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THE ROLE OF ZINC IN RAT BETA CELL FUNCTION

Dianne P. Figlewicz

This dissertation is dedicated to
the loving memory of Raymond C. Figlewicz.

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ABSTRACT

A definitive role for zinc in the synthesis, storage, or release of insulin from the pancreatic β cell has been suggested but not established. Early histochemical studies have demonstrated a high concentration of zinc in the islets of Langerhans, almost exclusively localized to the granules, and this zinc content has been roughly correlated with the insulin content of the islet. In order to elucidate the role of zinc in insulin synthesis or storage, we have examined zinc uptake kinetics, zinc content, and zinc localization in a granule-enriched fraction prepared from islets of Langerhans cultured under different conditions.

An initial study assessed uptake of ^{65}Zn by islets cultured 24 hr in basal glucose. Net uptake was linear for the first eight hours, then continued at a slower rate, not reaching equilibrium by 24 hr. (This slow uptake is in sharp contrast to islet uptake of Ca^{2+}, another ion known to be important in insulin storage and secretion: using similar techniques in our laboratory, ^{45}Ca uptake plateaued at 2--4 hr.) Total zinc content in non-cultured islets, as measured by atomic absorption spectrophotometry was 11.1 pmol/islet; islet insulin content was 13.1 pmol/islet. If insulin in the islet were to be stored as a two zinc insulin hexamer

(the unit crystal), 4.4 pmol of zinc would be required. Thus islet zinc content is about 2.5 times that needed to account for insulin complexes alone.

A granule-enriched fraction of islets was prepared to assess localization to the granules of endogenous zinc, and ^{65}zinc taken up by the islet. In contrast to previous histochemical results, only about 15% of the ^{65}Zn taken up, and 25% of the islet endogenous zinc, was found in a granule fraction which contained approximately 40% of total islet insulin. Thus most islet zinc is extragranular. The specific activity of ^{65}Zn/Zn in the granule-enriched fraction was lower than that found in the soluble fractions; thus ^{65}Zn did not equilibrate with endogenous granular zinc over a 24 hr period. This slow equilibration suggests that zinc enters the granules during their de novo synthesis and does not exchange significantly with the zinc in already formed granules. To examine the possible dissociation of ^{65}Zn from insulin in our in vitro systems, ^{65}Zn--insulin complexes were characterized chemically. Simulation of both in vitro culture conditions, as well as the conditions under which islet fractionation was carried out, revealed a strong affinity of ^{65}Zn for insulin, with no significant dissociation of ^{65}Zn--insulin for the duration of the experimental time course. Culture of islets in high glucose medium (to

enhance insulin synthesis and secretion); high glucose but no added Ca^{2+} (to inhibit secretion while stimulating synthesis); or following *in vivo* treatment of rats with tolbutamide (to deplete islet granules) revealed, in general, an enhanced loss of islet zinc as compared to islet insulin. Fractionation of the islets showed that there was sufficient zinc associated with the granule fraction to account for two zinc hexamers, under all culture conditions used. Further, no change in zinc or insulin distributions was observed under the different culture conditions, however, culturing seemed to destabilize β granules, as the amount of insulin found in the granule fractions decreased as compared to insulin in the fractions prepared from fresh islets.

The influence of exogenous zinc on insulin secretion was examined in short term experiments in the perfused pancreas, and in islets cultured 8 hr and subsequently challenged with high glucose for insulin release. No influence of zinc at "no added" or 50 µM was observed (physiological zinc is 15 µM). A small but significant inhibition of insulin secretion was observed when 200 µM zinc was added both to the culture medium, and to the incubation medium in which islets were stimulated with high glucose.

In conclusion, although chemical analyses suggest a strong affinity of zinc for insulin, only small

quantities are found associated with granule-enriched islet fractions, and no significant influence of exogenous zinc (or lack thereof) on insulin secretion was observed. The enhanced loss of islet zinc as compared to insulin under different culture conditions suggests that zinc may serve an additional role in islet metabolic processes besides crystallization with the insulin molecule itself, and that islet zinc fluxes may not be solely related to the phenomenon of insulin secretion.

INTRODUCTION

Zinc is a trace metal which is ubiquitously distributed throughout plant and animal tissues. Its role as an essential nutrient in humans was established in the early 1960's, at which time a zinc-deficiency syndrome in men was first identified and characterized. Zinc has several critical roles in the body. However, they cannot be linked via one common mechanism of action at this date; most investigators suggest that it acts either as a coenzyme or chelator. Zinc is necessary for the preservation of the integrity of epithelial surfaces, i.e., the skin and hair; it participates in the maintenance of normal immune function; it is needed for normal function of the senses of taste and smell; it is vital for growth and appears to facilitate wound healing; and it is required for sexual maturation and function in males (1).

These actions of zinc appear to be unrelated; a possible requirement of zinc in integrative functions of the body has not been explored. One recent study (2) implicates zinc in the central nervous system: stimulation of hippocampal mossy fibers in zinc-deficient rats resulted in synaptic responses which declined in amplitude with successive stimuli. (It was suggested that zinc may be a requisite coenzyme for transmitter synthesis.) Further, a definitive role for

zinc in the endocrine system has not been investigated extensively: neither zinc content nor its requirement for normal function of the various endocrine glands have been established. Its involvement in male reproduction has been studied; it appears to be required for both normal testosterone synthesis and/or secretion (not yet clarified) (3), as well as conversion of testosterone to the dihydro- derivative at a normal rate by target organs, most notably, the prostate (4). (This manifests itself at the clinical level as maintenance of normal potency and libido.) Isolated studies of the hypothalamic-pituitary axis have shown no major perturbation of the axis by zinc deficiency (3); decreases in TRH (5), TSH, and GH, with no change in prolactin levels have been reported (3).

In contrast to the other endocrines, a possible role for zinc in the endocrine pancreas has been investigated more extensively. However, the role of zinc in insulin synthesis, storage, and secretion has not been clarified. Many studies have examined zinc--insulin molecular interactions; the metabolic effects of zinc, or its absence, *in vivo*; and the histochemical localization of zinc in the endocrine pancreas. This has resulted in the amassing of a volume of indirect, and sometimes artifactual, evidence which suggests that β cell zinc is confined to the secretory granules, and

plays an important role in insulin synthesis and storage. However, systematic examination of zinc content and subcellular distribution in islets has not been undertaken. In the studies described here, these issues have been addressed more directly. Specifically, the following questions have been asked:

1. How much zinc is in the rat pancreatic islet?

2. How much zinc (^{65}zinc) does the islet take up, under basal conditions?

3. How much of ^{65}zinc and endogenous zinc is associated with an insulin secretory granule-enriched fraction? How much zinc is extragranular?

4. Are uptake, content, or distribution of islet zinc altered under conditions which alter insulin synthesis or secretion?

LITERATURE SUMMARY

The intracellular pathway of insulin as it is synthesized, stored, and secreted is believed to be similar to the scheme which has been characterized for zymogens in the exocrine pancreas (6). Insulin is synthesized at the ribosomes, by mRNA translation, in the form of a pre-proinsulin (7). The pre- portion of the molecule differentiates it from molecules which are not inserted into the endoplasmic reticulum for further processing, and ultimate packaging. Once inserted into the rough er, the pre- portion of the molecule is removed (8), and resultant proinsulin molecules are transferred via microvesicles to the Golgi apparatus. In this organelle, conversion of proinsulin to the final insulin molecule begins, with cleavage of an external portion of the proinsulin molecule, the 'C-peptide.' The insulin molecule thus has the same 3-dimensional configuration as the proinsulin molecule, minus the C-peptide, which is located exteriorly to the A and B chains. C-peptide and insulin are "packaged" in the Golgi in equimolar amounts (9) and are released from the Golgi as immature granules. It is believed that proinsulin, and perhaps the converting enzyme, are also contained in immature granules. "Maturation" of the beta granule involves both further conversion of proinsulin to insulin, as well as a condensation of

insulin molecules within the granular sac, resulting in a characteristic appearance of a granule with a dense core surrounded by a clear space, or halo (10). Upon exposure of the beta cell to a secretagogue, granules migrate to the region of the cell membrane, a process which is Ca^{+2}-dependent (11) and involves participation of microtubules and/or microfilaments (12), and fuse with the membrane. The granular contents are expelled into the interstitial spaces, and diffuse to the capillaries for *in vivo* circulation (13).

Evidence from several lines of investigation suggests that islet zinc may have an important role in the synthesis, storage or secretion of insulin. Chemical studies have shown a strong affinity of zinc for both the insulin and proinsulin molecules. Histochemical studies have localized islet zinc to the β-granules. *In vivo* manipulation of the total body zinc content has been shown to effect carbohydrate tolerance, with a putative action at the level of the islets. A correlation between the insulin secretory activity of the islet, and its zinc content, have been demonstrated. Finally, a unique physiologic role for a zinc-insulin moiety in circulation has been postulated. Put together, these findings seem to argue strongly for a role of zinc in the islet. However, closer inspection of the data reveals certain methodological artifacts;

thus, interpretation of those results must be limited. The critical studies which implicate a role for zinc in the islet will be summarized below.

Equilibrium dialysis experiments have revealed that the insulin molecule has multiple classes of binding sites with different affinities for zinc (14). In fact, both insulin and proinsulin interact with zinc. Such zinc binding is strongly pH dependent, increasing with an increase of pH between 6.0 and 8.0 (15,16). Between pH 6 and 7, only strong binding of zinc to the imidazole groups of the B-10 histidines occurs, resulting in the formation of a "unit cell" of two zincs per insulin hexamer (an aggregate of six insulin molecules, showing symmetry around both a 2-fold and 3-fold axis) (17). Above pH 7, weaker sites in the insulin and proinsulin hexamers begin to bind zinc (18). Hence, at a physiologic pH of 7.2--7.4, both molecules should be able to bind zinc extensively. The nature of this binding was studied by Grant, Coombs, and Frank (19). Equilibrium dialysis experiments showed that, whereas addition of zinc in excess of that needed to form a 2-zinc hexamer caused precipitation of insulin, proinsulin remained in solution as large polymers, with aggregates of as many as 10 or 11 proinsulin molecules. Each proinsulin molecule showed a capacity to bind as many as 30 zinc molecules. Frank and Veros (20) sug-

gest that the proinsulin-high zinc polymer may have a unique physiologic role: as the proinsulin molecule is converted to insulin, the presence of excess zinc molecules beyond the two per hexamer causes the insulin to aggregate and precipitate, preventing further degradation of the insulin molecules, and shifting the reaction equilibrium towards further conversion of proinsulin to insulin. This speculation is substantiated by the evidence that insulin containing small quantities of zinc is very resistant to digestion by trypsin and carboxypeptidase (21); an enzyme with tryptic and carboxypeptidase-like activities is responsible for the conversion of proinsulin to insulin. Further, the zinc-insulin hexamer is protected before it precipitates, as the trypsin-sensitive bond in insulin is shielded in the three dimensional configuration.

Such a teleologic construct is purely speculative. Neither the pH nor the zinc concentration in the rough endoplasmic reticulum or Golgi apparatus are known, and, in fact, the pH of the insulin secretory granule is acid (22), which would argue against binding of zinc to insulin in excess of two-zinc hexamers (16). Although early crystallization studies by Schlichtkrull showed that a minimum of two zincs per hexamer were necessary for the formation of rhombohedral crystals (23), insulin has been crystallized in the absence of

zinc (24,25), and can aggregate in zinc-free media, at pH 7, forming a heterogenous group of polymers, ranging from dimers to hexamers. Such self-associative behavior in the absence of zinc is very dependent on the local concentration of insulin (16). As will be discussed below, the zinc content of granule-enclosed insulin has not been measured; furthermore, changes in ionic composition of the surrounding medium can permit changes in the zinc content of the insulin crystal (e.g., from 2-Zn to 4-Zn hexamers) (26).

The fate of the putative zinc-insulin hexamer upon secretion is also purely speculative at this time. As stressed by Frank et al. (18), prevailing zinc and insulin concentrations in the serum would suggest that insulin exists as a zinc-free monomer, dissociating from the hexamer form rapidly upon secretion. However, as summarized by Blundell (16), zinc-insulin interactions are strongly dependent on the composition of the surrounding medium and, as serum is a very complex solution, it is impossible to predict the exact structure of the insulin molecule in solution. Provided such a complex may survive the brief passage from pancreas to liver, Arquilla et al. (27) provide evidence for its unique physiologic activity: both _in vivo_ and _in vitro_ they were able to demonstrate enhanced membrane binding, and inhibited degradation of a soluble

zinc-insulin complex by the liver.

Insulin *in vivo* has been found as a precipitate in the β-cell secretory granules. However, a crystalloid precipitate is neither the only type of precipitate nor the most frequently occurring. In fact, very few high resolution studies of insulin granule substructure have been carried out. Sato et al. (28) examined β-cell granules from several species. As might be expected, granules from the only species examined whose insulin is known to be precipitated in the absence of zinc, and whose islets appear to be zinc-free--the guinea pig--were unique in structure. Insulin was not condensed as a definitive 'core' but occurred in sparse deposits throughout the granule in addition to a major precipitate. Rat and cat β granular cores were generally spherical and of homogeneous density. Rectangular or bar-shaped precipitates in the dog and pig appeared crystalline, with a subunit periodicity of approximately 15 angstroms. Additional fibrillar material, between the crystalline core and the granule membrane, was frequently visualized. In the dog, a second type of smaller granule, containing a densely packed mass, was often observed. In the turkey and chicken, most granules contained needle- or bar-shaped crystalloids, occasionally enmeshed in fibrils (as were observed in the dog). Finally, representative amphibians--the

tiger salamander and Congo eel--had β granules with crystalline cores, in the shape of rectangles or parallelograms. Human islets from pancreatic autopsy samples were studied by Like (29). The β-cell granules had rectangular or hexagonal cores, and an inner repeating substructure was visible in some samples, but was not analyzed quantitatively. Like speculates that in amorphous or "non-crystalline" granules insulin may be in a different chemical or physical form. However, Greider et al. (30) suggest that, additionally, a periodicity may not be observed in some granules due to the orientation in the electron beam. Their high resolution examination of an isolated β-granule fraction from rat islets revealed a 50 angstrom periodicity within the granular cores-- in discord with the 15 angstrom periodicity observed by Sato. Such a spacing would be consistent with the packing of zinc-insulin hexamers in a rhombohedral or cubic cell (31). The cores had a heterogeneity of shapes, with no true spheres or polygons. A 50--60 angstrom periodicity was also observed in chick beta granules (32), whose cores showed a heterogeneity of shape *in vivo*. Although Howell (33) concludes that "the overall packing and morphology of the granule appear to be consistent with the presence of rhombohedral crystals or of the similar rhombic dodecahedral structure," the variation in the

few species of granules which have been studied cannot be ignored. It seems safe to conclude that insulin is precipitated within its storage granules. However the nature of the precipitate has yet to be determined: biogenic amines, present in the granules (34,35,36), and other proteins, or enzymes, (putatively present) may affect the milieu of the insulin molecule or may interact directly with insulin. No existing evidence allows one to conclude that insulin is present as a hexamer, either 0-zinc, 2-zinc, or 4-zinc.

Accumulation and turnover of islet zinc are relatively slow processes, in contrast with rapid zinc handling by most other tissues. As a result, high concentrations of zinc are found in the islets, when compared with the zinc concentration of other tissues. About 3% of a total dose of ^{65}Zn was found in normal rat pancreas 24 hr after administration; this amount dropped to 0.3% in pancreases from alloxan diabetic rats (37). In ^{65}Zn studies contemporary with our investigations, Ludvigsen et al. (38) found a very slow accumulation of ^{65}zinc by the islets; the uptake mechanism at low (and physiologic) zinc levels was characterized as carrier-mediated transport by several criteria. In an autoradiographic study following iv administration of ^{65}Zn, McIsaac (39) observed higher accumulation and longer retention of ^{65}Zn by pancreatic islets as compared to

the surrounding pancreatic acini, as well as all other organs examined. Whereas initially the specific activity of the islets was lower than the acini (7 hr), by 92 hr acinar radioactivity was essentially background whereas the islets still retained much of the ^{65}Zn taken up, with the highest specific activity of any organ examined. Dencker and Tjalve (40) observed ^{65}Zn in whole body X-ray sections of mice, rabbits, and guinea pigs. Islets from mice and rabbits were still accumulating ^{65}Zn at 48 hr and the radioactivity was frequently still detectable at 30 and 60 days. Unfortunately, limitations in methodology did not allow differentiation of the ^{65}Zn in α vs. β cells. Guinea pig islets did not accumulate zinc.

This slow turnover of islet zinc argues for the presence of a large and not very mobile pool in the islets: if islet zinc were localized solely in secretory granules, one would anticipate a more rapid turnover, as the granules represent about 10% of the β cell content, and are constantly being synthesized and secreted, at least at basal rates, under normal circumstances. Further, one might anticipate a more rapid turnover in islets exposed to insulin secretagogues, as the insulin-associated zinc would be secreted. However, Ludvigsen et al. found no differences in either acute (1--70 min) or more long term (24 hr) uptake of

zinc upon high glucose incubation of the islets.

Histochemical studies of the islets have revealed the presence of zinc; its possible localization to the secretory granules; and its correlation with the metabolic state of the islets. In initial studies by Okamoto (41), an *in vivo* dithizide staining technique was used to demonstrate the presence of zinc in the islets, and it was further noted that stainable material increased or decreased in rabbits following diets designed to increase or decrease the need for insulin. In a series of studies from Kadota et al. (42,43,44), the possibility that the diabetogenic action of certain organic compounds was due to their zinc-binding capacity was examined. Dithizone, a histochemical stain and zinc chelator, was shown to have a specific β-cytotoxic action. Histologic examination of the islets revealed normal α-cells. As α-cells as well as β-cells are known to contain zinc (vide infra), it seems surprising that a specific action on the β cells was observed if the mechanism indeed is zinc chelation. Further, Kadota and Midorikawa (43) note that dithizone is capable of chelating other heavy metals, e.g., nickel, cobalt, and cadmium. Data from these studies suggest that "dithizone diabetes" may be due to the zinc-chelating behavior of the molecule, but are by no means conclusive. Lazaris and Meiramov (45) injected

dithizone into rabbits and observed that after a short period of time, the islets from a tissue resection were no longer filled with colored granules. A subsequent injection of dithizone was given and the colored granules reappeared. These results suggest that dithizone does not permanently complex with islet zinc, and, from a technological viewpoint, that tissue from an animal treated <u>in vivo</u> with dithizone needs to be removed and examined promptly.

McNary (46) observed that much islet zinc is readily extractable, and diffuses upon lengthening of the time of islet fixation in alcohol or other water-soluble fixatives (Kadota's method); thus a great variance in the amount of stained material occurred in blocks prepared from the same animal. A positive reaction--purple "granules" on a red or pink background--was observed in unfixed (frozen or freeze-dried) tissue sections and was localized to the islets, with no striking reaction in the pancreatic acinar tissue. (This was in contrast with the original Okamoto findings of a strong reaction--extensive "stained granules" in the acini.) Staining was also observed in islets from dogs, rhesus monkeys, humans, rats, and mice. No staining was observed in guinea pig islets. The ability of dithizone to stain "zinc--insulin complexes" themselves was tested by staining a gel (5%

gelatin) containing zinc--insulin. Whereas the gel remained uncolored, a red-purple color was observed in gels containing zinc insulin. Microscopic examination of this stained material revealed that the stain was distributed as "very small granules."

Kawanishi (47) used *in vivo* dithizone staining for localization of intracellular zinc, and characterized the time course of dithizone β-cytotoxicity. As toxic changes were seen as early as 15 min post-injection, tissue samples for localization of the stain were taken at 10 min. The stain was found mainly on granule membranes, and the intragranular "halo" space, with little localization on granular insulin cores, or other organelles. Further, the amount of zinc-staining was shown to gradually increase during the course of a prolonged fast (although cell ultrastructure demonstrated that insulin synthesis and packaging into granules was severely curtailed). In a similar study by Logothetopoulos et al. (48), dithizone staining in fresh-frozen islets was examined following chronic insulin treatment of rats. Islets were stained with aldehyde-fuchsin in order to localize β-granules; staining was faint to nondistinguishable in islets from insulin-treated rats. Alpha cells from insulin treated rats were filled with dithizonate precipitate granules as described above whereas beta cells showed no precipitate; when β-cells

were regranulated, zinc staining reappeared. However, it was observed that the dithizone-stained "granules" did not coincide with insulin-containing, aldehyde-fuchsin stained granules. Rather, precipitation of the dithizone *in situ* appeared to take the form of "granules" (as was observed also by McNary) whose substructure could not be resolved by the microscopic techniques used. In dithizone stained rabbit islets, Maske (49) also observed that dithizone granules were not identical with insulin granules, and that changes in zinc staining could be dissociated in time from changes in insulin staining.

