

The Mechanism of Zinc Uptake in Isolated Islets of Langerhans

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SUMMARY

The uptake of zinc⁶⁵ (Zn) was determined in isolated pancreatic islets employing a dual isotope procedure with sucrose as an extracellular marker. Islets slowly accumulated Zn over long periods of time (24 h or longer) reaching three to five times the extracellular concentration by 70 min and a 30-fold differential over extracellular concentration by 24 h. Zn uptake at low concentrations (0.5–7 μM) demonstrated saturation kinetics with the K_m and V_{max} , 1.5 μM and 11.1 $\text{Fmol}/\text{islet}/\text{min}$, respectively. At higher Zn concentrations (10–3000 μM), a linear relationship between initial rates of uptake and Zn concentration was observed. Zn at a low concentration (2 μM) was countertransported by 2 mM ZnCl_2 pretreatment and inhibited by the presence of cadmium (2–20 μM). Countertransportation and cadmium inhibition of Zn uptake was not found at a higher Zn concentration (44 μM). Zn uptake at either 2 or 44 μM was not inhibited by pretreatment and presence of 300 μM dinitrophenol. Lowering of temperature from 37 °C to 20 °C and 4 °C depressed Zn uptake at 44 μM by 65% and 82%, respectively. However, at 2 μM Zn, the uptake was equivalent at 20 °C and 37 °C and showed a decrease of 70% at 4 °C. Zn uptake at either 44 or 2 μM in short (1–70 min) or long (1–24 h) studies was unaffected by concentrations of glucose that stimulated insulin secretion.

The results present the evidence that a facilitated mechanism of Zn transport predominates at low Zn concentrations and a diffusion mechanism of entry predominates at higher Zn concentrations. **DIABETES 28: 570–576, June 1979.**

Zinc was initially suspected to be an important component in packaging insulin, after Scott et al.¹ observed that this trace element facilitates the crystallization of insulin in solution. Zinc has since been firmly established as an integral part of the insulin

crystal of the beta granule by histochemical, electron microscopic, and x-ray diffraction techniques.^{2–6}

Studies by Maske et al.⁷ established a correlation between zinc concentration and insulin secretion by demonstrating that administration of either glucose or epinephrine to rabbits decreased the histologic staining for zinc within the beta cells of the rat. Bander et al.⁸ reported that several sulfonylurea agents administered to rats in vivo caused a loss of histochemically detectable zinc that appeared to parallel the degranulation of the beta cells. Autoradiographic studies by McIsaac et al.⁹ showed that the loss of zinc⁶⁵ from the rat pancreas was different for acinar and islet tissue. The acinar cells lost the radioactive isotope rapidly, whereas the concentration in the islet cells remained high and fairly constant for 92 h. These studies indicate that zinc accumulates in the insulin-storing granules of the islets in vivo and the intracellular zinc concentrations appear related to the functional state of the islet.

The purpose of the present study was to characterize in vitro the uptake and modulation of zinc⁶⁵ in isolated pancreatic islets.

METHODS

Islets were isolated from the pancreas of male Wistar rats (200–300 g) by use of a collagenase technique.¹⁰ These islets were further separated from acinar tissue by Ficoll separation as outlined by Sharp et al.¹¹ Briefly, the final acinar-islet-rich pellet obtained by collagenase digestion was mixed with 2 ml of Ficoll, 25% (w/v), dissolved in Hank's balanced salt solution (HBSS), which consisted of 136.8 mM NaCl, 5.36 mM KCl, 1.26 mM CaCl_2 , 0.81 mM MgSO_4 , 0.33 mM Na_2HPO_4 , and 0.44 mM KH_2PO_4 at pH 7.4. The Ficoll was previously dialysed for 24 h against distilled H_2O and then lyophilized. On top of the 25% Ficoll–HBSS islet mixture the 23%, 20.5%, and 11% (w/v) Ficoll–HBSS were layered. The discontinuous gradient was centrifuged for 10 min at 800 g and the islets were collected with a siliconized Pasteur pipette from the 11%:20.5% Ficoll interface. The islets were washed in a petri dish with 30 ml of Krebs-Ringer bicarbonate media to free them from the small amount of adherent Ficoll.

