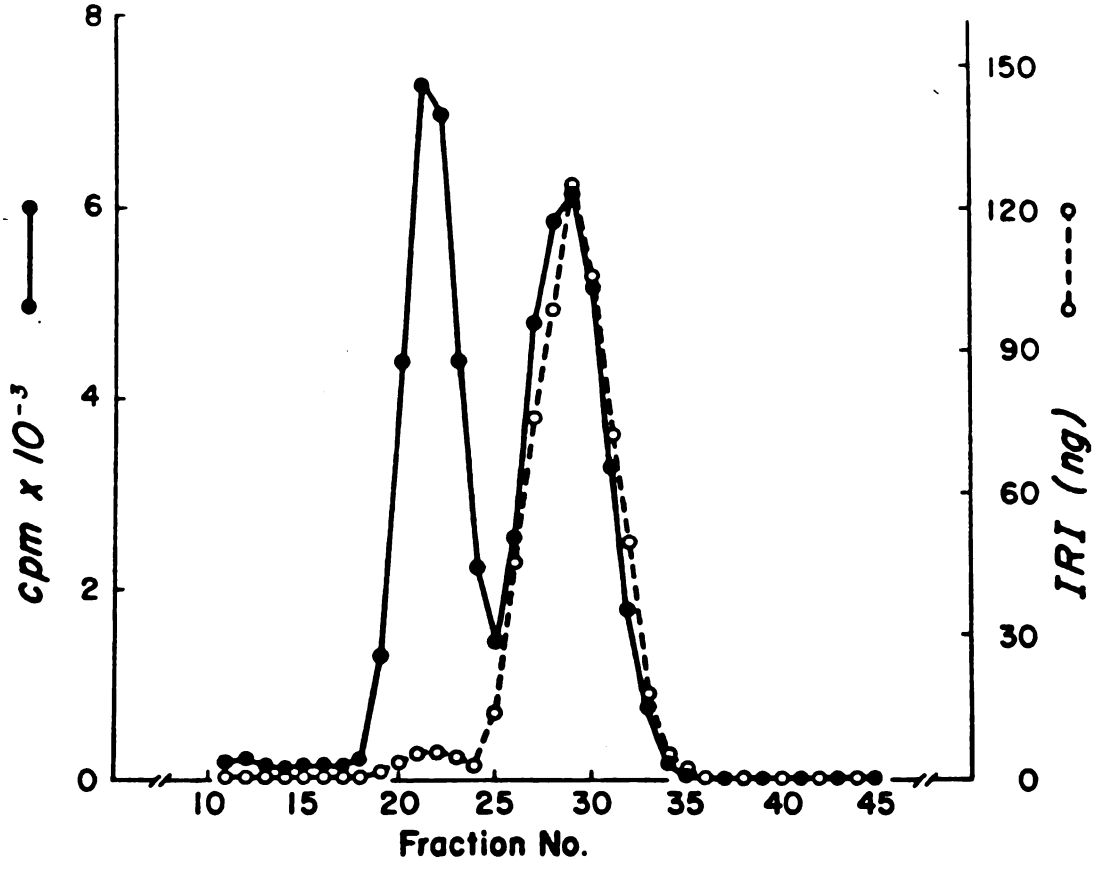


Figure 38 Profile of a typical sample eluted from a Biogel P-30 column. Islets were incubated continuously in KRB + 20 mM glucose beginning at -45 min, exposed to ^{3}H-leucine between 0 to 15 min, and extracted at 95 min with acidic ethanol. Proinsulin and insulin in these extracts were purified by antiinsulin-Sepharose chromatography. Purified samples were applied to a 1- x 110-cm column of Biogel P-30 and eluted at room temperature with 3 M acetic acid + 0.05 % bovine serum albumin. Fractions are 2.0 ml each.