A second major histochemical technique used to localize zinc *in vitro* is the sulfide-silver staining technique, modified for use in the islets by Pihl (50). A comprehensive study examined islet intracellular zinc localization by this technique in a number of species: man; rabbit; Chinese hamster; mouse; rat; guinea pig; coypu; cat; dog; and reindeer. With the exception of the guinea pig, the coypu, and the reindeer, whose islets showed trace zinc at best in the α and β cells, a heavy staining was observed in the β-cells with somewhat lesser staining in the α cells. Silver grains appeared localized almost exclusively to secretory granules, with some lysosomal deposits as well. Okamoto and Kawanishi (51) applied the sulfide-silver

technique and several modifications, to rabbit islets, and found silver grains predominantly over granules, with some grains in the endoplasmic reticulum. Coore et al. (52) demonstrated heavy metal content in an isolated granule fraction.

As initial studies with alcohol fixation prior to sulfide-silver staining led to granular dissolution (and, presumably, washout of soluble zinc), Falkmer and Pihl (53) used a modified fixation-staining procedure involving buffered glutaraldehyde. Even this technique resulted in a selective autolysis of β-granules in islets from species whose insulins are known to contain zinc. Other β- and all α-cell organelles remained intact. Thus the sulfide silver technique in conjunction with fixation cannot be legitimately used in any quantative study which would attempt to assess correlative changes of zinc and insulin contents. In fact, Bander and Schesmer (54) treated rats with either the sulfonylureas glibenclamide or tolbutamide, or with alloxan. A decrease in histochemically detectable zinc was observed following an oral sulfonylurea dose, with identical variations in aldehyde-fuchsin stained β granules. No change was observed in the α cells. However, a dissociation in the decrease of insulin granules, as compared to zinc "granules" was observed in islets from alloxanized rats: zinc-staining

material disappeared before the insulin granules (as was reported by Maske with dithizone staining). The degree of ultrastructural preservation in this study was not reported.

Rat islet zinc content was studied by Yoshinaga and Ogawa (55) following administration of chlorpropamide for acute insulin release. Zinc was found only in β-granule cores of control islets; however, problems in fixation led to severely damaged ultrastructure such that only β-granules remained intact. Following administration of chlorpropamide, silver grains were localized to the cell membranes and intercellular spaces, interpreted as co-secretion of zinc with the insulin. Finally, zinc content in β granules of genetically diabetic yellow KK mice was studied by Shino and Iwatsuka (56). Zinc was localized only in dense ("mature") secretory granules, and not in the large pale granules, without a definitive core, which predominate in the fed animal.

In conclusion, early histochemical studies seem to localize zinc to islet β-granules (and to α-granules, to some extent). However, problems with a potential loss of soluble zinc during fixation, and poor ultrastructural preservation in most of the studies make it difficult to state definitively that islet zinc is solely localized to the granules. Hence, development

of more rigorous approaches to the issue was necessary.

With the advent of electron-probe microanalysis and atomic absorption spectrophotometry, more accurate quantitations of islet zinc--and its intracellular localization--have become feasible. Havu et al. confirmed early histochemical studies which claimed that the guinea pig (57) and the hagfish (58) islets contain low amounts of zinc; rat islet zinc was found to be approximately 22 pmol/islet, based on atomic absorption spectrophotometry. In an early study by Maske (49), crude subfractions from flounder islets were prepared; stained with dithizone; and the zinc and insulin contents of the various fractions were compared. The highest insulin containing fraction (significantly contaminated with mitochondria) contained 47% of total insulin and 24% of the zinc. Notably, the majority of islet zinc was not localized with the insulin. In a recent preliminary study, Andersson et al. (59) measured zinc by flameless atomic absorption spectrophotometry in islet subfractions. Their results confirmed the findings presented in this dissertation: the majority of islet zinc was not localized to secretory granules. Further, calculating from their data, the zinc localized with the granules was marginally sufficient for formation of the 2-Zn hexamer. Total islet insulin and zinc were found to be approximately equimo-

lar in ob/ob (genetically obese) mice (60); db/db (genetically diabetic) mice whose islet insulin content was virtually depleted stil retained approximately 50% of the zinc found in a normal mouse islet. As with the two studies described above, this supports the hypothesis that islet zinc is not confined to the secretory granules (and is present in a quantity greater than that needed to form a 2-Zn hexamer).

In vivo and *in vitro*, physiologic and pharmacologic manipulations of the islet's environment have been carried out in an effort to determine if zinc plays a significant role in insulin synthesis, storage, or secretion. In early studies by Maske, rabbit islet zinc was found to decrease following administration of the insulin secretagogues, glucose or adrenalin. 24--48 hr after fasting, more zinc was histochemically demonstrable. As cited by Quarterman and Florence (61), the rat pancreas shows a greater decrease in zinc content than other organs in the zinc-deficient animal. Quarterman et al. (62) demonstrated a reduced tolerance to ip glucose; decreased plasma insulin; and decreased sensitivity to exogenously administered insulin in zinc-deficient rats as compared to their pair-fed controls. Huber and Gershoff (63) reported a significant decrease in serum insulin in zinc-deficient rats as compared with *ad lib* fed controls (but not as compared

to pair-fed controls). Glucose-stimulated insulin release from the zinc-deficient rats' incubated pancreas pieces was significantly reduced, but basal release was not affected. Falkmer et al. (64) observed impaired ip and iv glucose tolerances in zinc deficient Chinese hamsters. No overt diabetes or glucosuria were found in the animals. Histochemical staining for insulin revealed a decreased granulation, but other β-cell organelles, as well as α_1 and α_2 cells appeared normal. Boquist and Lernmark (65) speculate that the decrease in granules observed in the zinc-deficient hamster β-cells may be responsible for impairment of glucose tolerance, however, sufficient insulin is still present to prevent a full-blown diabetic state from developing. Wolters et al. (66) used dithizone staining in order to observe changes in islet zinc following either a 72 hr fast, or tolbutamide administered by gavage (to acutely deplete islet insulin). 72 hours of fasting resulted in a slight decrease of (aldehyde-fuchsin stained) islet insulin; zinc staining in the islets gave such variable results that no significant effect could be observed. Tolbutamide treatment dramatically depleted both stainable insulin and zinc contents in the islets. Wolters et al. emphasize that dithizone staining does not represent total islet zinc, as either completely ionized, or very tightly bound (e.g., to an ion

exchange resin) zinc will not react with the dithizone. In gelatin films, they observed that dithizone would stain zinc only if bound within the matrix of the gelatin, i.e., to albumin or insulin; diffusable zinc would not stain. Howell et al. (67) depleted zinc in the culture medium of isolated islets, either electrochemically, or with a chelating agent. Depletion up to 9 days did not adversely affect proinsulin biosynthesis, or insulin secretion. Possibly, conversion of proinsulin to insulin was somewhat retarded, as was visualized by a predominance of large, pale, "uncondensed" secretory granules, in electron micrographs. They conclude that the only significance of zinc in the islets is in forming condensed, well-packed secretory granules.

In contrast to the findings discussed above which suggest that extracellular zinc maintains normal insulin secretion, Ghafgazi et al. (68) exposed islets to pharmacologic levels of zinc and were able to demonstrate a significant inhibition of insulin secretion in response to several secretagogues. This inhibition was coupled, at the highest levels of zinc utilized, with a significant depression of islet glucose metabolism. A competition with Ca^{2+} ions, either for entry into the cell, or in intracellular translocation, is postulated to account for the inhibition of insulin secretion.

Thus, pharmacologic and pathophysiologic manipulations of the zinc environment of the islet suggest that, indeed, islet zinc is important for maintenance of normal insulin secretion. In addition to its putative role in the efficient storage of proinsulin and its conversion to insulin, zinc may be localized in other cell organelles, serving other functions. A few speculative functions--as studied in other systems--are described here. 1. Intracellular zinc affects plasma membrane--cytoskeleton interactions. Maro and Bornens (69) observed a significant inhibition of B lymphocyte immunoglobulin "capping" upon addition of low pharmacologic levels (50 µM) of zinc to the experiment. Zinc is known to stabilize microtubules. 2. Zinc can inhibit the mitochondrial electron transport system, between cytochromes b and c_1, even at physiologic (plasma: 15 µM) levels (70). Such an effect may help to explain the Ghafgazi finding of high extracellular zinc inhibiting islet glucose oxidation. 3. Zinc stabilizes plasma and lysosomal membranes (70). This effect is postulated to be due to reaction of zinc with SH groups (in membrane proteins), and subsequent formation of stable mercaptides. This reactive ability of zinc may have further consequences in the islets, as the ratio of reduced to oxidized glutathione within the beta cell is thought to play a role in stimulus-

secretion coupling. 4. Zinc can inhibit RNA polymerase I *in vitro*; thus zinc can inhibit initiation of RNA synthesis, without affecting RNA chain elongation (71). Further, zinc is required for DNA synthesis, RNA elongation and stabilization, and nuclear division (72). Zinc is a critical ion for maintenance of a normal cell cycle. 5. Zinc inhibits both basal, and calmodulin-stimulated, Ca^{2+}-ATPase activity (73). Whether zinc interacts solely with the ATPase, or if it can specifically interact with calmodulin has not been determined. 6. (Cytosolic) zinc can stabilize secretory granules if present at a sufficiently high concentration (52,74).

Clearly zinc may have a significant role in many cellular activities, either for secretion or for "basic maintenance." In the studies described below, we conclude that most islet zinc is extragranular, and that its quantity and distribution do not always correlate with insulin synthesis and secretion. As the β-cell secreting insulin has stimulated metabolism, Ca^{2+} fluxes, and protein synthesis, it is important to consider an alternative role for the zinc ion in the β cell.

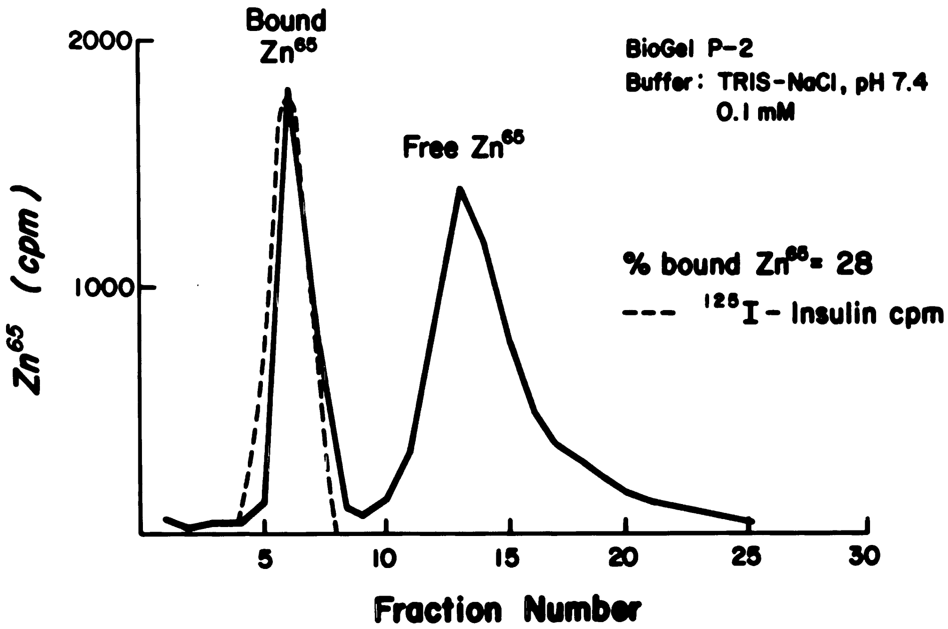
METHODS

Chemical Experiments.

Binding kinetics of zinc to insulin were assessed at 37°C, pH 7.4. Complexes of ^{65}Zn and insulin were formed by addition of .001 mM zinc with added trace ^{65}Zn to various concentrations of zinc-free sodium insulin (courtesy of Eli Lilly and Co., Indianapolis, Ind.) in 125 mM KCl--20 mM Tris, pH 7.4 at 37°C. At 30 sec, 90 sec, 5 min, 120 min, and 24 hr, 100 μl aliquots were removed for assessment of bound and free ^{65}Zn by chromatography on Biogel P2 (Biorad; Richmond, CA). Biogel columns were 20 x 0.7 cm (inner diameter) and were equilibrated and eluted with 0.1 M Tris--0.1 M NaCl, pH 7.4. ^{125}I--insulin was eluted at 5--7 ml (Fig. 1). Bound ^{65}Zn was eluted at 4.5--6.5 ml (i.e., coincident with the ^{125}I--insulin) and free ^{65}Zn was eluted at 12--18 ml (Figure 1).

The effect of dilution on dissociation of a 0.1 mM Zn--1 mM insulin (estimated insulin concentration in islets) complex was assessed after a 1:10 dilution of the complex with Tris-NaCl buffer at 25°C. Immediately prior to dilution, as well as at 90 sec, 5 min, 10 min, and 24 hr, bound and free ^{65}Zn were determined on the Biogel column. Dissociation was also assessed after addition of unlabelled $ZnCl_2$, either at pH 7.4 or pH 6.0, at 4°C. A concentrated $ZnCl_2$ solution was added

FIGURE 1


CHARACTERISTIC SEPARATION OF BOUND AND FREE Zn^{65}

to a 0.1 mM insulin--.001 mM Zn complex, to raise the final total zinc concentration to 0.01 M without significant dilution. Bound and free ^{65}Zn were determined in the complex before, and at 3 hr, 25 hr, and 5 days after addition of zinc. The experiment was repeated with the zinc added in an acid solution to bring the final buffer to pH 6.0, 0.01 mM zinc. Bound and free ^{65}Zn were determined before, and at 30 min, 90 min, 25 hr, and 5 days after addition of zinc.

Islet isolation.

Islets were isolated according to the method of Lacy and Kostianovsky (75) with some modifications. Pancreases from male Long Evans rats, 300--350 grams, were inflated with Hank's balanced salts solution, pH 7.3 (Table 1) via a cannula in the bile duct. The pancreas was excised from the rat, minced, suspended in 4 ml buffer, and digested with collagenase (Sigma, Type V, prepared especially for isolation of pancreatic islets, St. Louis, MO) in two steps. After an initial 10 minute manual digestion, at 65 strokes/min., in a 37°C H_2O bath with 4 mg/ml collagenase, the partially digested pancreas was washed and sedimented in a desk--top centrifuge (International Equipment Co., Needham, Mass.) three times. The washed digest was resuspended in 4 ml buffer, with collagenase at a concentration of

TABLE 1

INCUBATION BUFFERS

	Stock Soln.	"Hank's"		"Krebs Ringer Bicarbonate"		"Perfusate"	
Salt	Conc.	ml Soln.	Final Conc.	ml Soln.	Final Conc.	ml Soln.	Final Conc.
H_2O	---	681.0	---	185.0	---	175.3	---
KCl	11.5 g/L	34.8	5.37 mM	9.0	4.32 mM	9.0	4.38 mM
Ca-gluconate	5.0 % w/v	---	---	6.6	2.30 mM	6.7	2.35 mM
KH_2PO_4	21.1 g/L	2.8	0.44 mM	3.0	1.45 mM	3.0	1.47 mM
$NaHCO_3$	13.0 g/L	27.2	4.21 mM	60.0	28.90 mM	60.0	29.34 mM
NaCl	41.76 g/L	191.6	136.91 mM	50.0	111.20 mM	50.0	112.89 mM
$MgSO_4-7H_2O$	38.2 g/L	---	---	2.5	1.21 mM	2.5	1.22 mM
$CaCl_2$	12.2 g/L	11.6	1.28 mM	---	---	---	---
$Na_2HPO_4-7H_2O$	2.0 g/L	45.0	0.34 mM	---	---	---	---
Other Additions							
Bovine Serum Albumin		200 mg	0.2 % w/v	963.0 mg	0.3 % w/v	---	---
Glucose		400 mg	2.2 mM	58.0 mg	1.0 mM	---	---
Hepes		---	---	1.91 g	25.0 mM	---	---
Human Serum Albumin		---	---	---	---	3.16 g	1.0%
Dextran T-40		---	---	---	---	9.50 g	3.0%
pH		7.3		7.4		7.4	
Final Volume		1000 ml		322.5 ml		316.5 ml	

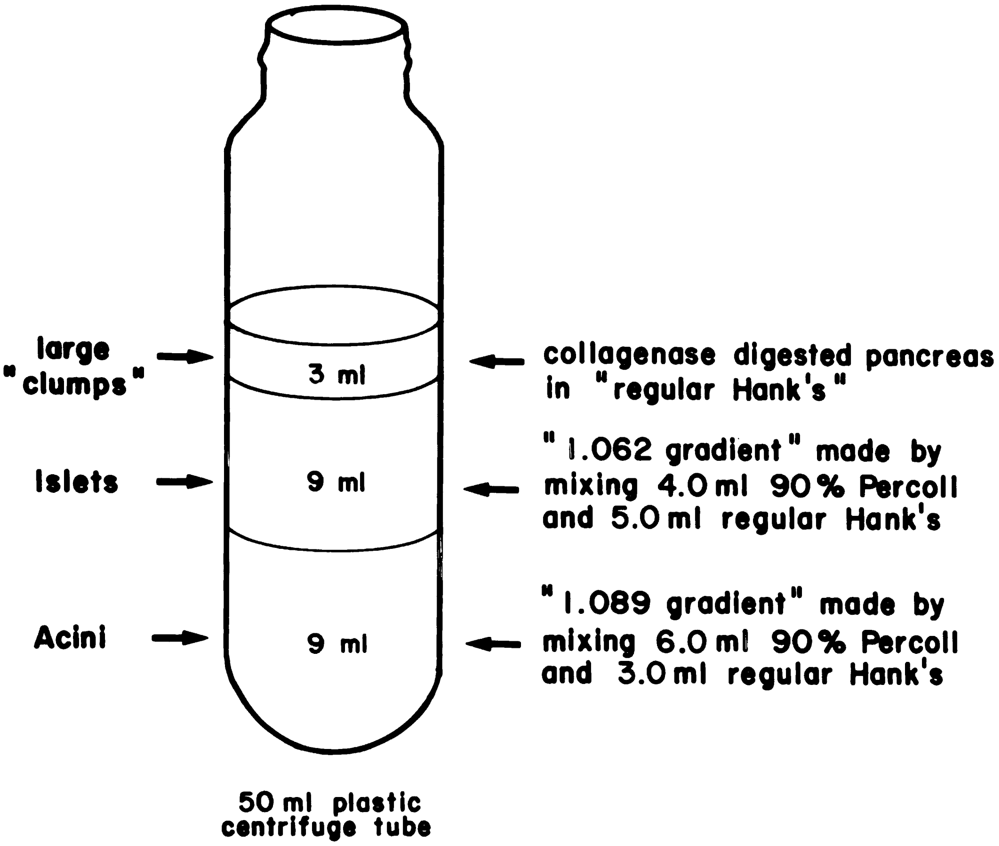
2 mg/ml digested an additional 5--10 min., also at 65 strokes/min.

The final digest was washed twice, resuspended in 4 ml Hanks' buffer and layered over a discontinuous Percoll gradient (Figure 2) (76). The gradient was prepared from a 90% Percoll (Pharmacia) solution (Percoll, 88 ml; 10x concentrated Hanks', Gibco, 10 ml; 7.5% $NaHCO_3$, 0.97 ml; Hepes, 0.238 gm; deionized water, 1.53 ml; pH 7.4). 6 ml 90% Percoll and 3 ml regular Hanks' were mixed in the bottom of a 50 ml plastic centrifuge tube (Sybron/Nalge, Rochester NY); 4 ml 90% Percoll and 5 ml Hanks' were mixed and layered over the bottom solution; and the pancreatic digest was layered over the Percoll gradient. Gradients were prepared at 4°C and centrifuged at 27°C, 800 x g, for 15 min. Islets were localized at the interface of the Percoll layers. The interface was removed with a Pasteur pipet, diluted with Hanks in a 75--ml capacity Petri dish, and islets picked with a mouth pipet.