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All incubations were accomplished with a modified Krebs-Ringer bicarbonate (KRB) medium containing 115 mM NaCl, 5.0 mM KCl, 2.5 mM CaCl₂, 24 mM NaHCO₃, 1.0 mM MgCl₂, and albumin (0.5% bovine plasma albumin, Armour Pharmaceutical, Chicago). The media were equilibrated to pH 7.4 with a mixture of O₂ (95%) and CO₂ (5%) and were maintained at a temperature of 37 °C unless indicated otherwise. Incubations were performed in a Dubnoff metabolic shaker (50 strokes/min) at a temperature of 37 °C unless otherwise stated. In all uptake studies, the islets were preincubated 20 min in KRB with 5.4 mM glucose.

Chemicals and materials were obtained from the following sources: ZnCl₂ and Ficoll from Sigma Chemical, St. Louis. D-glucose (dextrose) from the National Bureau of Standards, Washington, D.C. The radioisotopes, zinc⁶⁵ (carrier free) and ¹⁴C-sucrose, were obtained from New England Nuclear. CMRL 1066 (tissue culture medium), to which was added 1% glutamine, 100 μU/ml penicillin, and 100 μU/ml streptomycin, and 10% heat-inactivated fetal calf serum, was purchased from Grand Island Biological Co.

UPTAKE STUDIES

The uptake studies were based on a dual isotope procedure in which ¹⁴C (500 mCi/mmol)-sucrose and Zn⁶⁵ (carrier free) were both present in the incubation media. Sucrose, which does not penetrate the beta-cell membrane,¹²⁻¹⁶ was used as an extracellular marker to determine the uptake of zinc. Previous studies showed that the t_{1/2} for sucrose equilibration into the extracellular space of an islet is 9.14 s,¹⁷ allowing complete equilibration with the extracellular space by 1 min. In general, 25 pancreatic islets were incubated in glass vials (11 mm i.d. × 20 mm height) containing 250 μl KRB medium. The media was removed with the aid of a micropipette and a dissecting microscope. After the final incubation in media containing the radioactive isotopes, the media were removed and the vials containing the islets were frozen immediately in liquid nitrogen and were subsequently lyophilized. The lyophilized islets were transferred individually at room temperature by means of a microdissecting needle into scintillation vials, digested in 100 μl of hyamine hydroxide-10× for 1 h at 37 °C, and then neutralized with 10 μl of 20 mM ZnCl₂ in glacial acetate acid. These vials were then counted in the ³H, ¹⁴C channel of a Packard 3255 liquid scintillation spectrophotometer.

The islet content of the cellular marker was calculated by subtracting the extracellular marker content of ¹⁴C-sucrose. The calculations for determining cellular uptake were derived from the following equations:

cellular distribution space =

$$\frac{\text{Zn}^{65} \text{ cpm/islet}}{\text{Zn}^{65} \text{ cpm}/\mu\text{l medium}} - \frac{{}^{14}\text{C-sucrose cpm/islet}}{{}^{14}\text{C-sucrose cpm}/\mu\text{l medium}}$$

picomole uptake = (picomole Zn/μl medium) × (cellular distribution space). These calculations were similar to those described previously by Bloom et al.¹⁸ The sucrose correction for nonspecific uptake represents less than half of the total zinc uptake at the earliest interval (60 s) measured and yields an acceptable 2:1 ratio.