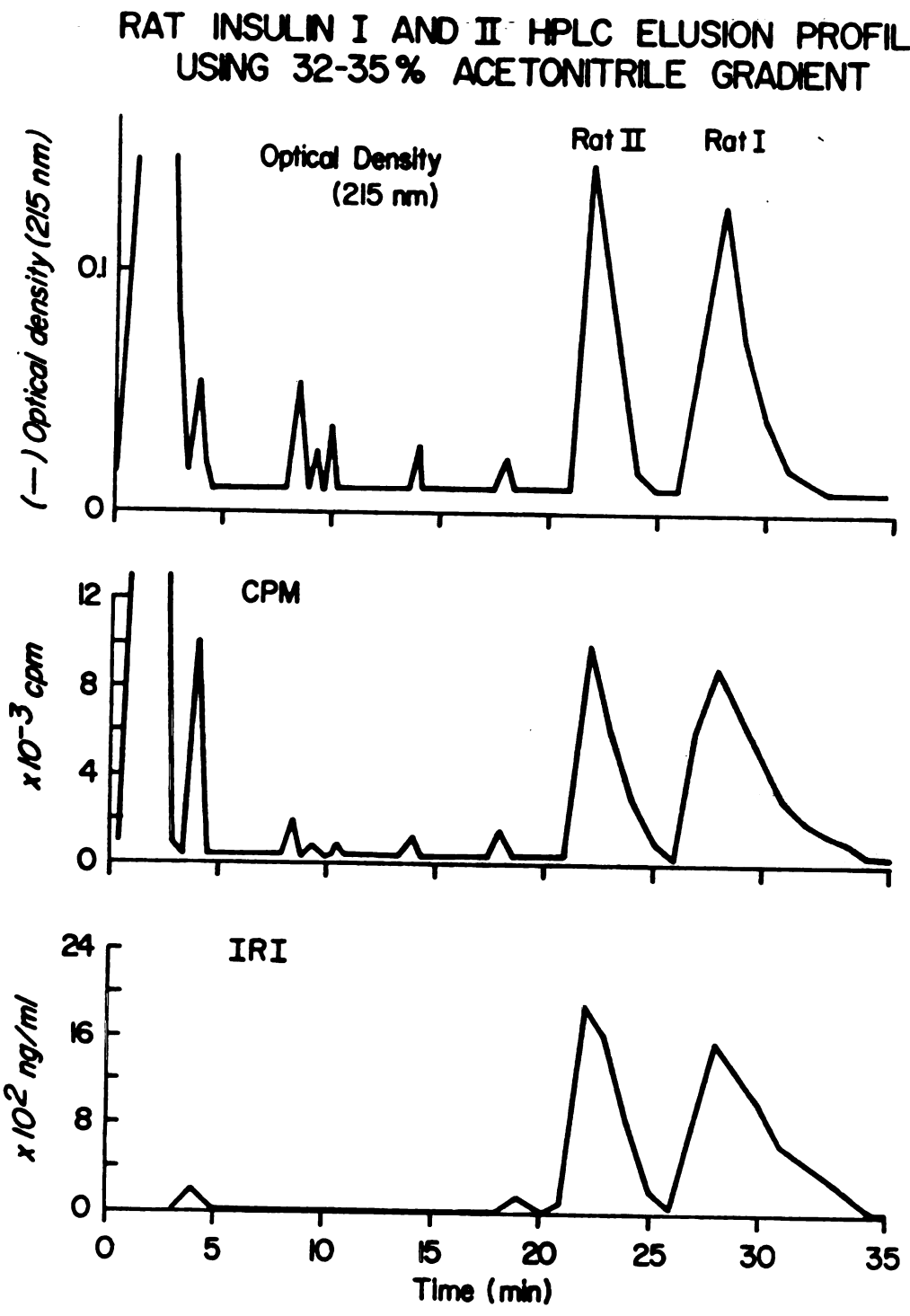


FIGURE 39 Schematic representation of rat insulin I and II separation in pulse-chased islets using reverse-phase HPLC with a 32-35% acetonitrile gradient over 35 min. The top panel depicts the optical density elution recording at a wavelength of 215 nm. The middle panel represents the radioactivity elution profile and the bottom panel is the corresponding IRI measurements for each fraction. Under these conditions rat insulin II elutes first at 21 to 25 minutes followed by a broader rat insulin I peak at 27 to 34 minutes.