Islet Culture.

Islets were cultured according to the method of Buitrago et al. (77). Groups of 100 islets were passed through several rinses of sterile-filtered culture medium, and placed in 3 ml of the final medium in 35 x 90 mm plastic disposable culture plates (Falcon, Oxnard, CA). Culture medium was Tissue Culture Medium

FIGURE 2

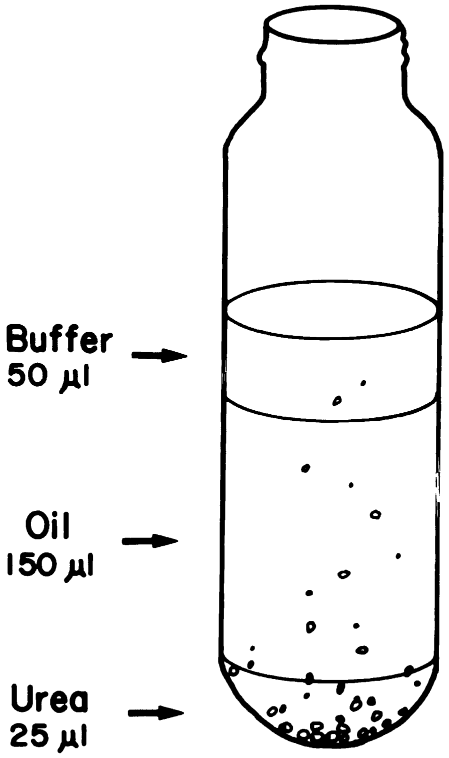

large "clumps"
Islets
Acini
3 ml
9 ml
9 ml
collagenase digested pancreas in "regular Hank's"
"1.062 gradient" made by mixing 4.0 ml 90% Percoll and 5.0 ml regular Hank's
"1.089 gradient" made by mixing 6.0 ml 90% Percoll and 3.0 ml regular Hank's
50 ml plastic centrifuge tube

199 (Gibco, New York) supplemented with 2 µg/ml penicillin-- streptomycin; 1% glutamine; and 10% (v/v) fetal calf serum. When islets were cultured for longer than 24 hr, a 1% (v/v) solution of fungizone (2.5 µg/ml; Amphotericin B, Gibco) was added to the medium to control contamination by molds. This concentration of antibiotic has been shown to have no influence on insulin syn thesis or islet development in the embryonic pancreas (78). In experiments in which insulin secreted during the culture period was measured, the fetal calf serum was replaced with an equivalent volume of heat--inactivated fetal calf serum. Use of this serum resulted in an inhibition of degradation of secreted insulin, thus allowing measurements of medium insulin content to more accurately reflect the true quantity of insulin secreted. Finally, in a separate experimental series, islets were cultured in TCM 199 with the Ca^{++} omitted ("low Ca^{++}" medium), and their controls in regular TCM 199.

Culture Controls.

A. ^3H--Sucrose Uptake. (Figure 3) As described by this laboratory (79) and others (80), ^3H-sucrose distributes throughout the extracellular (interstitial) space of the islet, a space of approximately 3--4 nl, with a "total islet volume" of approximately 8 nl. Damage or necrosis of cells, with subsequent plasma membrane

FIGURE 3



Islet Isotope Uptake

Islets are isolated from buffer by centrifugation through oil.

leakiness, results in an increase in islet sucrose space volume. In order to insure that islets were not becoming significantly necrotic and leaky over the 24 hr culture period, ^{3}H-sucrose space was assessed at 2 and 24 hr of culture. Extracellular fluid was instantly reduced by centrifugation through oil as follows: 250 µl microcentrifuge tubes (Beckman, Fullerton, CA) were layered from the bottom as follows: 25 µl 6 M urea; 150 µl oil (Harwick Versilube F-50), and 50 µl of a modified Krebs--Ringer Bicarbonate buffer (Table 1) containing 4 µCi ^{3}H-sucrose. Groups of ten islets were added to the tubes, at 37°C, in 10 µl of buffer. The tubes were incubated for 10 min (previously determined in this laboratory as the time of equilibration of uptake of ^{3}H-sucrose by the islet--with longer times, endocytosis of the ^{3}H-sucrose occurs at 37°C). The islets were spun through the oil layer into the urea by a 20 second centrifugation on a Beckman Microfuge B. The bottoms of the tubes containing the urea and islets were cut off, placed in liquid scintillation vials with 10 ml Aquasol (New England Nuclear; Boston, Mass.), and counted for ^{3}H radioactivity in a Packard Bicarb Liquid Scintillation Spectrometer. Counts in the pelleted islets were converted to a volume, based on the ^{3}H activity of the incubation buffer.

E.g., 141628 cpm / 10 µl medium = 14.16 cpm / nl

^{3}H cpm in 10 islets = 169 cpm

169 / 14.16 = 11.97 nl/10 islets or 1.20 nl/islet.

B. Responsivity to a glucose challenge. Cultured islets lose their sensitivity to glucose (81,82), and their dose--response curve for insulin secretion shifts to the left under the culture conditions employed here (77). We examined the glucose responsivity of islets in our culture system, and compared them to the glucose response of freshly isolated islets. Islets were removed in 100 µl of culture medium and "washed" in 3 ml of modified KRB, 1 mM glucose. Washed islets were transferred in groups of 10 in 10 µl to 990 µl of the KRB in 1.5 ml Beckman microcentrifuge tubes, and incubated 30 min at 37°C under 95% O_2/5% CO_2. At t=30 min, 900 µl of the buffer was removed without disturbing the islets and 900 µl of KRB with 22 mM glucose was added, bringing the glucose concentration of the medium to 20 mM. Incubation was continued for 30 min, then a second 900 µl sample of the buffer was removed. Buffer samples were frozen and assayed for IRI (immunoreactive insulin) content.

^{65}Zn Net Uptake.

Groups of 10 islets were cultured in 2.5 ml of the medium described above, containing either 5.6 mM or

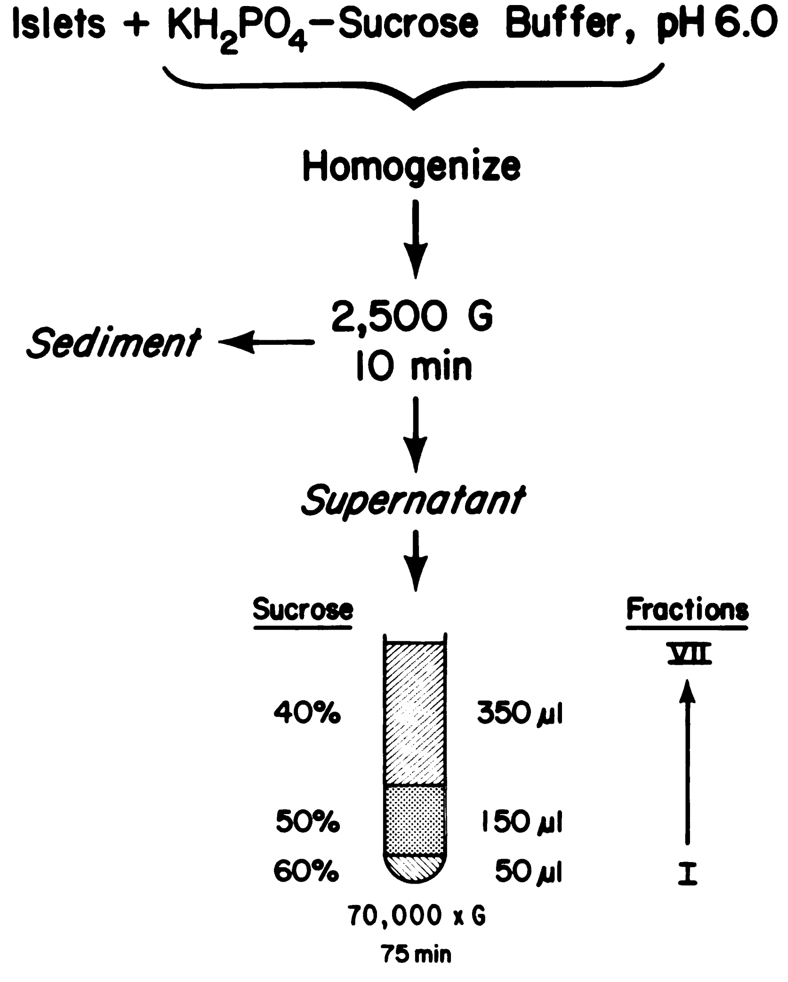
25.6 mM glucose. 10 µl of ^{65}Zn (approximately 1 mCi/ml; 6 µg/ml, New England Nuclear) were added to each plate, for a final total zinc concentration of 1.85 µM. (The zinc concentration of the culture medium with all additions except the ^{65}Zn was 1.5 µM.) At 1, 2, 3, 4, 8, 12, 16, 24, and 26 hours, a group of islets from medium containing 5.6 or 25.6 mM glucose were removed in 10 µl of buffer. The islets were placed in 250 µl microcentrifuge tubes, and handled as described above for determination of ^{3}H-sucrose uptake. The tips of the tubes were cut off and placed in disposable plastic culture tubes (Sarstedt; W. Germany). ^{65}Zn radioactivity was measured in a gamma ray well spectrometer (Amersham, Arlington Heights, Ill.). Uptake data is expressed as percent of the total dose of ^{65}Zn; an aliquot of medium was taken to determine total dose.

Islet Fractionation.

A granule enriched fraction of the islets was prepared as outlined in Figure 4, using the method of Formby and Capito (83,84). The method involves loading of a supernatant, with large particulate matter precipitated, over a discontinuous sucrose gradient (40%; 50%; 60%). A milky band, containing a high yield of insulin secretory granules, can be visualized in the 50% sucrose layer. Islets were pooled in a 2 ml glass Potter Elvehjem tissue grinder (Wheaton Scientific,

FIGURE 4

ISLET FRACTIONATION PROCEDURE



Millville, N.J.) and buffer was drawn off with a fine-tipped Pasteur pipet. 1 ml homogenization buffer (8 mM KH_2PO_4; 0.3 M sucrose, pH 6.0) was added as a wash. Islets were allowed to settle by gravity, and the wash buffer was discarded. For every 100 islets, 100 µl of the homogenization buffer was added to the tube and the islets were homogenized using a motor-driven (Tri-R Instruments, Rockville Centre, New York) Teflon pestle, 25 strokes. The homogenate was transferred to 0.5 ml microcentrifuge tubes (West Coast Scientific, Oakland CA) and centrifuged in an SE-12 Sorvall (Newtown , Conn.) rotor, 2500 x g, 10 min. The sediment was resuspended in 50 µl of the homogenization buffer for subsequent analysis. The supernatant was layered over discontinuous sucrose (Mallinckrodt, St. Louis, MO) gradients, with approximately 100 µl supernatant per gradient. The gradients were layered from the bottom in 600 µl cellulose nitrate ultracentrifuge tubes (Beckman, Palo Alto, CA) as follows: 50 µl 60% sucrose; 150 µl 50% sucrose; and 350 µl 40% sucrose. All sucrose solutions were prepared in 8 mM KH_2PO_4 and the pH was adjusted to 6.00--6.04. (Isolated granules are optimally stable at this pH; once released upon homogenization they remain in an optimal pH environment for the duration of the fractionation.) The gradients were centrifuged at 70,000 x g, 75 min, with an SW 50.1

rotor (Beckman Spinco, Palo Alto, CA) on a Beckman L ultracentrifuge. Fractions were collected by puncturing the bottom of the centrifuge tube and collecting 2 drops per fraction. (Data from fractions 6 and 7 were pooled when considering their significance, because of slight variations in the total gradient volume; occasionally fraction 7 would be either miniscule, or 3 drops in volume.)

Characterization of Fractions.

A. Protein Determination. The microprotein assay adaptation of the Bradford method (85) of protein determination was used. Aliquots of the fractions, up to a 30 µl volume, as well as standards, were mixed with 300 µl of a Coomassie Blue binding dye (100 mg Coomassie Blue G250; 50 ml 95% ethanol; 100 ml 85% w/v phosphoric acid; and distilled water for a final volume of one liter) and read at an absorbance of 595 nm on a Beckman DU Spectrophotometer (Fullerton CA). The standards of fatty acid free, crystalline bovine albumin (Sigma; St. Louis, MO) were prepared in distilled water and in the various fractionation buffers, to correct for interference of the high concentrations of sucrose where necessary. Blanks of distilled water, or the various fractionation buffers, were read with the appropriate samples or standards.

B. Monoamine Oxidase. Monoamine oxidase activity was used as a marker for the presence of mitochondrial contamination. It was assayed via a modification of the McCaman (86) method, as outlined below.

Substrate for the assay was prepared by diluting ^{14}C-tyramine (Amersham Searle, Arlington Heights, Ill.) with 0.1 M KH_2PO_4 , pH 7.2, 1:20, v:v. 10 µl of this substrate was incubated with 10 µl of the islet fraction(s) at 37°C for 60 min. The reaction was stopped by the addition of 10 µl of 6 N HCl and rapid vortexing. 225 µl ethyl acetate was added to each tube, and the tubes were vortexed again. The tubes were centrifuged 1 min on a Beckman microcentrifuge. 150 µl of the upper organic phase of each sample was transferred to another tube containing 125 µl 0.3 N HCl. The tubes were vortexed as a wash, and centrifuged for 1 min on the Beckman microcentrifuge. 90 µl of the upper phase were added to 2 ml Aquasol, and the ^{14}C activity counted on the Packard Liquid Scintillation Spectrometer. Data was calculated with homogenate activity equaling 100%. Corrections were made for the aliquot of the ethyl acetate counted, to obtain total activity of each of the fractions. A correction factor was used to correct for suppressed activity in the fractions containing 40, 50, and 60% sucrose; addition of final corrected activities in the fractions resulted

in recoveries of 85--90% of total homogenate activity.

C. RNA. RNA content of islet fractions was determined by a modification of the method of Keleti and Lederer (87) as described below:

Aliquots of islet fractions were mixed with 50 µl reagent A (0.6 N $HClO_4$) and incubated at 4°C 10 min in 1.5 ml Beckman microcentrifuge tubes. The tubes were centrifuged 15 min on the Beckman Microfuge, 4°C. The supernatant was discarded, and the wash was repeated with 50 µl reagent B (0.2 N $HClO_4$) after resuspension of the precipitate. After a second centrifugation, the precipitate was resuspended in 40 µl reagent C (0.3 N KOH) and was incubated in a shaking H_2O bath, 37°C, 1 hr. The tubes were removed and chilled at 4°C for 15 min. The initial washes with reagents A and B were repeated, and the supernatants were reserved. They were combined with the supernatant from the third and final wash with 50 µl reagent B. The supernatants were brought to a total volume of 300 µl with H_2O. Samples were read in the Beckman DU Spectrophotometer at 260 nm. Standards were prepared from ribosomal RNA (Sigma, St. Louis, MO; 5 U/mg) in H_2O.

D. IRI. Insulin was assayed in all samples and extracts by solid phase radioimmunoassay, as outlined by Lundquist et al. (88). Islet extracts were prepared by homogenization of islets in 300 µl acid-ethanol (750

ml 100% ethanol; 15 ml concentrated HCl; 235 ml H_2O). The extracts were kept at 4°C for 18--24 hr, then vortexed and centrifuged 2 min on the Beckman microcentrifuge. The supernatant was diluted with perfusate (Table 1) and the pH adjusted to 7.2--7.4 prior to insulin assay. Extracts of the culture medium were prepared in a similar fashion. Several dilutions (e.g., 1:10, 1:50, 1:100) of each extract were prepared in order to obtain IRI values within the range of the standard curve.

Fractionation with ^{65}Zn.

Islets were cultured as described above in groups of 100 with 10 µl of ^{65}Zn for 24 hr. At the termination of the culture period, islets were removed in a minimal volume and transferred to the tissue grinder. The islets were allowed to settle and the culture medium was drawn off. The islets were rinsed, homogenized and fractionated as described above. The total homogenate, sediment, and sucrose fractions, as well as an aliquot of the supernatant, were counted in the gamma counter. A 10 µl aliquot of the culture medium was counted to obtain the total dose of ^{65}Zn.

^{65}Zn Fractionation Controls.

In order to assess non-specific distribution of ^{65}Zn during the fractionation procedure, ^{65}Zn was added

to a group of freshly isolated islets and the fractionation was carried out instantly, as described. In a second control protocol, in order to evaluate distribution of free zinc in the gradients, ^{65}Zn was added to 100 µl of homogenization buffer and layered over the sucrose gradients. The gradients were centrifuged 75 min at 70,000 x g; the fractions were collected and counted in the gamma counter. In an attempt to discern whether zinc redistributes significantly during the fractionation procedure, a homogenate of islets cultured with ^{65}Zn was prepared. The homogenate was split into two aliquots; one aliquot was carried through the fractionation immediately. The other half was fractionated 3 hr later, being kept at 4°C until the time of fractionation.

Measurement of Endogenous Islet Zinc.

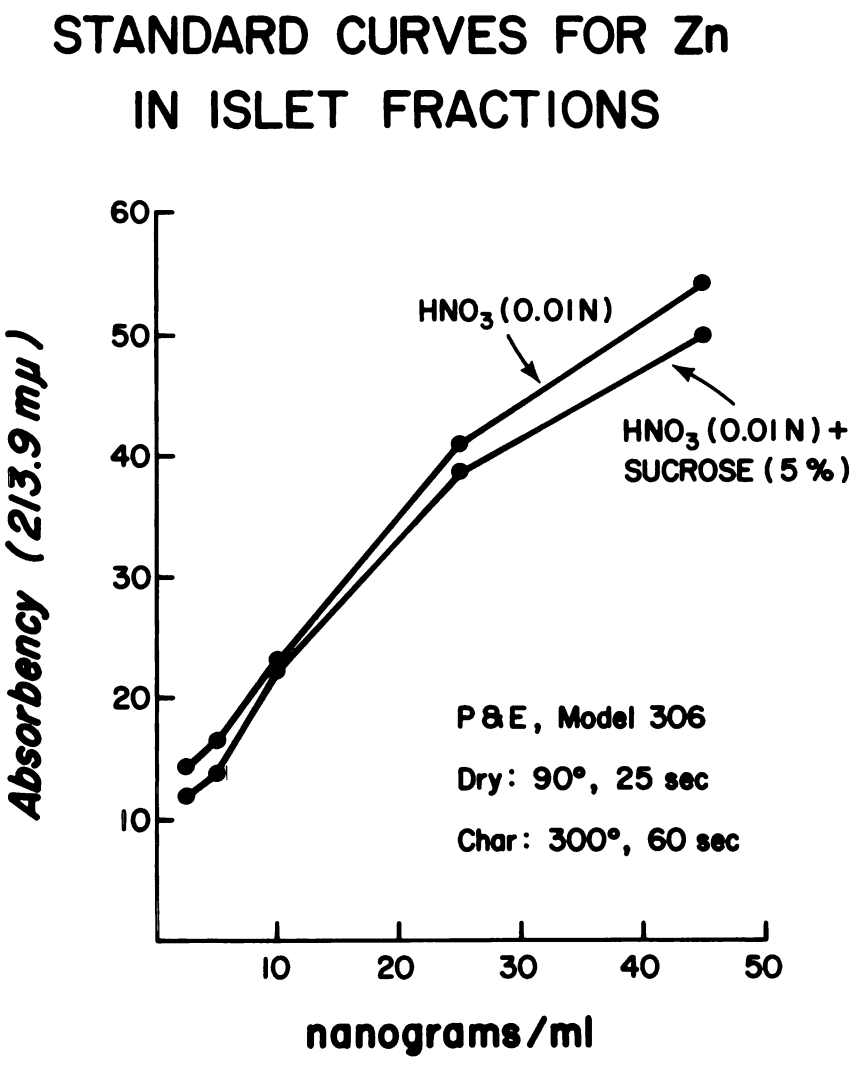
Total content of endogenous zinc in fresh islets, cultured islets, and cultured islet fractions were assessed using a Perkin Elmer atomic absorption spectrophotometer Model 306 (with deuterium arc background correction; HGA 400 graphite furnace; and AS-1 Auto Sampler; Norwalk, Conn.). Absorption of zinc was read at 213.9 nm, with a sensitivity of 5 ng/ml. An initial problem was encountered with interference of the concentrated sucrose solutions and a significant reduction of sensitivity. Sensitivity was optimized by adjust-

ment of times and temperatures chosen for sample processing prior to analysis of light absorption. Samples were dried at 90°C, for 25 seconds, and charred at 300°C for 60 seconds. Standards were prepared from a 1000 µg/ml standard zinc solution (Scientific Products, McGaw Park, Ill.) in 0.01 N HNO_3. All islet fractions were diluted twenty-fold in 0.01 N HNO_3. The reduced sucrose concentrations (and optimal sample processing protocol) minimized interference with zinc absorption; as shown in Figure 5 zinc standards prepared in .01 N HNO_3 + 5% sucrose showed the same absorbance as zinc standards prepared in .01 N HNO_3 alone.