Islet enrichment of zinc over extracellular concentration was calculated making the assumption that the average

intracellular space (IS) of an islet was 3.5 nl^{12,13} and applying the following formula:

$$\frac{\text{Zn uptake in islet}}{\text{IS (3.5 nl)}} = [\text{Zn}] \text{ in islet:}$$

$$\frac{[\text{Zn}] \text{ islet}}{[\text{Zn}] \text{ medium}} = \text{fold increase}$$

COUNTER TRANSPORT STUDIES

These experiments were performed by incubating half the number of the vials containing pancreatic islets with ZnCl₂ (2 mM) for 30 min. The reaction vessels were then pulsed for either 1 or 2 min with labeled zinc at 2 or 44 μM. The islet content of the labeled compound was determined as described in the UPTAKE STUDIES. If the uptake of Zn⁶⁵ reflects primarily membrane binding, the islets preincubated with unlabeled zinc should inhibit subsequent uptake of Zn⁶⁵ as compared with the nonpreincubation conditions. Conversely, if uptake predominantly reflects facilitated diffusion, then uptake of Zn⁶⁵ will be enhanced in islets preincubated (i.e. preloaded) with ZnCl₂ as compared with control.¹⁹

TISSUE CULTURE STUDIES

Zinc uptake over 24 h was assayed in a similar way as the acute uptake studies. Islets for long-term experiments were isolated under sterile conditions and were placed in CMRL 1066 medium, to which 1% glutamine, 10% fetal (heat-inactivated) calf serum, 100 μU/ml penicillin, and 100 μU/ml of streptomycin were added. Islets were preincubated for 20 min at room temperature, preincubation media were removed, and 500 μl of labeled media with the appropriate additions of unlabeled ZnCl₂ and glucose was then added with sterilized micropipettes. The incubation vessels were plugged with sterile foam stoppers and were placed in a 37 °C incubator gassed continuously with 95% air and 5% CO₂.

STATISTICS

Data were evaluated by an unpaired *t* test when necessary. All curves were drawn by inspection, and when two sets of data were not significantly different (*P* < 0.05) one curve was drawn to represent both sets of data.

RESULTS

Time studies. Initial studies were undertaken to determine the time dependency of zinc uptake in pancreatic islets. Zinc uptake was measured at time intervals of from 1 to 70 min and at two zinc concentrations (2 and 44 μM). Zinc concentrations were chosen to reflect those found in whole rat blood (about 44 μM) and a lower value that may reflect ionized (free) zinc levels in rat blood. The pattern of zinc uptake under these conditions was linear through 5 min and reached a four- to sixfold increase in concentration over the extracellular zinc concentration at 70 min (Figures 1 and 2). These calculations were made assuming the intracellular space of an islet is about 3.5 nl¹² and using formulas detailed in the METHODS. Complete isotopic equilibrium was not reached at the latest time point measured (70 min), since a slow increase in radioactivity was still apparent. At later time points (90 and 120 min) in several experiments,

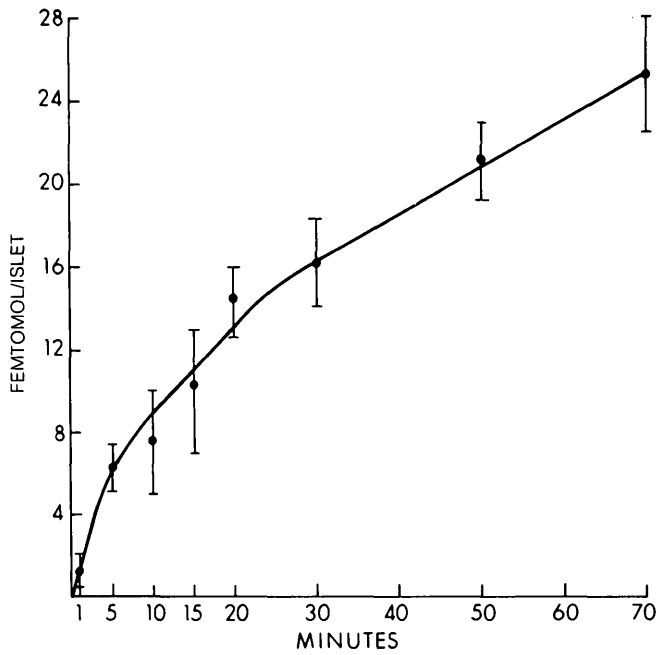


FIGURE 1. Time course of uptake at 2 μM zinc. Isolated islets were preincubated in KRB and 5.7 mM glucose and were gassed with 95% O_2 and 5% CO_2 for 20 min. The preincubation medium was removed and labeled medium [^{65}Zn (2 μCi per vial) and ^{14}C (1 μCi per vial) sucrose], containing 5.7 mM glucose, was added for the above time intervals. Values are expressed as the mean of four values for each point \pm SEM.

there was a further increase in zinc concentrations (data not illustrated).