TABLE 1

Average time of 20 min secreted sample (min)	% cpm eluting in PI peak (% cpm PI/ cpm PI+I) mean ± SEM
30	71.3 + 7.5
68	62.6 + 6.2
111	32.3 + 4.2
162	15.6 + 2.1

Effect of time on the percentage of radioactive proinsulin in secreted, newly synthesized hormone. The radioactivity eluting from columns of Biogel P-30 in the insulin peak was multiplied by 11/6 to correct for the difference in leucine content between insulin and proinsulin. Numbers represent the mean ± SE from 8 experiments and are an elaboration of data used for figure .

Table 2

STIMULUS	n	SPECIFIC ACTIVITY secreted insulin	SPECIFIC ACTIVITY RATIO secreted/islet
20 mM glucose	10	15.1± 2.1	3.36± 0.24
	9*	32.9± 5.9	3.55± 0.46
20 mM glucose + 1 mM IBMX	11	16.6± 2.4	3.69± 0.32
20 mM glucose + 25 uM forskolin	6	13.1± 4.1	3.04± 0.51
5 mM glucose	9	16.0± 1.8	2.60± 0.33
5 mM glucose + 100 ug/ml tolb.	8	18.6± 1.7	3.25± 0.42
2 mM glucose + 50 mM K^+	9*	28.7± 4.4	3.47± 0.44

Table 3

	2 mM glucose 225 min	2 mM glucose 180 min 20 mM glucose 45 min	20 mM glucose 225 min
ecific Activity (cpm/ng IRI)	secreted 9.6± 1.3	31.8± 4.2	180.0± 23.8
	islet 3.1± 0.7	12.0± 2.1	19.7± 2.6
ratio creted/islet	3.1± 0.3	2.65± 0.4	9.1± 0.9
creted insulin g IRI/20 min)	0.96± 0.12	1.00± 0.3	0.48± 0.08
IRI secreted per 20 min	2.58± 0.42	2.68± 0.6	1.71± 0.6
ial islet sulin content ng IRI/islet)	36.3± 5.3	36.2± 4.6	26.6± 4.1

TABLE 4

PROINSULIN BIOSYNTHESIS AND CONVERSION TO INSULIN IN TUMOR CELLS VS CONTINUOUSLY GLUCOSE-STIMULATED ISLETS

MEASUREMENT	TUMOR CELLS	ISLET CELLS
% labeled, cellular proinsulin remaining intact at 152 min	(16) 19.6 ± 4.9	(8) 17.4 ± 1.91
% labeled proinsulin secreted intact in windows		
Window A (90 - 110 min)	(16) 39.1 ± 5.7[a]	(19) 28.0 ± 3.3
Window B (111 - 131 min)	(16) 31.8 ± 5.5[a]	(10) 15.9 ± 2.0[b]
Window C (132 - 152 min)	(16) 22.2 ± 4.3[a]	(10) 15.1 ± 0.68[b]
Specific activity of cellular insulin at 153 min (cpm/ng I.R.I.)	(16) 34.3 ± 7.9	11.0[c]

[a]Percentage of labeled proinsulin secreted from tumor cells in windows A vs B vs C were significantly different ($P < .001$) from each other by paired analysis.

[b]Percentage of labeled proinsulin secreted from continuously glucose-stimulated islets was significantly different ($P < .02$) in window A vs B or window C by unpaired analysis.

[c]Specific activity of insulin in continuously glucose-stimulated islets at min 152 (taken from reference 9)