Zinc content of whole islets was assessed by the method of standard additions. The method involves addition of small aliquots of unknown to a blank, and to absorption standards, generating a new "standard curve" which, when plotted, is parallel to the original standard curve. The new y--intercept is a positive number; the new x--intercept is obtained by extrapolation of the standard curve (which therefore must be linear) and represents the negative inverse of the unknown's zinc concentration.

Groups of islets were frozen in modified KRB, approximately 100 islets/100 µl. The islets were thawed and sonicated (Biosonik II, Bronwill) for 45 sec. An aliquot of the same buffer was handled identi-

FIGURE 5


STANDARD CURVES FOR Zn
IN ISLET FRACTIONS
60
50
40
30
20
10
Absorbency (213.9 mμ)
10
20
30
40
50
nanograms/ml
HNO3 (0.01N)
HNO3 (0.01N) + SUCROSE (5%)
P&E, Model 306
Dry: 90°, 25 sec
Char: 300°, 60 sec

cally and served as a blank. The sensitivity of the atomic absorption spectrophotometer was increased three-fold to "spread out" this portion of the standard curve. 30 µl of each sonicate was diluted with 1.5 ml quartz-distilled water. 500 µl of this was mixed with 500 µl H_2O; 500 µl of 10 ng/ml standard in H_2O; or 500 µl of 20 ng/ml standard in H_2O. The three absorbances for each sample yielded a linear plot which was extrapolated to the abscissa, from which a concentration of the dilute unknown could be read. Data was converted from ng/ml to pmol/islet according to the following calculation:

(ng/ml of sample-ng/ml of blank) x dilution x sample volume = ng/sample

(ng/sample) / (# islets/sample) = ng/islet

(ng/islet) x (1000 pmol / 65 ng) = pmol/islet

<u>Islet Culture Protocols</u>.

Islet zinc, ^{65}zinc, and insulin content and distribution were assessed in islets cultured as described above, but with various experimental modifications. Islets were cultured in TCM 199 with 5.6 mM glucose, either with added ^{65}Zn, to assess net uptake, or without ^{65}Zn, for measurement of endogenous zinc content and distribution. When available, additional islets were extracted for insulin content immediately

upon isolation. In one group, islets were cultured in medium containing heat-inactivated fetal calf serum. The medium was sampled at 24 hr, and the samples were extracted for insulin content. The modified protocols are detailed below.

A. High vs. Low Glucose Culture. In order to examine if enhanced insulin synthesis and secretion can influence zinc uptake, zinc content, or its localization with insulin granules, groups of 100 islets were cultured 24 hours in either 5.6 (low) or 25.6 (high) mM glucose in TCM 199 as described.

B. Arginine--glucose vs. Glucose. Arginine acts as another strong stimulant of insulin secretion, yet is believed to cause secretion via a different mechanism than glucose. Groups of 100 islets were cultured 24 hr in either 10 mM arginine--5.6 mM glucose or 5.6 mM glucose in TCM 199 as described above, in order to observe possible changes in the distribution of islet zinc upon exposure to a different secretagogue.

C. Low Ca^{++}--High Glucose vs. High Glucose. Groups of 100 islets were cultured in the modified TCM 199 as described above, with a final glucose concentration of 25.6 mM glucose. As omission of extracellular calcium results in an inhibition of insulin secretion, with no effect on synthesis, additional islets were cultured in a TCM 199 from which $CaCl^{2+}$ had been omitted also

containing 25.6 mM glucose. These islets should be stimulated to synthesize insulin, yet be unable to secrete it. In an initial series of experiments, no differences in net uptake or distribution of ^{65}Zn were observed at 24 hr. Therefore it was decided to extend the culture time to 48 hr. At 24 hr, islets were transferred after several rinses into fresh medium. At 48 hours, islets were fractionated as described. Additional islets from both groups had been cultured to assess glucose responsivity (insulin secretion) at 48 hr. Also, 1 ml medium samples taken at 24 and 48 hours, were extracted with 4 ml acid--ethanol, and assayed for insulin content.

D. Tolbutamide Treatment. Rats received 500 mg/kg sodium tolbutamide (compliments of the Upjohn Co., Kalamazoo, MI) in water via a stainless steel feeding tube, twice daily for three days. This dosage has been shown to significantly deplete islet insulin (66). Control rats received a comparable volume of water. Approximately twelve hours after the last treatment, rats were sacrificed, and their pancreases were digested in order to isolate the islets. In contrast to previous protocols, where islets from several rats served as a common pool from which 'control' and 'experimental' islets were randomly selected, each rat in this experiment served as an individual experimental

value.

Influence of Exogenous Zinc on Islet Insulin Secretion.

The influence of acute changes in extracellular zinc concentration on insulin secretion was assessed in the *in vitro* perfused pancreas. Pancreases, with a portion of duodenum attached, were removed from male Long-Evans rats (300--370 grams). Cannulae were placed in the celiac axis and portal vein. The mesenteric artery was ligated and cut, and the entire intestine with a portion of duodenum was separated and removed from the rat. The esophagus and stomach were ligated and cut above the ligature. A loose ligature was placed, but not tied, around the entire gastrohepatic ligament. The aorta was exposed through the crura of the diaphragm well above the point of origin of the celiac axis. The ligature around the gastrohepatic ligament was then tied, and the ligament cut. The aorta was cut and the preparation was lifted out of the animal. The arterial cannula was inserted and tied in place. Circulation through the preparation was checked for outflow from the portal vein. After a minute or two, the flow was stopped and a cannula was inserted into the portal vein and tied in place. Flow was then resumed continuously. The perfusion apparatus used was a modification of the Anderson--Long apparatus (89),

with constant oxygenation of the perfusate (see Table 1 for composition). Total flow was 4 ml/min, primarily from one of two parallel reservoirs. $ZnCl_2$ (prepared from a 100 mM stock solution in 0.1 M Tris) was added to perfusate in one of the reservoirs. The main flow was switched from one reservoir (no added zinc) to the other (zinc added as indicated; Table 2 lists zinc concentration protocols). Glucose was introduced as indicated in perfusate through an indwelling sidearm at 0.05 ml/min to give a final concentration of 11.1 mM.

To observe the effect of longer exposure to various zinc concentrations, islet responsivity to a glucose challenge was assessed after 8 hr culture in TCM 199 with 5.6 mM glucose, and either no added; 50 µM; or 200 µM zinc. Islets were transferred in approximately 100 µl into plates containing approximately 5 ml KRB with 1 mM glucose, as a wash. Islet glucose responsivity was assessed as described above, with islets incubated in KRB containing 1 mM glucose, then 20 mM glucose. In a third series of experiments, islets were transferred to plates containing KRB with the same amount of zinc as the islets were exposed to during the 8 hr culture. The glucose challenge protocol was carried out, with zinc added at the same concentrations to which the islets were exposed in culture.

TABLE 2

ZINC CONCENTRATIONS FOR PANCREAS PERFUSIONS WITH 11.1 mM GLUCOSE

	Zinc. Conc. (μM)		
Time (min.) of perfusion:	0-10	10-20	20-30
	10	no added	10
	10	50	10
	50	no added	50
	50	200	50
Time (min.) of perfusion:	0-20	20-40	40-60
	no added	200	no added
	200	no added	200
	200	200	200
	50	200	50
(N = 3 or greater)			

RESULTS

Zinc and Insulin Measurements in Isolated Islets.

As shown in Table 3, insulin content in acid--alcohol extracts of freshly isolated islets (as the 6000 dalton monomer) is 13.1±0.9 pmol/islet (n=7). Endogenous zinc content as measured by the method of standard additions is 11.1±0.9 pmol/islet (n=20). This value is compared with the amount of zinc needed to create 2-Zn insulin hexamers, approximately 4.4 pmol. Thus, the islet contains about 2.5 times the amount of zinc needed to form insulin hexamers.

Chemical Studies.

Binding of 0.001 mM (i.e., trace) zinc to 0.1 mM insulin was studied at 37°C, pH 7.4. This amount of insulin is in excess of the amount needed to form zinc hexamers with .001 mM zinc, i.e., virtually all zinc should be bound. As shown in Table 4 and Figure 6, binding was extremely rapid, being approximately 78% at 30 seconds, and not increasing beyond 87% at 5 min. Thus free insulin in islets should form a complex immediately when exposed to free zinc. Dissociation of this complex by addition of more cold zinc (hence, competition and exchange of the ^{65}Zn for cold zinc) at pH 7.4 resulted in a very slow and gradual decline of ^{65}Zn binding, with 97% of initial binding at three hours--

TABLE 3

INSULIN AND ZINC CONTENTS OF ISOLATED ISLETS

Insulin content/islet	13.1 pmol
Zinc content/islet	11.1 pmol
Zinc required for 2-zinc hexamer	4.4 pmol

TABLE 4

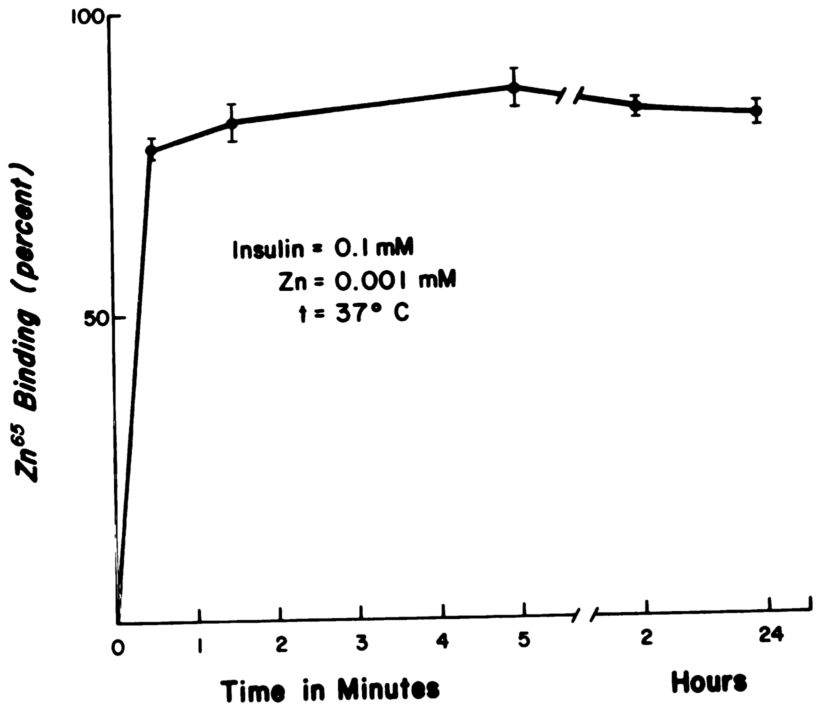
FORMATION AND DISSOCIATION OF ZINC-INSULIN COMPLEXES

Percent Binding

Time:	0	30 sec	90 sec	5 min	10 min	30 min	2 hr	3 hr	24 hr	5 days
A. 0.1 mM Insulin: 0.001 mM Zinc										
X	---	77.9	81.6	86.8	---	---	83.6	---	85.2	---
±SEM	---	±1.4	±1.8	±2.8	---	---	±0.7	---	±1.5	---
(N)	---	(4)	(3)	(3)	---	---	(4)	---	(5)	---
B. After Increase of [Zn] to 0.01 mM Zinc										
X	84.2	---	---	---	---	---	---	81.7	64.1	33.4
±SEM	±4.0	---	---	---	---	---	---	±2.2	±2.9	---
(N)	(5)	---	---	---	---	---	---	(4)	(4)	(1)
C. After Increase of [Zn] to 0.01 mM, Lowering pH to 6.										
X	83.2	---	---	---	---	86.1	---	64.4	16.4	17.7
±SEM	±2.6	---	---	---	---	±3.8	---	±7.4	±2.5	---
(N)	(6)	---	---	---	---	(4)	---	(4)	(3)	(1)
D. 1:10 Dilution of 1 mM Insulin: 0.1 mM Zn Complex										
X	72.6	---	28.6	25.1	27.7	---	---	---	29.2	---
±SEM	±4.3	---	±0.6	±3.3	±0.7	---	---	---	---	---
(N)	(3)	---	(3)	(3)	(3)	---	---	---	(2)	---

FIGURE 6

EFFECT OF TIME ON FORMATION OF Zn^{65}-INSULIN COMPLEX



the approximate time frame of an islet fractionation experiment--and 76% of the initial binding after 1 day of dilution (Fig. 7). Thus, zinc dissociation from insulin is slow. Simultaneously lowering the pH to 6.0 and increasing the available cold zinc--a simulation of conditions under which islet fractionations are carried out--resulted in a more rapid and extensive dissociation of the ^{65}Zn. However, binding was still at 77% of original at 3 hr, although it dropped to 18% by 25 hr. It is emphasized that a change of extragranular pH may or may not cause a similar change in pH inside the granule. A 1:10 dilution of a 1 mM insulin--0.1 mM Zn complex (yielding final concentrations of 0.1 mM insulin and 0.01 mM zinc) resulted in virtually immediate dissociation of the complex: binding fell from 73% to 29% at 90 sec after dilution. No further dissociation was observed at 24 hr. Thus dilution of a preformed complex presumably causes partial dissociation of the insulin polymers and rapid dissociation of zinc. As long as studies are restricted to intact granules, such insulin dilution should not occur.

Islet Culture Controls.

In order to ascertain if islets become significantly leaky over the culture period, ^{3}H-sucrose distribution in the islet was measured at 2, 12, and 24 hr of culture. As shown in Table 5, the ^{3}H-sucrose space

FIGURE 7

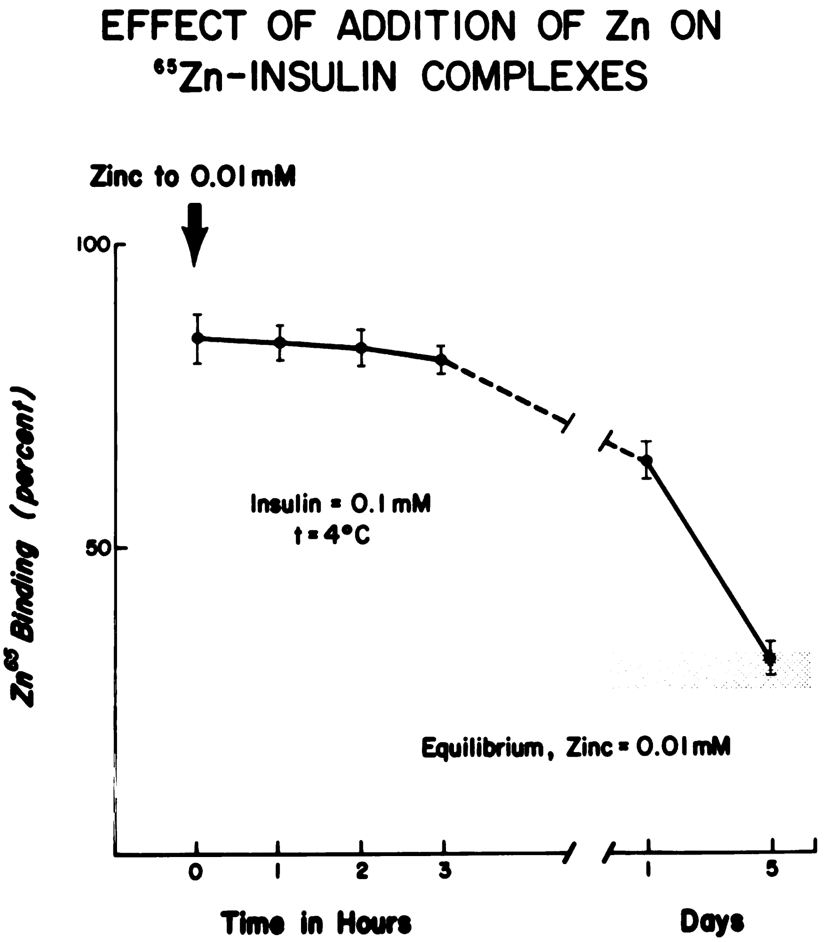

EFFECT OF ADDITION OF Zn ON ^{65}Zn-INSULIN COMPLEXES

TABLE 5

VIABILITY PARAMETERS IN FRESH vs. CULTURED ISLETS

A. ^{3}H-Sucrose Uptake

	^{3}H-Sucrose Space (nl/islet)		
Time of Culture:	2 hr	12 hr	24 hr
X ±SEM	1.2 ±0.2	1.0 ±0.2	1.3 ±0.4
(N)	(9)	(4)	(9)

was unaltered during 24 hr of culture, being approximately 1 nl/islet at 2, 12, and 24 hr. Islet response to a glucose challenge after 24 hr was approximately 4-fold, as compared to the response of fresh islets in the same system, which give an approximate 5-fold response (Table 6).

Characterization of Fractions.

Distributions of insulin; total protein; monoamine oxidase (MAO: a mitochondrial marker enzyme); and RNA (a marker of rough endoplasmic reticulum in nuclei--free fractions) were assessed in the sucrose gradients. Data was obtained from fractionations of fresh islets. Approximately 50% of homogenate insulin; 40% of homogenate total protein; 11.5% of homogenate monoamine oxidase activity; and 42% of homogenate RNA remained in the supernatant after the initial Sorvall spin and were layered over the sucrose gradient. Table 7 shows the distribution of these activities throughout the sucrose gradient. Approximately 71% of total gradient IRI was localized to fractions 2 and 3 (Fig. 8), which will be designated the "granule--enriched" or "insulin" fractions, containing about 40% of total islet insulin. These fractions also contained about 2% of total islet MAO, indicating that there was little mitochondrial contamination. A significant quantity of RNA was localized to the granule fraction: 24% of total homo-

TABLE 6

VIABILITY PARAMETERS IN FRESH vs. CULTURED ISLETS

B. Basal and Glucose-Stimulated Insulin Secretion

	Insulin Secretion (ng/islet x 30 min)			
Time of Culture:	0 hr		24 hr	
Glucose Conc.:	1 mM	20 mM	1 mM	20 mM
X ±SEM	0.3 ±0.1	1.4 ±0.2	0.4 ±0.1	1.3 ±0.3
(N)	(12)	(12)	(9)	(9)

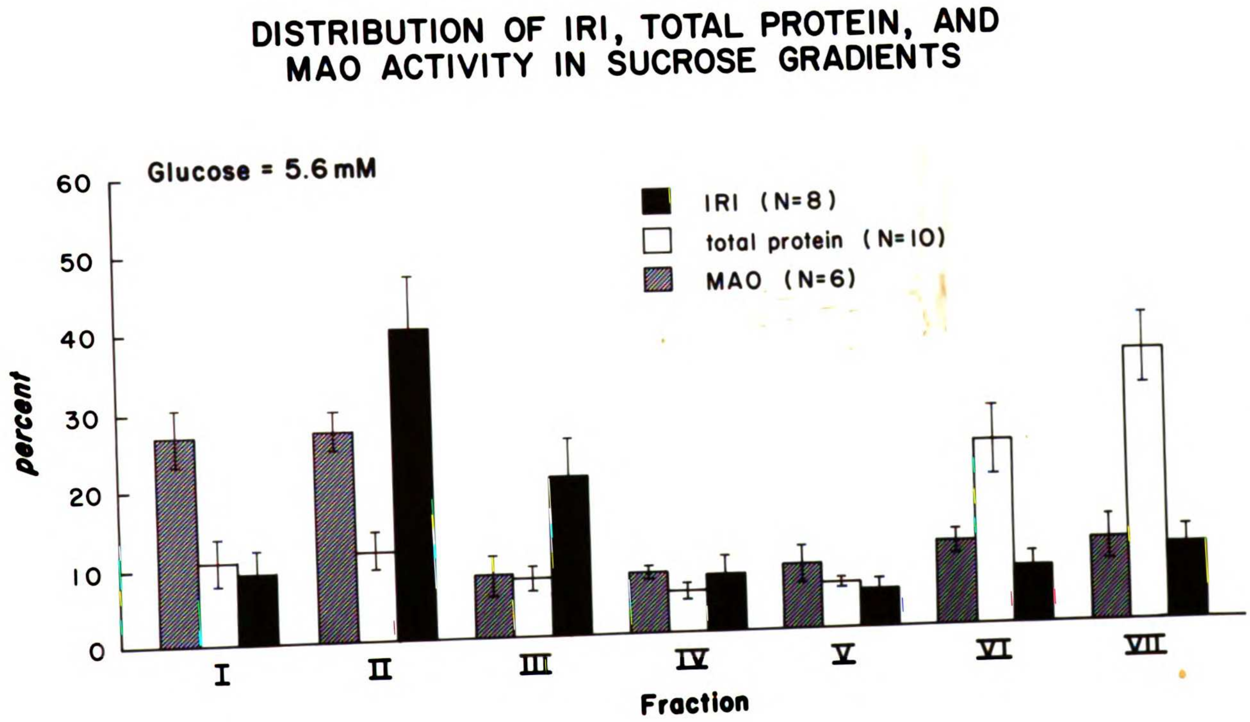
TABLE 7

CHARACTERIZATION OF FRACTIONS

		Percent of Gradient Activity*						
Fraction:		1	2	3	4	5	6	7
	(N)							
A. Insulin								
X	(5)	6	42	29	5	4	5	9
± SEM		±1	±10	±4	±0.5	±1	±1	±3
B. Protein								
X	(10)	11	11	7	5	6	24	35
±SEM		±3	±2	±2	±1	±1	±4	±11
C. Monoamine Oxidase								
X	(6)	26	27	8	8	8	11	11
_SEM		±3	±3	±2	±1	±2	±2	±3
D. RNA								
X	(4)	8	39	9	9.5	7	8.5	4
±SEM		±2	±13	±1	±1	±3	±2.5	±2

* Activities in fractions 1 through 7 total 100%.