Kinetic studies. To characterize the kinetics of uptake, the relationship of the initial rates of zinc uptake to zinc concentration was studied. One-minute time points were used to reflect initial rates, since the uptake of zinc was linear through 5 min (Figures 1 and 2). At low concentrations (0.5 to 7 μM), a curve suggesting saturation kinetics was observed

FIGURE 2. Time course of uptake at 44 μM zinc. Isolated islets were treated identically to those experiments represented in Figure 1. Values are expressed as the mean of four values for each point \pm SEM.

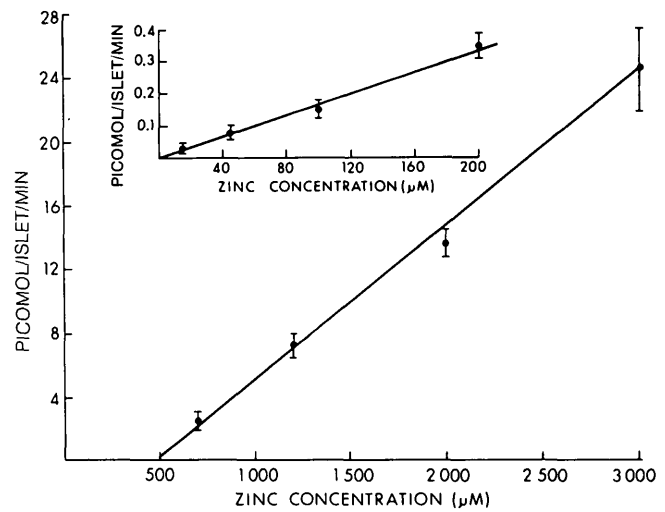
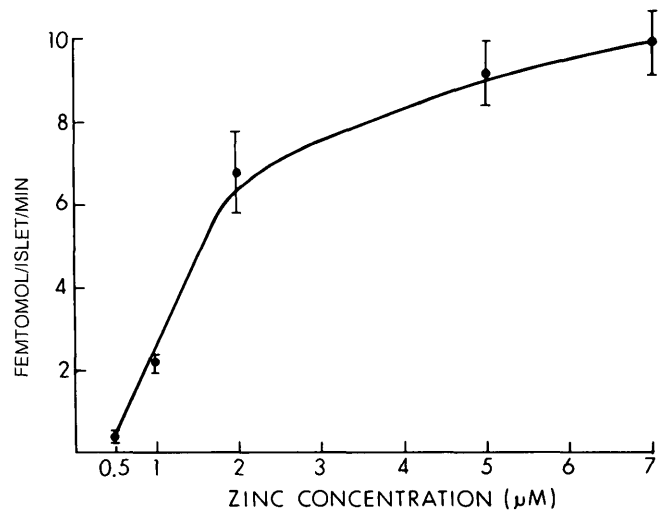
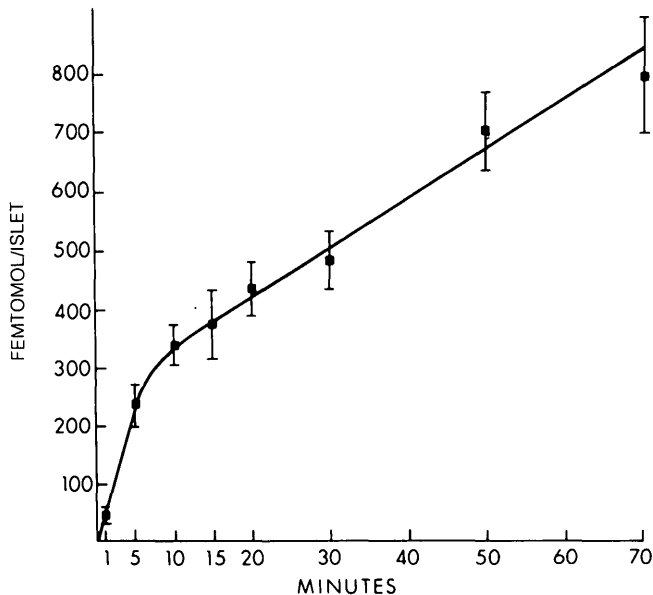