FIGURE 8


DISTRIBUTION OF IRI, TOTAL PROTEIN, AND MAO ACTIVITY IN SUCROSE GRADIENTS
Glucose = 5.6 mM
IRI (N=8)
total protein (N=10)
MAO (N=6)
percent
0
10
20
30
40
50
60
I
II
III
IV
V
VI
VII
Fraction

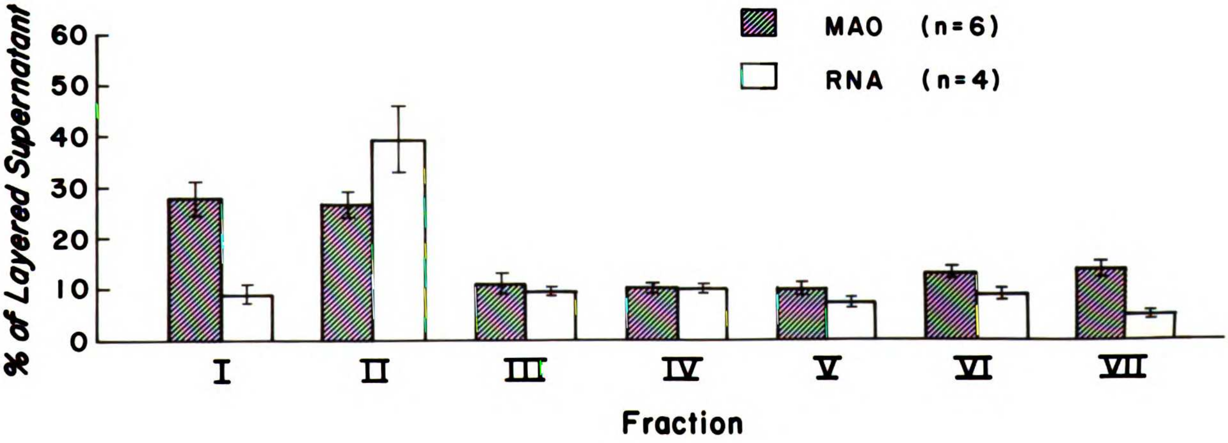
genate activity, suggesting a significant contamination of the fraction with RER (Fig. 9). This contamination has also been a problem for previous investigators attempting to prepare a purified granule fraction (49,52), and is shown in the electron micrograph (Fig. 10) of the combined fractions 2 and 3.

^{65}Zn Uptake Controls.

Non-specific distribution of ^{65}Zn and its possible redistribution following homogenization were assessed in several control experiments. As shown in Table 8, virtually 100% of ^{65}Zn, added in an aliquot of homogenization buffer, remained at the top of the sucrose gradients following centrifugation at 70,000 x g, 75 min. In a second experiment ^{65}Zn was added to freshly isolated islets. They were homogenized and fractionated and the distribution of ^{65}Zn cpm was compared with the distribution of ^{65}Zn cpm in islets which had been cultured 24 hr in the presence of ^{65}Zn (vide infra). A very low percentage of ^{65}Zn cpm was found in the insulin--enriched fractions from fresh non-cultured islets. In gradients from both fresh and cultured islets, the majority of ^{65}Zn cpm were localized to the upper fractions.

As redistribution of ^{65}Zn loosely associated with cellular components might occur following the dilution and mechanical disruption that result from homogeniza-

FIGURE 9



DISTRIBUTION OF MAO ACTIVITY AND RNA IN SUCROSE GRADIENTS

FIGURE 10

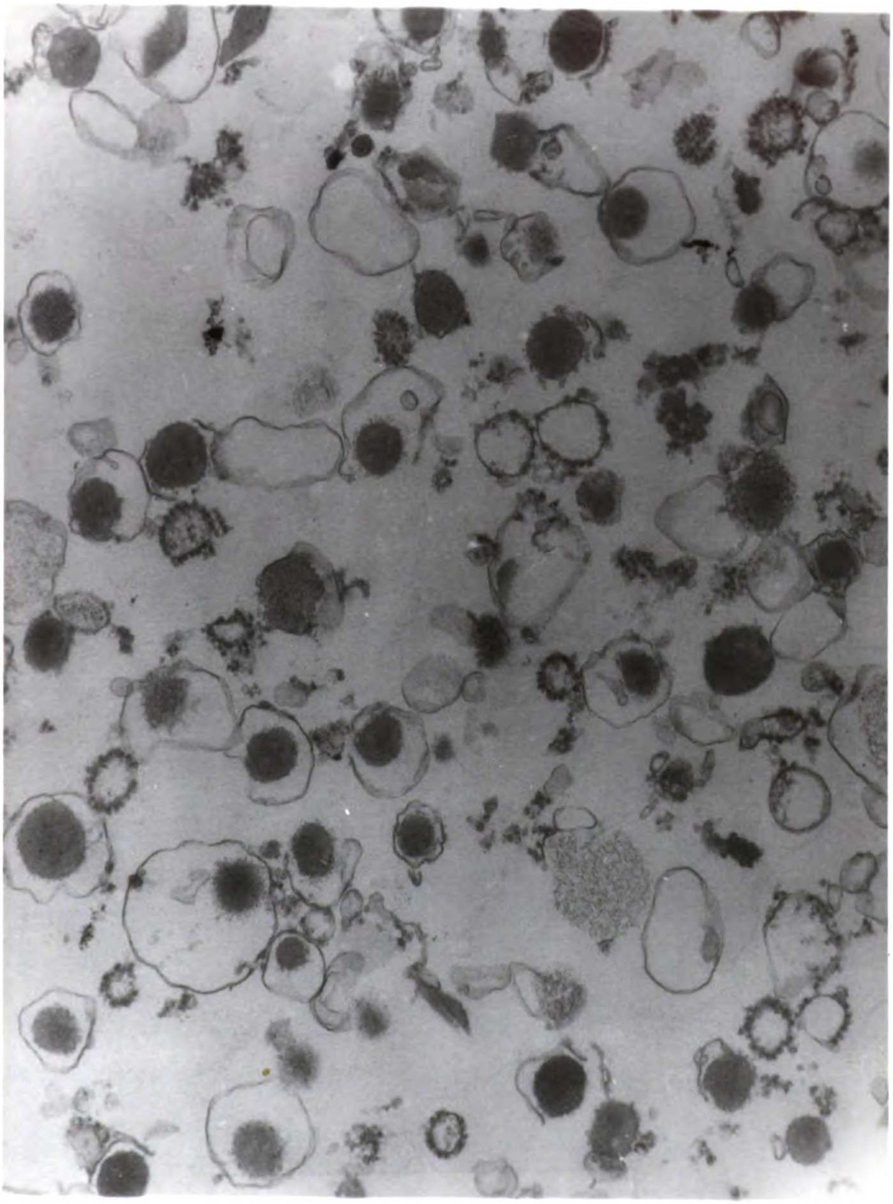


TABLE 8

^{65}ZINC DISTRIBUTION UPON FRACTIONATION

Fractionation Conditions:		^{65}Zn Distribution (Percent)*	
		^{65}Zn in Homog. Buffer	Fresh Islets, Homog with ^{65}Zn
(N)		(8)	(6)
Supernatant (% Homog. cpm)	X	—	64
	±SEM	—	±6
Sediment (% Homog. cpm)	X	—	28
	±SEM	—	±7
Fraction 1	X	0.015	0.255
	±SEM	±0.003	±0.069
Fraction 2	X	0.012	0.552
	±SEM	±0.003	±0.188
Fraction 3	X	0.017	0.670
	±SEM	±0.003	±0.253
Fraction 4	X	0.10	1.15
	±SEM	±0.02	±0.39
Fraction 5	X	7	3
	±SEM	±3	±1
Fraction 6	X	56	37
	±SEM	±9	±5
Fraction 7	X	37	57
	±SEM	±11	±6

---

* Activities in fractions 1 through 7 total 100%.

tion, we split a homogenate of islets cultured with ^{65}Zn into two aliquots, one fractionated immediately and the other after 3 hr. As shown in Table 9, no difference in the distribution of ^{65}Zn cpm was observed after 3 hr, suggesting that either re-distribution of the zinc is rapid and occurs immediately upon homogenization, or that no significant redistribution of ^{65}Zn occurs within the time frame of the fractionation period.

^{65}Zn Net Uptake into Islets.

Figure 11 and Table 10 show the net uptake of ^{65}Zn by isolated islets over a 24 hour culture period. Net uptake of the ^{65}Zn was slow, being almost linear over the first eight hours, then increasing at a slower rate for the remainder of the culture period. Uptake did not reach equilibrium by 24 hr, and only about 0.1% of the total available ^{65}Zn was taken up by the islets. Calculation of islet zinc content at 24 hr based on ^{65}Zn uptake and the specific activity in the media yielded a value of 0.5 pmol, in contrast with the value of 9--10 pmol obtained by atomic absorption spectrophotometry. Culture of islets in high (25.6 mM) as opposed to basal (5.6 mM) glucose resulted in a decrease in net ^{65}Zn uptake. This decrease was first significant by paired analysis at 4 hr, and also achieved significance at 12, 24 , and 26 hr.

TABLE 9

ZINC DISTRIBUTION AT TIME 0 AND 3 HR AFTER HOMOGENIZATION

		^{65}Zn Distribution (Percent)*	
Time:		0	3 hr
(N)		(4)	(4)
Supernatant	X	43	46
(% Homog. cpm)	±SEM	±2	±7
Sediment	X	29	29
(% Homog. cpm)	±SEM	±2	±8
Fraction 1	X	4	5
	±SEM	±2	±3
Fraction 2	X	3	2
	±SEM	±0.5	±1
Fraction 3	X	2	2
	±SEM	±1	±1
Fraction 4	X	3	2
	±SEM	±1	±0.5
Fraction 5	X	3	2
	±SEM	±1	±1
Fraction 6	X	22	16
	±SEM	±5	±6
Fraction 7	X	64	70
	±SEM	±9	±10

* Activities in fractions 1 through 7 total 100%.

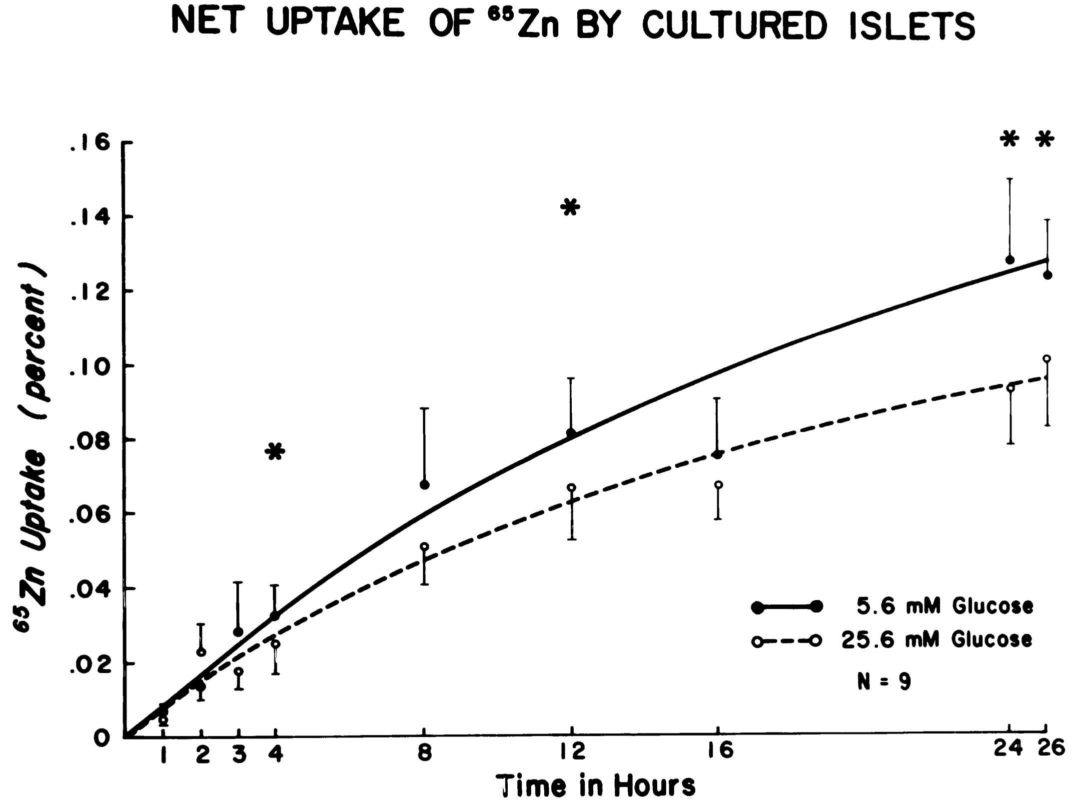
TABLE 10

NET UPTAKE OF ^{65}ZINC BY CULTURED ISLETS

	Percent ^{65}Zn Dose/Islet	
Glucose Conc.:	5.6 mM	25.6 mM
(N)	(9)	(9)
Time (hr)		
1	0.007 ± 0.002	0.005 ± 0.001
2	0.014 ± 0.004	0.023 ± 0.008
3	0.029 ± 0.013	0.018 ± 0.005
4	0.033 ± 0.008	0.025 ± 0.008*
8	0.068 ± 0.021	0.051 ± 0.010
12	0.082 ± 0.015	0.067 ± 0.014*
16	0.076 ± 0.015	0.068 ± 0.009
24	0.129 ± 0.022	0.094 ± 0.015*
26	0.125 ± 0.015	0.102 ± 0.018*

* $p < .05$ as compared to basal glucose value

FIGURE 11


NET UPTAKE OF 65Zn BY CULTURED ISLETS
65Zn Uptake (percent)
.16
.14
.12
.10
.08
.06
.04
.02
0
1
2
3
4
8
12
16
24
26
Time in Hours
*
*
* *
5.6 mM Glucose
25.6 mM Glucose
N = 9

Zinc and Insulin Distributions in Cultured Islets.

A. Low and High Glucose. In the initial study, the distribution of ^{65}Zn in islet fractions was measured after 24 hr culture in low glucose medium. As shown in Figure 12, only a small percentage--about 12%--of total gradient zinc was localized to the granule fractions: most islet zinc was extragranular. This percentage, however, was significantly greater than that found in fresh islet controls, suggesting a specific incorporation of ^{65}Zn into the granule fraction during 24 hr culture.

In a paired series of experiments, islets were cultured for 24 hr in either basal (5.6 mM) or high (25.6 mM) glucose. 24 hr culture in basal glucose resulted in a diminution of islet content of both insulin and zinc (compare Table 11 with Table 3). If cultured in high glucose, islets displayed an even further, and significant diminution of insulin and zinc contents. In addition, as shown in Fig. 11, ^{65}Zn net uptake was significantly decreased in comparison with the uptake of islets in basal glucose. Figure 13 shows the distributions of ^{65}Zn, and endogenous islet zinc, in sucrose fractions from islets cultured in basal glucose. Low percentages of both ^{65}Zn and endogenous zinc are localized to granule fractions 2 and 3: only about 10% of total gradient ^{65}Zn and approximately 20% of

FIGURE 12

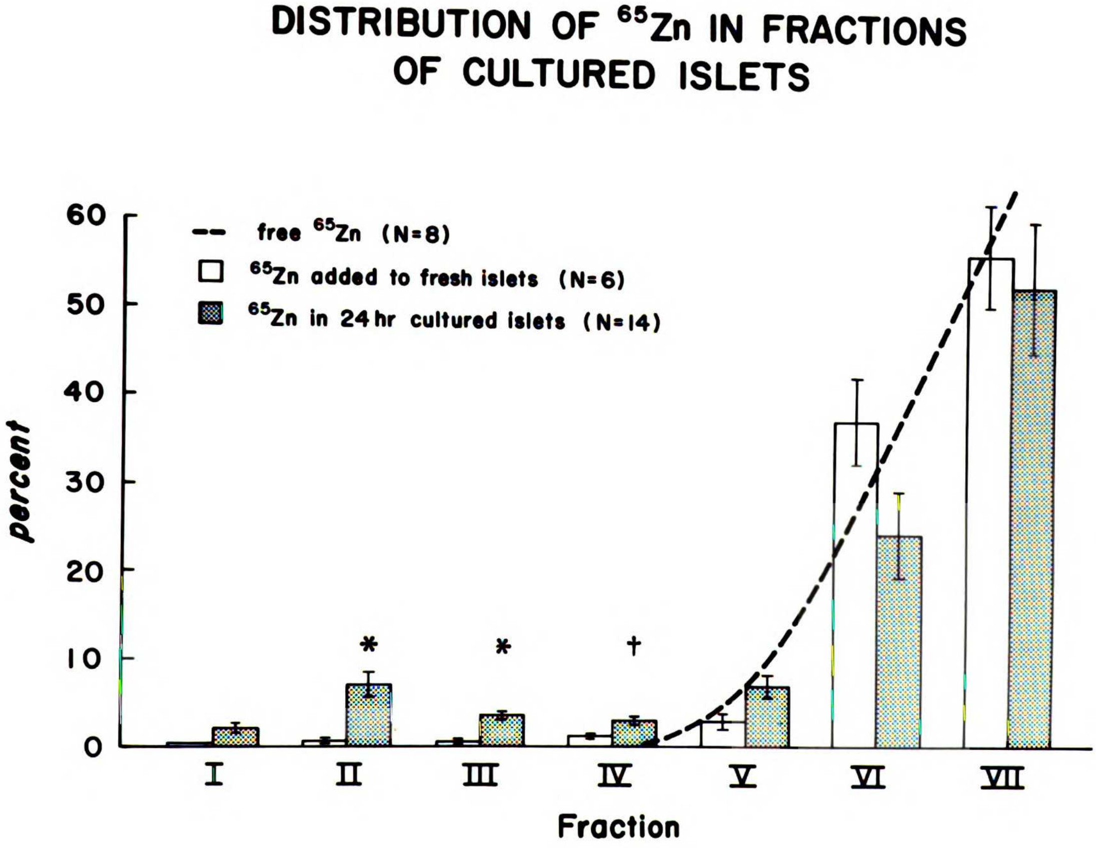
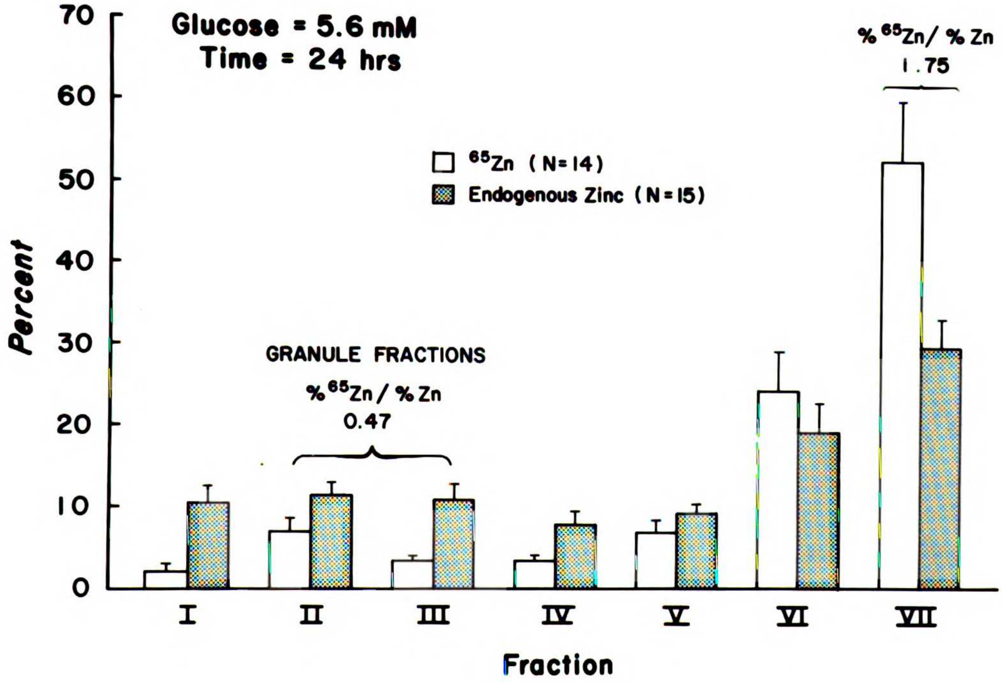

DISTRIBUTION OF ^{65}Zn IN FRACTIONS OF CULTURED ISLETS
free ^{65}Zn (N=8)
^{65}Zn added to fresh islets (N=6)
^{65}Zn in 24 hr cultured islets (N=14)
percent
60
50
40
30
20
10
0
*
*
+
I
II
III
IV
V
VI
VII
Fraction

TABLE 11

INSULIN AND ZINC CONTENTS OF 24-HR CULTURED ISLETS

	(pmol/islet)	
Glucose Conc. in Medium:	5.6 mM	25.6 mM
A. Insulin		
X ±SEM	8.8 ±1.3	4.2 ±0.2
(N)	(8)	(3)
B. Zinc		
X ±SEM	9.2 ±2.3	4.3 ±0.5
(N)	(4)	(3)

FIGURE 13



^{65}Zn AND ENDOGENOUS ZINC DISTRIBUTION IN FRACTIONS OF CULTURED ISLETS gradient zinc, occur in fractions containing a high percentage of islet insulin.

A comparison of % ^{65}Zn/% Zn (Table 12; Figure 13) in the fractions reveals a relatively low specific activity of the zinc in the granule fractions as compared to the upper "soluble" fractions, suggesting that ^{65}Zn has not equilibrated with the granular zinc pool over the 24 hr culture period. The specific activity of granule fractions from islets cultured in high glucose (0.60) (Fig. 14) was slightly higher than that of basal glucose islets (0.47) but was still quite low as compared to the specific activity of soluble fractions (1.63; 1.94).

Although the insulin content of islets cultured in high glucose was significantly depleted as compared to basal glucose controls, no difference was observed in the percentage distribution of the insulin in the sucrose gradients (Table 13; Figure 15). Thus, of the insulin present, it was consistently found primarily in the storage granule fraction. In addition, no change in the low percentage of endogenous zinc in the granule fraction was observed following islet culture in high, as compared to low, glucose.

B. Arginine--glucose vs. glucose. Culture of islets in 10 mM arginine with basal glucose resulted in a significantly diminished uptake of ^{65}Zn as compared to

TABLE 12

ZINC "SPECIFIC ACTIVITY":
ISLETS CULTURED IN BASAL vs. HIGH-GLUCOSE

	%^{65}Zn cpm/%Zn ng	%^{65}Zn cpm/%Zn ng
Glucose Conc.:	5.6 mM	25.6 mM
	(N = 14)/(N = 5)	(N = 8)/(N = 3)
Fraction 1	1.94 / 10.63 = 0.18	3.23 / 11.62 = 0.28
Fraction 2	6.94 / 11.35 = 0.61	9.38 / 9.78 = 0.96
Fraction 3	3.63 / 10.99 = 0.33	3.29 / 13.46 = 0.24
Fraction 4	3.27 / 7.93 = 0.41	3.17 / 13.46 = 0.23
Fraction 5	7.18 / 9.73 = 0.74	7.61 / 11.31 = 0.67
Fraction 6	24.52 / 19.28 = 1.27	30.45 / 18.65 = 1.63
Fraction 7	52.78 / 30.09 = 1.75	42.14 / 21.71 = 1.94

FIGURE 14

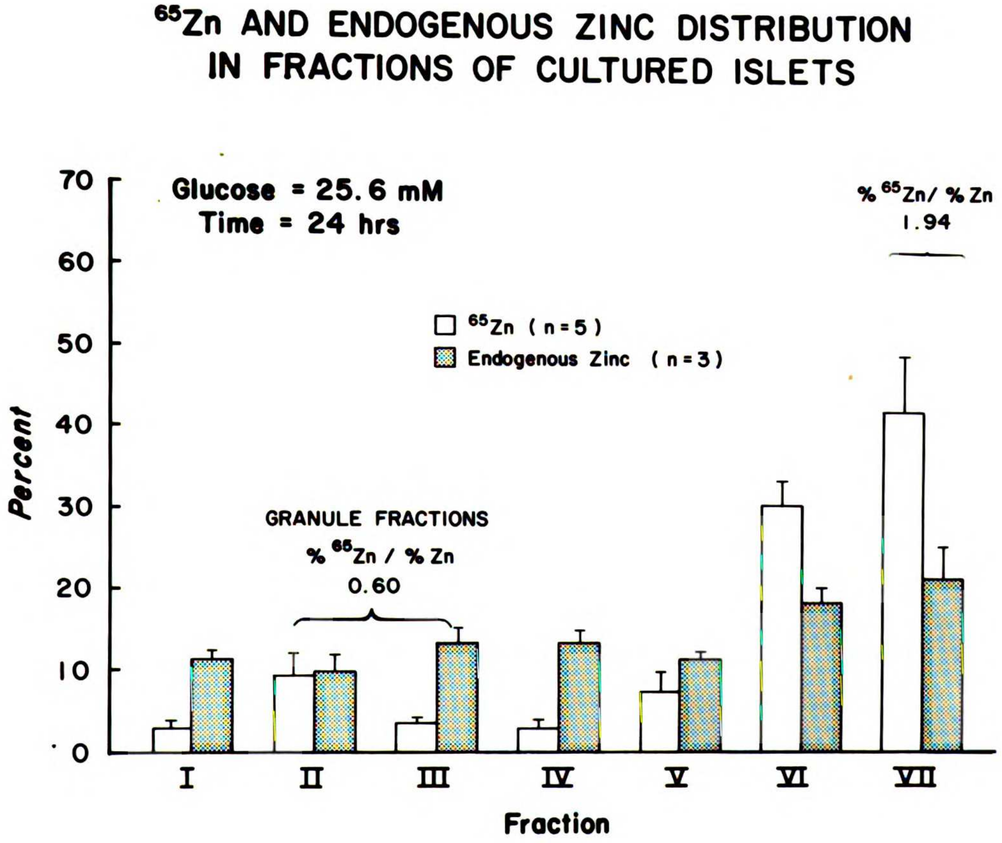

65Zn AND ENDOGENOUS ZINC DISTRIBUTION
IN FRACTIONS OF CULTURED ISLETS
Glucose = 25.6 mM
Time = 24 hrs
% 65Zn / % Zn
1.94
□ 65Zn (n = 5)
▨ Endogenous Zinc (n = 3)
GRANULE FRACTIONS
% 65Zn / % Zn
0.60
Percent
70
60
50
40
30
20
10
0
I
II
III
IV
V
VI
VII
Fraction

TABLE 13

DISTRIBUTION OF ^{65}ZN, ZINC, AND INSULIN IN FRACTIONS.

A. Islets Cultured in High vs. Basal Glucose

		Percent*					
		^{65}Zn (cpm/islet)		Insulin (ng/islet)		Endogenous Zinc (pmol/islet)	
Glucose Conc.:		25.6 mM	5.6 mM	25.6 mM	5.6 mM	25.6 mM	5.6mM
(N)		(5)	(5)	(3)	(8)	(3)	(5)
Homogenate	X	202	243**	25	53**	4	9**
	±SEM	±18	±17	±1	±9	±0.1	±0.05
Supernatant (% Homog. activity)	X	62	49	46	48	61	65
	±SEM	±5	±3	±5	±9	±7	±17
Sediment (% Homog. activity)	X	35	40	38	55	19	41
	±SEM	±3	±1	±2	±10	±2	±7
Fraction 1	X	3	2	3	9	12	11
	±SEM	±1	±0.1	±0.2	±4	±2	±2
Fraction 2	X	13	13	26	44	10	12
	±SEM	±3	±2	±4	±15	±2	±1
Fraction 3	X	4	6	12	20	13	11
	±SEM	±1	±1	±1	±7	±3	±2
Fraction 4	X	4	5	5	9	13	9
	±SEM	±1	±1	±0.5	±4	±1	±1
Fraction 5	X	11	7	8	6	11	11
	±SEM	±4	±1	±2	±3	±2	±1
Fraction 6	X	28	21	14	5	19	18
	±SEM	±4	±6	±6	±1	±1	±3
Fraction 7	X	38	46	26	6	22	28
	±SEM	±11	±9	±1	±1	±3	±4

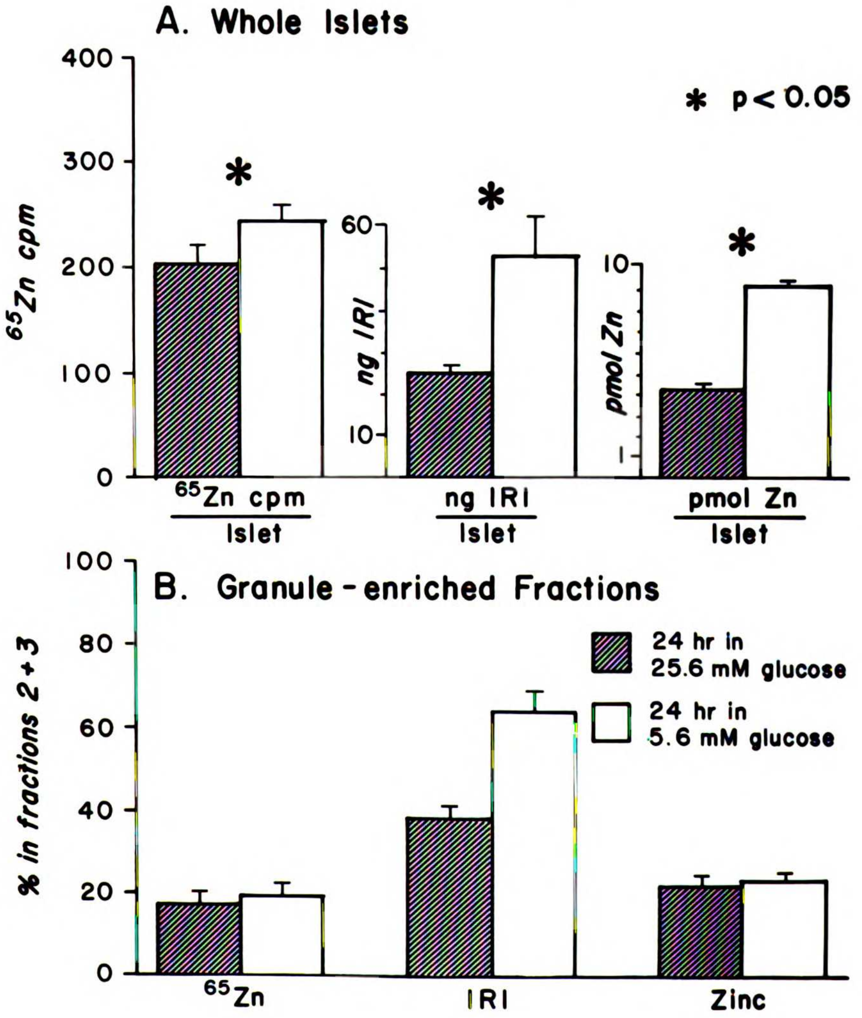
---

* Activities in fractions 1 through 7 total 100%.

** $p < 0.05$ compared to "high-glucose" value

FIGURE 15

CONTENT OF ^{65}Zn, ZINC AND INSULIN IN WHOLE ISLETS AND GRANULE-ENRICHED FRACTIONS:
High vs Low Glucose



basal glucose controls, as well as a lower insulin content (Table 14). A significantly lower percentage of both ^{65}Zn and insulin was found in one of the granule fractions from islets cultured with arginine--glucose as compared with granule fractions from islets cultured in glucose alone.

C. Tolbutamide--treated vs. Control. Treatment of rats with sodium tolbutamide, 500 mg/kg, for three days resulted in a significant depletion of islet insulin content (28% of control value; Table 15). Islets were fragile and translucent in appearance, and lower islet yields were obtained from rats treated with tolbutamide than rats treated with water (controls). After 24 hr of culture, tolbutamide-treated islets showed no further decline in total islet insulin content whereas their corresponding controls showed the usual decrease in insulin content (Table 16). Thus, by 24 hr, insulin content of tolbutamide islets was not significantly different from control islets. Control islets also demonstrated a decline in endogenous zinc content, as described previously, while zinc content of tolbutamide-- treated islets did not decrease during the culture period. No difference was observed in the amount of insulin secreted in response to the basal glucose in the culture medium (8.25 ng/tolbutamide--treated islet vs. 6.32 ng/control islet); the apparent

TABLE 14

DISTRIBUTION OF ^{65}ZINC AND INSULIN IN FRACTIONS.

B. Islets Cultured in 10 mM Arginine-5.6 mM Glucose vs. 5.6 mM Glucose

		Percent*			
		^{65}Zinc (cpm/islet)		Insulin (ng/islet)	
Culture Medium:		Arg.-Glucose	Glucose	Arg.-Glucose	Glucose
(N)		(6)	(6)	(4)	(4)
Homogenate	X	567	646**	53	38
	±SEM	±90	±85	±10	±4
Supernatant (% Homog. Activity)	X	45	55	33	42
	±SEM	±7	±7	±7	±12
Sediment (% Homog. Activity)	X	53	44	43	45
	±SEM	±9	±6	±13	±11
Fraction 1	X	4	3	4	5
	±SEM	±1	±1	±1	±0.5
Fraction 2	X	15	16	16	17
	±SEM	±4	±4	±3	±2
Fraction 3	X	7	8	13	16
	±SEM	±1	±1	±0.5	±2
Fraction 4	X	7	7	16	17
	±SEM	±1	±1	±0.5	±2
Fraction 5	X	12	11	19	19
	±SEM	±1	±2	±1	±2
Fraction 6	X	31	25	22	19
	±SEM	±5	±2	±2	±4
Fraction 7	X	21	29	10	6
	±SEM	±9	±7	±1	±1

* Activities in fractions 1 through 7 total 100%.

** $p < 0.05$ compared to arg.-glucose value

TABLE 15

DISTRIBUTION OF ZINC AND INSULIN IN FRACTIONS.

D. Fresh Islets Following *in vivo* Administration of Tolbutamide vs. Water

		Percent*			
		Insulin (ng/islet)		Endogenous Zinc (pmol/islet)	
Treatment:		Tolbutamide	Water	Tolbutamide	Water
(N)		(5)	(7)	(5)	(7)
Homogenate	X	19	64**	6	7
	±SEM	±4	±6	±1	±1
Supernatant (% Homog. Activity)	X	44	41	41	67
	±SEM	±5	±6	±7	±14
Sediment (% Homog. Activity)	X	41	37	17	22
	±SEM	±5	±3	±2	±4
Fraction 1	X	2	3	16	14
	±SEM	±1	±1	±7	±4
Fraction 2	X	17	22	25	21
	±SEM	±4	±4	±4	±2
Fraction 3	X	15	19	3	9
	±SEM	±4	±3	±2	±2
Fraction 4	X	13	12	5	7
	±SEM	±1	±2	±2	±2
Fraction 5	X	15	12	6	9
	±SEM	±1	±2	±5	±2
Fraction 6	X	21	17	9	14
	±SEM	±3	±2	±4	±2
Fraction 7	X	15	13	36	25
	±SEM	±7	±1	±10	±6

* Activities in fractions 1 through 7 total 100%.
** $p < 0.05$ compared to tolbutamide value

TABLE 16

DISTRIBUTION OF ^{65}ZN, ZINC, AND INSULIN IN FRACTIONS.

E. Islets Cultured 24 Hr. in Basal Glucose Following *in vivo* Administration of Tolbutamide vs. Water

				Percent*			
		^{65}Zn (cpm/islet)		Insulin (ng/islet)		Endogenous Zinc (pmol/islet)	
Treatment:		Tolbutamide	Water	Tolbutamide	Water	Tolbutamide	Water
(N)		(7)	(8)	(5)	(6)	(4)	(6)
Homogenate	X	314	236**	17	25	5***	6
	±SEM	±29	±21	±3	±3	±0.5	±1
Supernatant (% Homog. Activity)	X	61	68	64	63	54***	53
	±SEM	±3	±3	±23	±14	±4	±3
Sediment (% Homog. Activity)	X	32	26	38	49	23***	28
	±SEM	±3	±2	±19	±20	±3	±3
Fraction 1	X	5	6	11	11	16	13
	±SEM	±1	±2	±5	±5	±3	±5
Fraction 2	X	12	13	25	25	18	16
	±SEM	±2	±2	±7	±3	±3	±2
Fraction 3	X	5	9	9	18**	8	14
	±SEM	±1	±2	±1	±3	±3	±4
Fraction 4	X	6	7	7	9	4	6
	±SEM	±1	±2	±2	±1	±2	±1
Fraction 5	X	13	14	11	9	8	10
	±SEM	±3	±4	±1	±1	±2	±2
Fraction 6	X	42	25	22	13	20	15
	±SEM	±7	±6	±9	±1	±7	±3
Fraction 7	X	18	26	15	15	26	26
	±SEM	±7	±7	±1	±2	±5	±3

* Activities in fractions 1 through 7 total 100%.

** $p < 0.05$ compared to tolbutamide value

*** N = 8

enhanced synthetic activity of the tolbutamide islets was also reflected in enhanced net uptake of ^{65}Zn as compared to controls. However, no difference in the percentage of endogenous zinc, ^{65}Zn, or insulin localized to the granule fractions was observed between the two (Table 16; Figure 16).