FIGURE 3 (top) and 4. Saturation kinetics of zinc uptake. Isolated islets were preincubated in KRB with 5.7 mM glucose. The preincubation medium was removed and labeled media were added at the appropriate zinc concentrations for 1 min (5 min for those zinc concentrations below 2 μM to allow significant counts to enter; the answer was then divided by 5 to yield zinc uptake/min). Values are expressed as the mean of four or more values \pm SEM.

(Figure 3). The K_m and V_{max} , calculated by Woolf-Hofstee analyses,²⁰ for these data were 1.6 μM and 11.1 femtomole/islet/min, respectively. At higher zinc concentrations (10 μM to 3 mM), a linear relationship of initial rates of uptake to zinc concentration was observed (Figure 4).

Temperature studies. Studies were undertaken to determine the effect of temperature on zinc uptake at zinc concentrations of 2 and 44 μM at 1 to 70 min. Zinc uptake at 44 μM progressively decreased at 70 min by 75% and 82% as the temperature was lowered from 37 $^\circ\text{C}$ to 20 $^\circ\text{C}$ and 4 $^\circ\text{C}$, respectively (Figure 5). However, at 2 μM zinc, the uptake was identical at 20 $^\circ$ and 37 $^\circ$ and showed a decrease at 70 min of 70% at 4 $^\circ\text{C}$ (Figure 6).

Competition studies. To further differentiate between saturable and nonsaturable uptake processes of zinc by pancreatic islets, the effect of cadmium on zinc uptake was examined. Zinc and cadmium compete for binding to the metallothioneine protein of kidney and liver as well as other proteins.^{21,22} In this study, therefore, zinc uptake at 2 and 44

μM was determined in the absence and presence of cadmium. The uptake of zinc at $2 \mu\text{M}$ was inhibited 32% by an equimolar concentration of cadmium and inhibited 58% by a 10:1 cadmium to zinc concentration. The uptake of zinc at $44 \mu\text{M}$ showed no inhibition by cadmium concentrations of 44 or $100 \mu\text{M}$ as compared with control (Table 1). Zinc uptake was measured for 5 min at $2 \mu\text{M}$ zinc as opposed to 1 min at $44 \mu\text{M}$ zinc, because 5 min was needed to accumulate significant counts under cadmium inhibition of uptake.