D. Low Ca^{++}--High Glucose vs. Normal Ca^{++}--High Glucose. Islets were cultured 48 hr in a high glucose (25.6 mM)--low Ca^{++} medium in order to enhance insulin synthesis while inhibiting secretion. Measurement of insulin secreted into the culture medium at 24 and 48 hr revealed an inhibition of approximately 50% and 75%, respectively (Table 17). When removed from culture and tested for insulin response to a glucose challenge, islets previously cultured in low Ca^{++} showed an enhanced responsivity (Table 18). Islets in high glucose with regular calcium displayed a significant decline (approximately 40%) in insulin secretion between 24 and 48 hr of culture. Islets in low Ca^{++} had a significantly higher insulin content, and less of a decrement of zinc content (i.e., higher zinc content than islets cultured in high glucose--regular calcium but lower than the zinc content of fresh islets); however no difference in net uptake of ^{65}Zn was observed (Table 19; Figure 17). No consistent changes in distributions of zinc, ^{65}Zn, or insulin in the granule

FIGURE 16

CONTENT OF ^{65}Zn, ZINC AND INSULIN IN WHOLE ISLETS AND GRANULE-ENRICHED FRACTIONS:
Tolbutamide-treated vs Control Islets

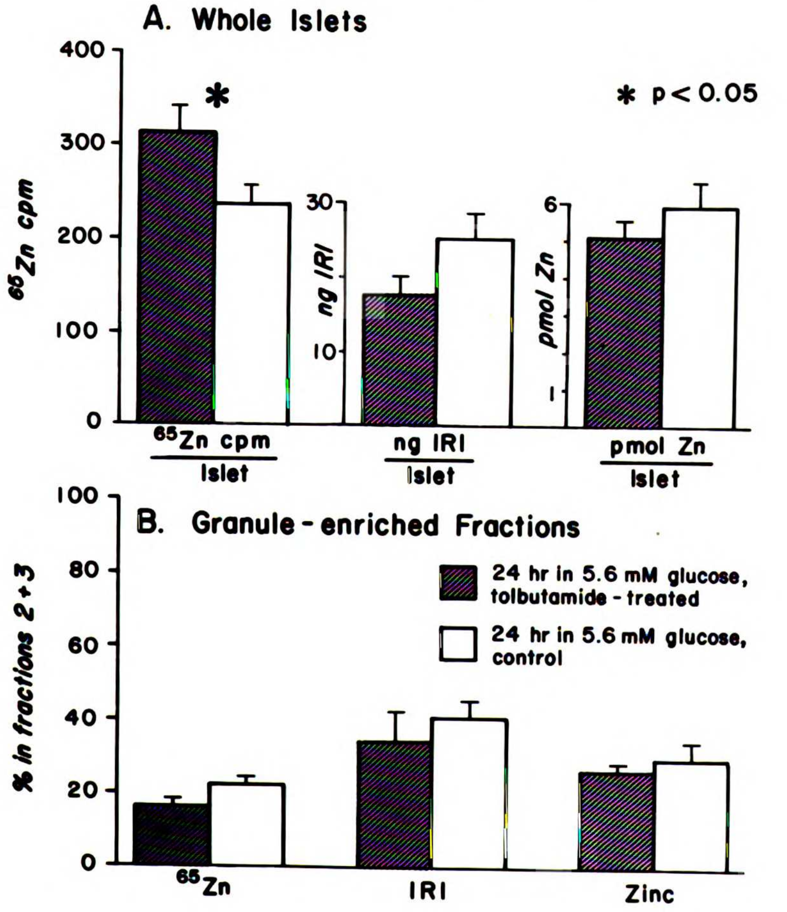


TABLE 17

INSULIN SECRETION FROM 48-HR CULTURED ISLETS

	Insulin Secretion (ng/islet)			
Time of Culture:	0-24 hr		24-48 hr	
Culture Medium:	Low Ca^{2+}- High Glucose	Reg. Ca^{2+}- High Glucose	Low Ca^{2+}- High Glucose	Reg. Ca^{2+}- High Glucose
X	29	52*	9	32*
±SEM	±4	±7	±2	±3
(N)	(11)	(11)	(12)	(12)

* $p < 0.05$ as compared to Low Ca^{2+}-High Glucose value

TABLE 18

GLUCOSE RESPONSIVITY OF 48-HR CULTURED ISLETS

	Insulin Secretion (ng/islet x 30 min)			
Culture Medium:	Low Ca^{2+}- High Glucose		Reg. Ca^{2+}- High Glucose	
Glucose Conc.:	1 mM	20 mM	1 mM	20 mM
X ±SEM	0.21* ±0.03	1.64** ±0.21	0.35 ±0.07	1.33*** ±0.28
(N)	(11)	(11)	(11)	(11)

* $p < 0.05$ as compared to regular Ca^{2+}-high glucose value

** $p < 0.05$ as compared to basal glucose value

*** $p < 0.05$ as compared to basal glucose value, and as compared to low Ca^{2+}-high glucose, 20 mM glucose value

TABLE 19

DISTRIBUTION OF ^{65}ZN, ZINC, AND INSULIN IN FRACTIONS.

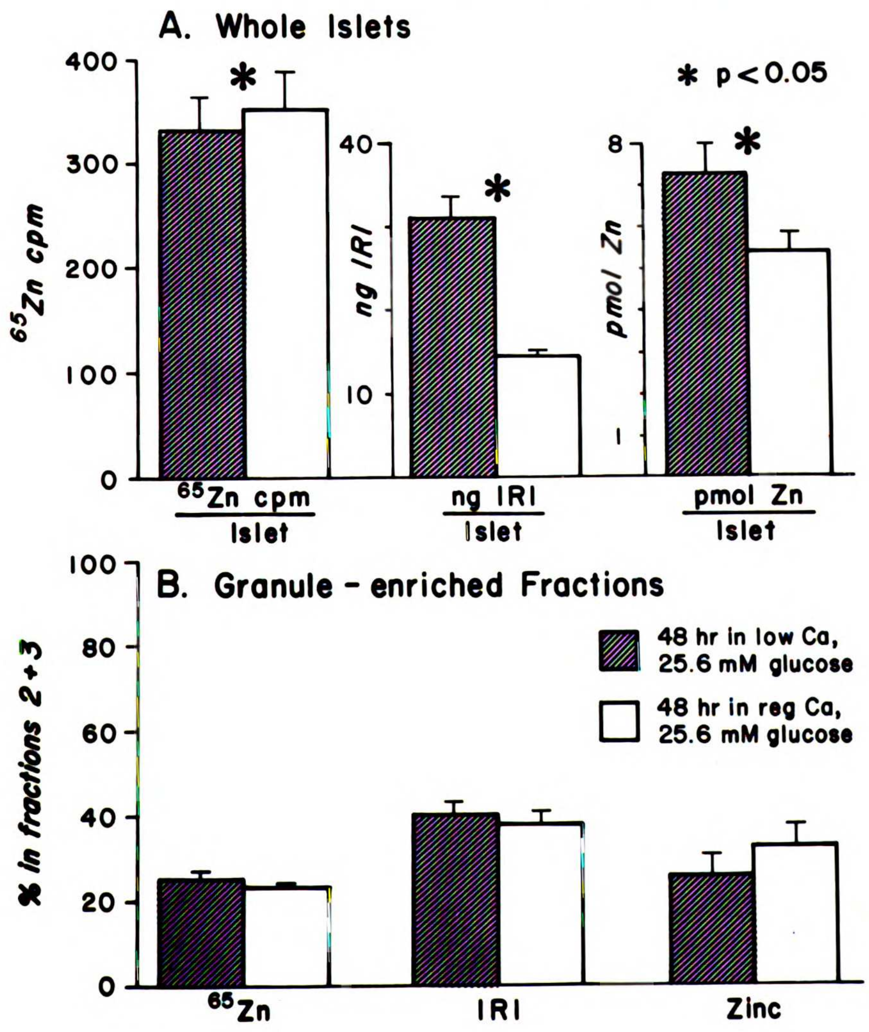
C. Islets Cultured 48 Hr. in High Glucose-Low Calcium vs. High Glucose-Regular Calcium

		Percent*					
		^{65}Zn* (cpm/islet)		Insulin (ng/islet)		Endogenous Zinc (pmol/islet)	
Calcium Conc.:		Low Ca^{2+}	Reg. Ca^{2+}	Low Ca^{2+}	Reg. Ca^{2+}	Low Ca^{2+}	Reg. Ca^{2+}
(N)		(7)	(7)	(6)	(6)	(6)	(6)
Homogenate	X	332	354**	31	15**	7	5**
	±SEM	±34	±36	±2	±1	±1	±0.5
Supernatant (% Homog. Activity)	X	60	49	49	49	—	—
	±SEM	±1	±4	±4	±4	—	—
Sediment (% Homog. Activity)	X	33	29	50	58	—	—
	±SEM	±2	±3	±8	±8	—	—
Fraction 1	X	7	10	4	5	7	8
	±SEM	±2	±2	±1	±1	±1	±2
Fraction 2	X	18	15**	23	24	17	21
	±SEM	±1	±1	±4	±4	±5	±7
Fraction 3	X	7	8	16	15	9	12**
	±SEM	±1	±1	±3	±2	±1	±2
Fraction 4	X	8	6	10	14**	7	10
	±SEM	±1	±0.5	±1	±1	±1	±2
Fraction 5	X	13	10	10	15	9	9
	±SEM	±3	±1	±2	±3	±1	±2
Fraction 6	X	31	26	17	20	19	15
	±SEM	±3	±4	±4	±2	±3	±2
Fraction 7	X	15	25	39	8	33	24
	±SEM	±6	±6	±3	±2	±5	±4

* Activities in fractions 1 through 7 total 100%.

** $p < 0.05$ compared to "low-Ca^{2+}" value

FIGURE 17



CONTENT OF ^{65}Zn, ZINC AND INSULIN IN WHOLE ISLETS AND GRANULE-ENRICHED FRACTIONS:
Low Ca - High Glucose vs High Glucose

fractions of the two groups were observed. Hence, although insulin synthesis was enhanced, no novel class of insulin granule was being formed.

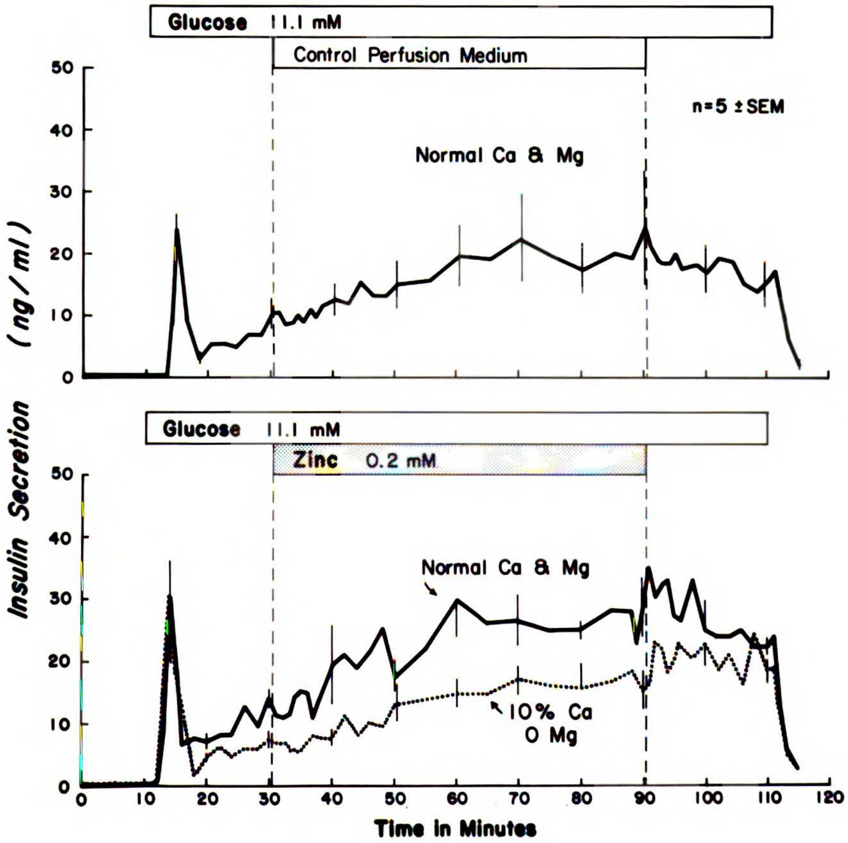
Influence of Exogenous Zinc on Insulin Secretion.

Table 2 summarizes the various concentrations of zinc used for pancreatic perfusions, ranging from physiologic (10--20 µM) to pharmacologic (200 µM) levels. Neither addition of zinc in single steps (e.g., 50 µM to 200 µM) nor perfusion at the same concentration throughout, significantly influenced the insulin secretory response to 11 mM glucose. In order to maximize the possible effects of zinc on insulin release from the perfused pancreas, Ca^{++} and Mg^{++} levels in the perfusate were manipulated. Mg^{++} was eliminated in order to reveal a possible suppressive effect of Zn; Ca^{++} was dropped to 10% of its normal concentration in order to demonstrate a possible enhancement of Zn on insulin secretion. Alterations of Ca^{++} and Mg^{++} concentrations did not reveal an influence of zinc on insulin secretion at any of the zinc concentrations utilized. Figure 18 shows, as an example, the insulin secretory pattern of pancreases exposed to the highest concentration of zinc used as compared to control perfusions. No influence of 200 µM zinc on insulin secretion could be observed.

FIGURE 18

LACK OF EFFECT OF ZINC ON INSULIN RELEASE

BY THE PERFUSED PANCREAS



In more long term experiments, pancreatic islets were cultured 8 hr in the presence of either 0.15 µM; 50 µM; or 200 µM zinc. Subsequent incubation of these islets in zinc-free KRB with 1 and 20 mM glucose resulted in a 5--6 fold response of the islets to the glucose challenge (Table 20); no difference in either basal or glucose--stimulated insulin release by islets which had been cultured with either 0.15, 50, or 200 µM Zn was observed.

In a second series of islet experiments, islets were cultured with the same zinc additions; further, islets were incubated in KRB containing the same zinc "concentrations" to which they had been exposed during culture. No change was observed in insulin release to 1 mM glucose; however a significant inhibition of glucose--stimulated insulin release by islets cultured and incubated in 200 µM Zn, as compared to 0.15 µM Zn, was observed (Table 21; Figure 19). Culture and incubation of islets in 50 µM Zn had no effect on insulin secretion.

TABLE 20

EFFECT OF EXOGENOUS ZINC ON ISLET INSULIN SECRETION.

A. 8-Hr Culture of Isolated Islets With Added Zinc

	Insulin Secretion (ng/islet x 30 min)					
Zinc Conc.:	---		50 µM		200 µM	
Time (min):	0-30	30-60	0-30	30-60	0-30	30-60
Glucose Conc.:	1 mM	20 mM	1 mM	20 mM	1 mM	20 mM
X ±SEM	0.11 ±0.04	0.51 ±0.09	0.08 ±0.02	0.52 ±0.10	0.10 ±0.04	0.55 ±0.13
(N)	(5)		(5)		(4)	

TABLE 21

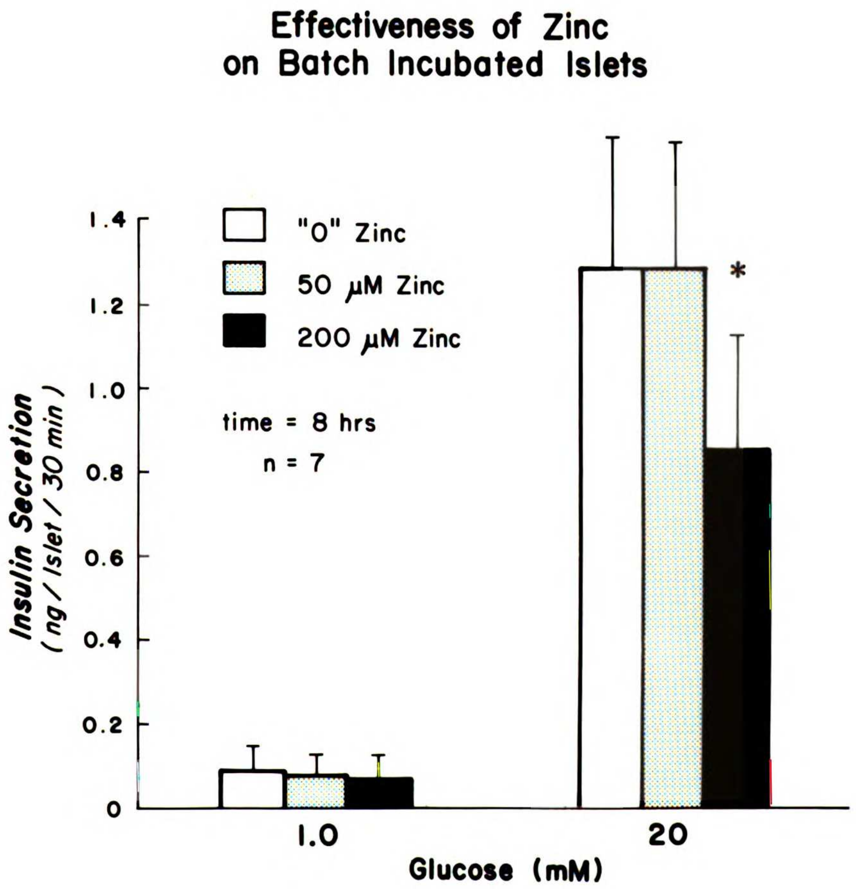
EFFECT OF EXOGENOUS ZINC ON ISLET INSULIN SECRETION.

B. 8-Hr. Culture of Isolated Islets
and Subsequent Incubation with Added Zinc

	Insulin Secretion (ng/islet x 30 min)					
Zinc Conc.:	---		50 µM		200 µM	
Time (min):	0-30	30-60	0-30	30-60	0-30	30-60
Glucose Conc.:	1 mM	20 mM	1 mM	20 mM	1 mM	20 mM
X	0.09	1.29	0.08	1.29	0.07	0.86*
±SEM	±0.06	±0.32	±0.05	±0.30	±0.06	±0.27
(N)	(7)		(7)		(7)	

* $p < 0.05$ when compared to 30-60 min, "no added zinc" value

FIGURE 19


Effectiveness of Zinc on Batch Incubated Islets
Insulin Secretion (ng / Islet / 30 min)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
"0" Zinc
50 μM Zinc
200 μM Zinc
time = 8 hrs
n = 7
*
1.0
20
Glucose (mM)

DISCUSSION

Zinc Content of Isolated Islets.

Measurement of the endogenous zinc content of whole rat islets reveals that, on a molar basis, the islet contains more than twice as much zinc as would be needed to form a two--zinc insulin hexamer. In fact, the rat islet contains approximately equimolar amounts of insulin and zinc, as was observed in mice by Berglund and Hellman (60). The value obtained by this laboratory is significantly lower than that obtained by Havu et al. (57) of approximately 22 pmol/islet in the rat: such a difference may be ascribed to differences in sample preparation and the selectivity of the atomic absorption spectrophotometer for the zinc atom, as well as the fact that Havu et al. measured zinc absorption at a different wavelength. In fact, our value is within the range of zinc contents associated with "zinc-deficient" animals (91). However, both our value and that of Havu et al. represent a significant quantity of zinc in the islet above that which would be crystallized with insulin as a two zinc hexamer.

Chemical Studies.

Insulin--zinc interactions were studied under conditions which simulated both the islet *in vivo* or *in vitro*, and the experimental conditions used for islet

fractionation. Simulation of in vivo conditions--pH 7.4, 37°C--resulted in an extremely rapid binding of insulin and zinc. This suggests that in vivo, where high concentrations of insulin, or proinsulin (19), and zinc may exist such as in the rough endoplasmic reticulum or the secretory granule, a rapid complexing of insulin and zinc should occur. That the binding of ^{65}Zn to insulin in our chemical studies is high affinity in nature is suggested by the fact that when preformed complexes were exposed to high levels of ("cold") zinc, the hot zinc which was already bound was not readily displaced. It would be anticipated that the extent of low affinity binding would parallel displacement of bound zinc. Thus results of our binding studies are in general agreement with the results of more rigorous binding studies (15,16) which show high affinity zinc binding at physiologic pH.

An additional significance of this "increased cold Zn" experiment lies in the fact that it simulates the islet fractionation conditions. Whole islet measurements suggest that there is more zinc in the islet than can be accounted for by binding to insulin alone, or binding to proinsulin also as a two zinc hexamer. Homogenization and handling of the islets in a relatively large volume might be expected to either break organelles which sequester some of the "excess" zinc,

or to cause a shift in equilibrium, with the addition of a large zinc-free volume of solution, such that the "excess" zinc is released as free zinc in the homogenization buffer. Based on our chemical data, we would expect that this increase in free zinc concentration should have no effect on the islet insulin--zinc complexes already formed, for the duration of the experiment. As the fractionation experiments were run at pH 6, we examined the influence of simultaneous lowering of the pH and increasing the free zinc concentration. This resulted in a more rapid dissociation of the ^{65}Zn from insulin, again corroborating previous binding studies which showed a loss in zinc binding affinity of the insulin molecule upon a decrease of pH. This control experiment essentially examined insulin--^{65}Zn binding under the least optimal conditions, since, as yet, no evidence exists to suggest that in pancreatic islets alteration of extragranular pH will cause a similar shift in intragranular pH; β-granules are thought to maintain an autonomous pH, and proton gradient, for the purpose of accumulating amines (22). Even under these least optimal conditions, dissociation of the complex was only about 20% during the time, and under the conditions, of fractionation. Further, if zinc indeed exists in a crystalline state with insulin--essentially out of solution--inside the

granules, which themselves remain intact through the fractionation procedure, then the complexes should not be influenced by the large volume dilution which occurs, and which would result in rapid dissolution of the unshielded complex in solution.