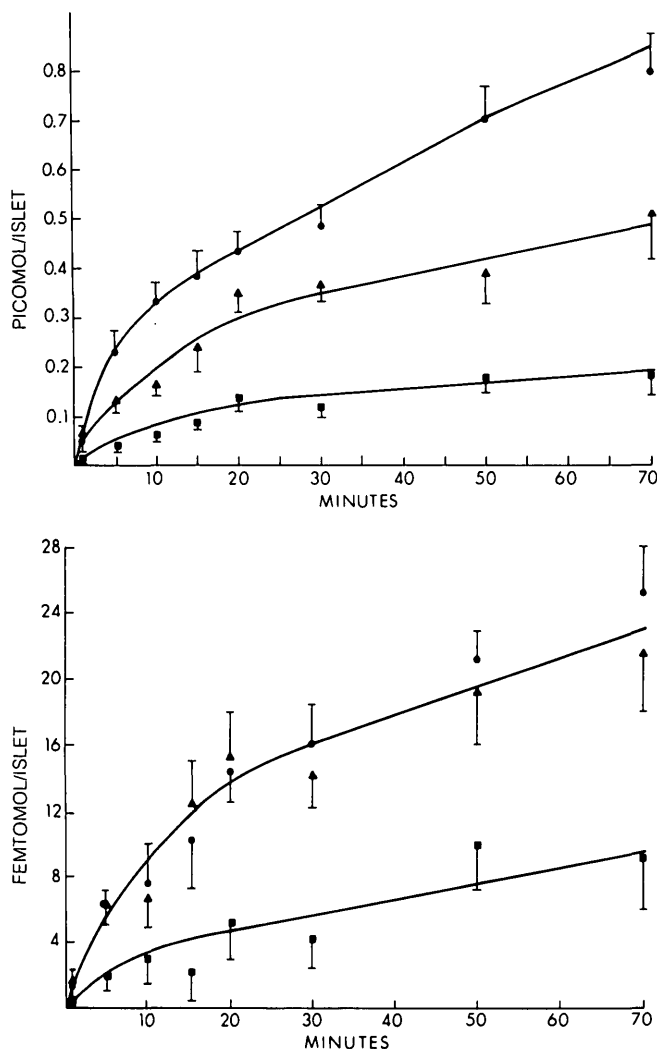
Counter transport studies. Further evidence of carrier-mediated uptake was sought by performing a counter transport experiment. Briefly, islets were preloaded for 30 min in $2 \mu\text{M}$ unlabeled ZnCl_2 and were then exposed to a brief pulse (1 or 2 min) of Zn^{65} at either $1/45$ or $1/1000$ ($44 \mu\text{M}$ or $2 \mu\text{M}$) of the preloading zinc concentrations. Counter transport was present at $2 \mu\text{M}$ zinc, as evidenced by the enhanced uptake of Zn^{65} by the preloaded islets, but not at $44 \mu\text{M}$ zinc (Table 2).
Oxidative phosphorylation dependency of uptake. In the uptake studies, zinc was significantly concentrated to be

TABLE 1
Cadmium effect on zinc uptake

Cadmium concentration, μM	$2 \mu\text{M}$ Zn Uptake	n
0	17.5 ± 2.1	(10)
2	12.0 ± 1.1	(10)
20	7.3 ± 0.6	(10)
44 μM Zn Uptake		
0	107.5 ± 9.0	(8)
44	112.2 ± 10.6	(8)
100	121.4 ± 10.3	(8)

Isolated islets were preincubated in KRB, containing 5.5 mM glucose, for 20 min. The preincubation medium was removed, and labeled media (^{65}Zn and ^{14}C sucrose), with appropriate zinc and cadmium concentrations, were added for 5 min for the $2\text{-}\mu\text{M}$ zinc uptake and for 1 min for the $44\text{-}\mu\text{M}$ zinc uptake. Values are expressed as femtomoles per islet \pm SEM. Amounts in parentheses are the number of observations.

FIGURE 5 (top) and 6. Temperature effects on zinc uptake. Isolated islets were treated identically to those in experiments represented in Figure 1. Zinc concentrations were $2 \mu\text{M}$ (Figure 5) or $44 \mu\text{M}$ (Figure 6). The temperatures are indicated as: (■) 4° ; (▲) 20° ; (●) 37°C . The values are expressed as the mean of four values \pm SEM.



above the extracellular levels. The relationship between oxidative phosphorylation and the ability to concentrate zinc was studied. Islets preincubated (30 min) with dinitrophenol ($300 \mu\text{M}$) and then incubated with zinc and dinitrophenol for 30 min showed no diminution of zinc uptake ($2 \mu\text{M}$ or $44 \mu\text{M}$) compared with controls (Table 3).