Islet Culture Controls.

The viability of islets following a 24-hr culture in basal glucose was assessed by two methods. Buitrago et al. reported that their islets showed an enhanced release of insulin at basal and low glucose levels. In order to insure that this was not the result of increased permeability of islet cell membranes (i.e., "leaking" of insulin as opposed to "secretion" of insulin), we measured ^{3}H-sucrose uptake in cultured islets, as well as in their respective controls, the newly isolated islets. ^{3}H-sucrose does not penetrate intact cell membranes, and has a characteristic volume distribution in the interstitial space of the islet (up to 4 nl (79,92)). Our results demonstrate that the islets, indeed, do not become significantly leaky over the culture period. Ability of the islets to respond to a glucose challenge was also assessed. The reduced responsivity of islets cultured in basal or low glucose has been documented; Andersson et al. (81,82) observed decreased insulin biosynthesis and islet content, as well as a decreased responsivity to a glucose stimulus

in islets cultured in low glucose. Our islets retained secretory sensitivity to glucose with only a slight decrease in responsivity at 24 hr.

Characterization of Islet Fractions.

A "granule-enriched" fraction was localized in the 50% layer of the sucrose gradient. Granules were isolated at pH 6, a pH at which granules exhibit maximal stability (33). The granule-enriched fraction prepared from newly isolated islets contained about 40% of total islet insulin (Figure 8), and presumably its associated zinc; the small amounts of insulin at the top of the gradient might represent insulin in smaller or less dense granules or vesicles, or perhaps insulin released from broken granules damaged by the fractionation procedure. Electron microscopy (Fig. 10) of the fraction revealed that the major contaminant of the fraction was rough endoplasmic reticulum (rer); a fair number of empty vesicles are also visible. Such vesicles might be granules, cut so that the core was not included; smooth er; or plasma membrane vesicles (an unlikely possibility). Measurement of RNA as a crude marker of rer in the sucrose fractions indicated that a significant amount of total islet RNA--and perhaps almost the total islet cells' rer--was migrating with the granules. Rough er contamination has been a long standing problem in the purification of a secretory granule

fraction. Coore et al. (52) attempted further purification by phase distribution in polyethylene glycol and dextran, following conventional sucrose gradients, with no success. Other methodologies, e.g., ultrafiltration, and isopycnic density gradient centrifugation have also been unsuccessful (90). Further purification attempts in this laboratory included use of finer gradations of sucrose in the gradient, an initial incubation with RNAase to dissociate ribosomes from the r.e.r. vesicles, (and presumably to decrease their density), and use of D_2O instead of H_2O in the gradients. No significant purification resulted from any of these attempts.

One consistent finding during the course of these studies was that, upon culture, islet granule fractions contained significantly less insulin, and, correspondingly, more insulin was found in the upper fractions of the gradient. This suggests that granules lose some stability--become more fragile--as a result of the culturing procedure. In contrast, the percentage of zinc associated with the granule-enriched fraction did not decline in islets as a function of the culture procedure. Perhaps this zinc is associated mainly with the rer and other cell organelles in the granule fraction, thus corroborating our original findings of low amounts of zinc associated with large quantities of

insulin in this fraction.

^{65}Zn Uptake Controls.

The possibility of significant distribution of ^{65}Zn (layered at the top of the sucrose gradient) throughout the sucrose layers during the time of centrifugation was eliminated. As is shown in Fig. 11, free ^{65}Zn does not diffuse down the gradient during the time course of the centrifugation; virtually 100% of this ^{65}Zn remains in the upper two fractions. A second possible artifact examined was that of loose interaction and binding of ^{65}Zn to islet components, upon homogenization. As with free ^{65}Zn layered over the sucrose gradient, very low percentages of ^{65}Zn were found in the bottom five fractions if islet tissue was included in the protocol, although slightly more ^{65}Zn was localized in these fractions. This suggests that only a very small amount of non-biologic association of ^{65}Zn with islet cell components may occur during fractionation. A final problem which was considered was the possibility that upon dilution and mechanical handling of the islets, already bound zinc might dissociate, and either remain free, or re-bind to other islet components. We observed no difference in the ^{65}Zn distribution whether islets were fractionated immediately, or 3 hours after, homogenization. It was concluded that either zinc redistribution is virtually

instantaneous, or that it occurs so slowly that virtually none occurs during the 3 hr isolation period. Andersson et al. subsequently (59) used EDTA to "freeze" zinc in the homogenate prior to fractionation and measurement of endogenous zinc. Their results were in fair agreement with the findings presented here, thus supporting the validity of our conclusion.

^{65}Zn Net Uptake and Distribution in the Islets.

^{65}Zn net uptake by whole islets was shown to be a slow process, in accord with the findings of Ludvigsen (38). Equilibrium was not reached by 24 hr (and equilibration of the newly taken-up ^{65}Zn with endogenous zinc pools had not occurred (vide infra)).

In contrast with Ludvigsen's findings, a diminution of net uptake by islets cultured in high glucose was observed, and was first apparent at 4 hr. Such a diminution may be due to either a decrease in uptake or an increase in efflux. The latter hypothesis is appealing if one visualizes newly taken up ^{65}Zn being associated, and ultimately released, with newly synthesized insulin, particularly since there was a 2--3 hr time lag before the effect was observed. (Although the "time lag" may be due to technical limitations of the uptake experiments, it should be noted that no significant differences could be observed within a few hours, whereas highly significant changes in calcium

uptake have been observed within 60 minutes in our laboratory.) There is no obvious reason underlying the disparity between these findings and those of Ludvigsen, but it is emphasized that the "glucose effect" (albeit small) was observed for both total endogenous zinc content, as well as net ^{65}Zn uptake.

^{65}Zn distribution was measured following 24 hr culture in basal glucose. As mentioned above, only 19% of the ^{65}Zn was found which was localized to the granule-enriched fractions, in contrast to 64% of the of insulin localized there (Table 13). This observation is in sharp contrast to previous histochemical observations, i.e., that islet zinc is almost completely localized to islet granules. As the percentage of ^{65}Zn was greater than that found when newly isolated islets were fractionated with ^{65}Zn added at "zero" time, the distribution of ^{65}Zn must be due to specific uptake or incorporation of ^{65}Zn by granules (and other organelles) during the culture period.

Even at 48 hr, islet uptake of ^{65}Zn was far from being equilibrated with the culture medium. As discussed above, and shown in Table 13, the %^{65}Zn/%Zn ratio in the granule fractions was significantly lower than that found in the upper fractions. Thus, zinc reaching the cytosol does not equilibrate rapidly with granular zinc, suggesting that zinc entry may occur

during de novo synthesis of the granule, rather than by rapid exchange across the granular membrane. Culture of the islets in high glucose for 24 hr did increase the specific activity of the granule fractions, although not to a very great extent. This increased specific activity suggests that a greater rate of incorporation of ^{65}Zn into new granules--and greater granule turnover due to stimulated secretion--results in a somewhat "hotter" pool of granular zinc. Although zinc specific activities in all the fractions were not the same (Table 23), the percentages of ^{65}Zn and endogenous zinc localized to the granules were approximately equal by 48 hr, suggesting that equilibration within the granule zinc pool had occurred. Thus, equilibration within (smaller) islet zinc pools appeared to be slow but much faster than the rate of entry of extracellular zinc.

Measurements of the endogenous zinc localized to the granule-enriched fractions show that only about 20% of the endogenous islet zinc is associated with granules. This finding is also in conflict with early histochemical findings that islet zinc is confined to the granules, although it seems likely that large amounts of zinc might either have been washed out, or were not detected, in the histologic studies. The insulin--zinc ratio in the granule fractions was deter-

mined to be about 0.70 under most culture conditions. This quantity of zinc is sufficient to account for 2-Zn insulin hexamers (ratio = 0.33). However limitations in the precision of the atomic absorption spectrophotometer and radioimmunoassay make it difficult to conclude this definitively. We can conclude that the existence of stable zinc-insulin complexes exceeding 2 zinc hexamers is highly unlikely.

Exposure of islets to 10 mM arginine in the presence of basal (5.6 mM) glucose results in a release of insulin and glucagon (93) as well. Arginine is believed to stimulate insulin secretion via a different mechanism than glucose (94). Similar to the high-glucose stimulus, 24 hr culture in arginine--glucose resulted in a net diminution of ^{65}Zn net uptake. However, at 24 hr there was no significant difference in the insulin content of the islets cultured in arginine as compared with their low glucose culture controls; perhaps the secretory rate with this stimulus was matched by the islets' synthetic rate. As with high glucose, no change in the percentage of ^{65}Zn cpm associated with granules was observed. Thus the storage forms and intracellular distribution of ^{65}Zn are affected by arginine as they are by (high) glucose.

In a previous study by Wolters (66), significant depletion of islet insulin, as well as zinc (stainable

by dithizone), was observed in islets following _in vivo_ tolbutamide treatment. This study was expanded here by following the same _in vivo_ treatment, with 24 hr' culture in basal glucose. In the freshly isolated islets, a 70% decrease of insulin content was accompanied by only a 10% drop in islet zinc as measured by atomic absorption spectrophotometry. Wolters et al. themselves point out the likely reason for the disparity in the data: "stainable zinc" by no means represents "total islet zinc." It is possible that the zinc which was depleted had been complexed with insulin. However, if one approximates that 30% of islet zinc is associated with insulin, then a 70% reduction in insulin should result in a 21% drop in islet zinc. Thus, our observation can be added to the few others (49,59) which suggest that quantitative changes in islet insulin content do not necessarily parallel changes in islet zinc. Perhaps granule-associated zinc is associated with the granular membrane, and is recycled with membranous material upon secretion, rather than being released with insulin. Such an interpretation of the data is consistent with our low Ca^{+2} data as well (vide infra), but does not explain the equimolar losses of zinc and insulin found in high glucose studies.

Although the tolbutamide protocol was designed to contrast islets with depleted insulin (95) and

suppressed insulin synthesis (96) with normal islets, in fact, the tolbutamide islets recovered function rapidly in the low glucose culture medium so that by 24 hr their insulin content was essentially not different from islets isolated from control rats and also cultured in low glucose. Significantly less insulin was localized in one of the two granule fractions from the tolbutamide islets: the physiologic significance of this finding is not clear, but the data suggests a possible structural change (decreased stability?) in the insulin storage compartments after prolonged tolbutamide treatment. Although insulin content was maintained in the tolbutamide islets, endogenous zinc content decreased further during the culture period, as did control islet zinc content. At all times, granular zinc content was still sufficient for the 2-Zn hexamer. Net uptake of ^{65}Zn over the culture period was significantly enhanced in the tolbutamide islets: this may be ascribed to an enhanced synthesis of insulin. Nevertheless zinc specific activity of the granule fractions was no greater in the tolbutamide islet fractions (Appendix) indicating that the equilibration rate of zinc had not been affected.

In a final series of experiments, a model with stimulated insulin synthesis and secretion (high glucose-normal Ca^{2+}) was contrasted to a model with

stimulated insulin synthesis and inhibited secretion (high glucose-low Ca^{2+}). As has been demonstrated previously, culture in low Ca^{++}-high glucose resulted in a significant depression of insulin release while in culture but a somewhat enhanced responsivity when removed from the low Ca^{++} medium (97). In our experiments, the inhibited release of insulin from the low Ca^{++} islets resulted in a significant enhancement of both total islet insulin and endogenous zinc contents as compared to their high glucose--regular Ca^{2+} controls. The islet zinc content/insulin content ratio in the high glucose-regular Ca^{2+} islets was higher than that found in fresh islets, as losses of insulin did not parallel losses of zinc. Net ^{65}Zn uptake was significantly lower in the low Ca^{++} islets, as compared to the regular Ca^{2+}--high glucose controls. Perhaps either low levels of Ca^{2+}, or the low secretory rate, may directly inhibit ^{65}Zn uptake. Isolated changes in the distributions of all three parameters--^{65}Zn, insulin, and endogenous zinc--were observed, but they did not follow any consistent pattern.

Granule-fraction contents of insulin, ^{65}Zn, and zinc, as well as changes in islet content of insulin and zinc, are summarized in tables on the following pages. Both ^{65}Zn and endogenous zinc are present as a low percentage of the fraction, not varying to a great

degree with either insulin content, or the culture condition. As discussed above, this leads one to question whether this zinc is, in fact, associated with insulin granules at all. That some zinc is associated with the granules is suggested by the peak of ^{65}Zn, with the insulin, in fractions from islets cultured under different conditions. A less pronounced peak of endogenous zinc is found; most of this zinc may be associated with other (contaminant) organelles which presumably would be distributed among a number of the sucrose fractions.

Islets lost both insulin and zinc as a result of the culture procedure. The losses varied under different culture conditions, in no uniform manner. For instance, islets removed from tolbutamide-treated rats lost 71% of their insulin, but only 10% of their zinc (insufficient to account for the loss as a two-Zn insulin hexamer, as discussed above). However, upon culture in basal glucose, insulin content was maintained, whereas a significant drop in zinc content occurred. As only very small amounts of insulin were secreted into the medium, it seems difficult to associate the zinc depletion with concomitant secretion of insulin. In contrast, zinc losses in islets cultured in either basal glucose (24 hr) or in high glucose-low Ca^{2+} are approximately one third of the amount of insulin depletion: the loss could be accounted for solely as secre-

tion as a two-zinc hexamer. It seems surprising that the low Ca^{2+}-high glucose islet, which is synthesizing, but not secreting insulin, is not similar to the tolbutamide-treated islet in culture, which also appears to be synthesizing, but not secreting, insulin. In fact, islet losses in the cultured, tolbutamide-treated, islet resemble those of the high-glucose cultured islet (24 and 48 hr), which is actively synthesizing and secreting insulin. In these islets, loss of zinc far exceeds that which would occur if zinc were localized solely in secretory granules. Perhaps a large quantity of zinc is lost "non-specifically" as a result of increased activity at the plasma membrane level in islets with enhanced secretion. Or perhaps highh glucose for extended periods of time is somewhat toxic to the islet: some increased membrane permeability ensues, and zinc is lost. Either of these may explain our observation that, in islets cultured in high glucose, a small diminution of ^{65}Zn net uptake occurs. The released zinc contributes to the extracellular pool of total zinc, and competes with the ^{65}Zn for (re-)entry into the islet. In conclusion, it seems difficult to present one consistent picture which accounts for the islet zinc and insulin data presented here. However, it seems reasonable to conclude that zinc loss during the culture period cannot be explained

by the simple phenomenon of zinc secretion as part of a two zinc hexamer.

Influence of Exogenous Zinc on Insulin Secretion.

Although a preliminary study by Ghafgazi et al. (56) reported a significant inhibition of insulin secretion from isolated islets by low pharmacologic levels (50 µM: serum levels are 15-20 µM) of exogenous zinc during a 30 min incubation, we found a total of 9 hr' exposure to higher pharmacologic levels (200 µM) were necessary to produce a significant effect. Neither laboratory was able to show an influence of high levels of zinc in the isolated perfused pancreas. One problem with both studies is that the free (and presumably "effective") concentration of zinc in the buffers used is not known. Most likely the majority of the added zinc in buffer or plasma is complexed by albumin, which can bind large quantities of zinc by high capacity, low affinity binding (98). (Biologic availability of this zinc is unknown.) It is interesting that Ghafgazi et al. observe an inhibition although their incubation buffer contains more albumin than the buffer used by this laboratory. A 9-day culture in zinc depleted medium was shown to have no significant effect upon granule stability or insulin secretion (67) although conversion of proinsulin to insulin was signi-

TABLE 22

GRANULE-ENRICHED FRACTIONS' CONTENT OF INSULIN AND ZINC UNDER DIFFERENT CULTURE CONDITIONS

Culture Condition	Percent ^{65}Zn	Percent Endogenous Zinc	Percent Insulin
5.6 mM Glucose, 24 hr.	19	23	64
25.6 mM Glucose, 24 hr.	17	23	38
10 mM Arginine, 5.6 mM Glucose, 24 hr.	22	—	29
Tolbutamide-treated, fresh	—	28	32
Tolbutamide-treated, 24 hr.	17	26	34
25.6 mM Glucose, 48 hr.	23	32	39
25.6 mM Glucose, low Ca^{2+}, 48 hr.	25	26	39

TABLE 23

CHANGES IN ISLET INSULIN AND ZINC CONTENTS UNDER VARIOUS CULTURE CONDITIONS*

Culture Condition	% Depletion of Insulin	% Depletion of Zinc
5.6 mM Glucose, 24 hr.	33%	17%
25.6 mM Glucose, 24 hr.	68%	62%
10 mM Arginine, 5.6 mM Glucose, 24 hr.	33%	—
Tolbutamide-treated, fresh	71%	10%
Tolbutamide-treated, 24 hr.	78%	54%
25.6 mM Glucose, 48 hr.	81%	52%
25.6 mM Glucose, low Ca^{2+}, 48 hr.	60%	34%

* Islet homogenate contents are compared to fresh islet values.

ficantly affected. It thus seems reasonable to conclude that either depletion, or enhancement, of exogenous zinc concentration within the physiologic range do not significantly alter islet insulin secretion.

CONCLUSION

Our studies corroborate previous findings that zinc is highly concentrated in the islets of Langerhans. However, in contrast to previous observations, we find only a small percentage of islet zinc associated with insulin in a granule-enriched fraction, although adequate to account for 2-zinc insulin hexamers. Under conditions which manipulate the insulin content of the islets, changes in zinc content, or net uptake, do not always correspond appropriately with the insulin content. We suggest that the majority of islet zinc does not appear to be associated with the insulin molecule or beta granule, although zinc may be involved in insulin synthesis, processing, or packaging. The possibility that zinc may be involved in other aspects of islet metabolism, which is stimulated in the presence of insulin secretagogues, should not be excluded. Finally, exogenous zinc over a wide range of concentrations does not appear to significantly influence the insulin secretory process.

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APPENDIX

TABLE 24

ZINC "SPECIFIC ACTIVITY": ISLETS FROM TOLBUTAMIDE-TREATED AND CONTROL RATS

	$\%^{65}$Zn/%Zn nq	$\%^{65}$Zn/%Zn ng
Treatemnt:	Tolbutamide	Saline
	(N = 7)/(N = 4)	(N = 8)/(N = 6)
Fraction 1	4.63 / 16.14 = 0.29	5.91 / 13.06 = 0.45
Fraction 2	11.71 / 17.62 = 0.66	13.10 / 15.53 = 0.84
Fraction 3	5.47 / 8.19 = 0.68	8.88 / 13.88 = 0.64
Fraction 4	5.68 / 4.35 = 1.30	7.07 / 6.40 = 1.10
Fraction 5	12.70 / 8.02 = 1.58	13.64 / 9.84 = 1.39
Fraction 6	41.89 / 20.12 = 2.08	24.84 / 15.36 = 1.62
Fraction 7	17.91 / 25.54 = 0.70	26.30 / 25.92 = 1.01

TABLE 25

ZINC "SPECIFIC ACTIVITY": ISLETS CULTURED 48 HR IN LOW CA^{2+}-HIGH GLUCOSE vs. REGULAR CA^{2+}-HIGH GLUCOSE

Culture Medium:	%^{65}Zn cpm/%Zn ng Low Ca^{2+}-High Glucose (N = 7)/(N = 6)	%^{65}Zn cpm/%Zn ng Reg. Ca^{2+}-High Glucose (N = 7)/(N = 6)
Fraction 1	6.64 / 6.71 = 0.99	9.98 / 7.88 = 1.27
Fraction 2	17.86 / 17.36 = 1.03	15.02 / 20.61 = 0.73
Fraction 3	7.27 / 8.64 = 0.84	8.09 / 12.12 = 0.67
Fraction 4	7.68 / 7.42 = 1.04	6.41 / 10.31 = 0.62
Fraction 5	13.22 / 8.72 = 1.52	10.16 / 9.46 = 1.07
Fraction 6	31.14 / 18.56 = 1.68	26.36 / 15.30 = 1.72
Fraction 7	14.95 / 32.58 = 0.46	24.99 / 24.30 = 1.03