Glucose modulation of zinc uptake. Glucose concentration is a major factor in regulating insulin release and biosynthesis and may be an important regulatory factor of zinc metabolism. Zinc uptake was studied under basal (5.2 mM) and insulin biosynthetic and secreting (27.5 mM) levels of glucose. Uptake at the two zinc concentrations (2 and $44 \mu\text{M}$) was studied. Paired experiments, performed at 1, 5, 10, 15, 20, 30, and 50 min, showed no consistent, significant difference in zinc uptake at either zinc concentration regardless of the glucose concentration (Figures 7 and 8).

LONG-TERM EXPERIMENTS

Tissue culture experiments were undertaken to study further the time course and the effects of glucose stimulation on zinc uptake at longer times. It was found that Zn^{65} uptake continued to and perhaps beyond 24 h. Isotopic equilibrium was not reached by this time. Glucose (5.2 mM or 27.5 mM)

TABLE 2
Counter transport of zinc by preloaded islets

Incubation time (min)	Control	Preloaded (2 mM zinc)	% Change
(femtomoles/islet)			
(2 μM Zinc)			
1	1.66 ± 0.41	4.16 ± 0.67	+150
2	2.67 ± 1.12	9.11 ± 1.84	+241
(44 μM Zinc)			
1	60.1 ± 7.3	72.0 ± 4.9	+19
2	100.5 ± 7.8	95.9 ± 4.1	-5

Isolated islets were preincubated in KRB \pm 2 mM ZnCl_2 . The preincubation medium was removed, and labeled medium at the appropriate zinc concentration was added at the indicated times. Values are femtomoles ^{65}Zn per islet \pm SEM.

* Not significant at $P = 0.05$.

TABLE 3
Effect of dinitrophenol (DNP) treatment on zinc uptake

Zinc concentration	Without DNP	With DNP
2 μ M	(4) 13.8 \pm 6.7	(4) 21.6 \pm 6.1
44 μ M	(4) 330 \pm 54	(4) 365 \pm 53

Isolated islets were incubated for 30 min in KRB, 0 mM glucose, with or without 300 μ M DNP. The medium was removed, and labeled medium, as described in Figure 1, with appropriate additions of ZnCl₂, with or without DNP, was added. After another 30 min the medium was again removed; the values are expressed as femtomoles per islet \pm SEM. The values in parentheses are the number of observations. The differences between the groups were not significant at the P = 0.05 level.

FIGURE 7 (top) and 8. The effect of glucose concentration on zinc uptake. Isolated islets were preincubated in tissue culture medium (see METHODS) containing 5.7 mM glucose for 20 min at room temperature. The preincubation medium was removed and labeled tissue culture medium [⁶⁵Zn (2 μ Ci/vial) and ¹⁴C sucrose (1 μ Ci/vial)], containing 5.7 mM (●) or 27.5 mM (▲) glucose, were added for the indicated time intervals. Islets were incubated at 37 °C and gassed with 95% air and 5% CO₂. Zinc concentrations were 2 μ M (Figure 7) and 44 μ M (Figure 8). Values are expressed as the mean of four values for each point \pm SEM.

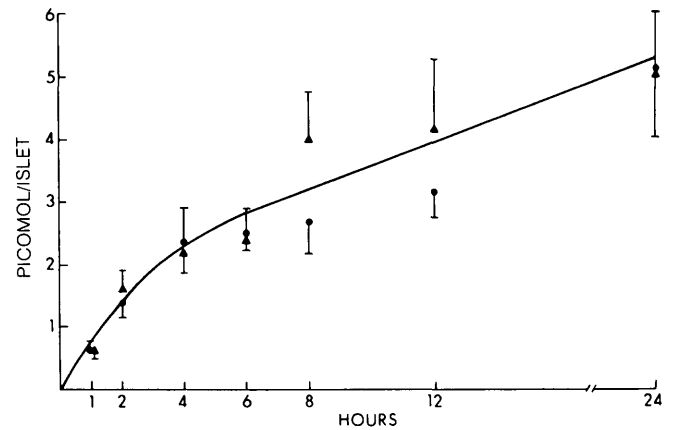
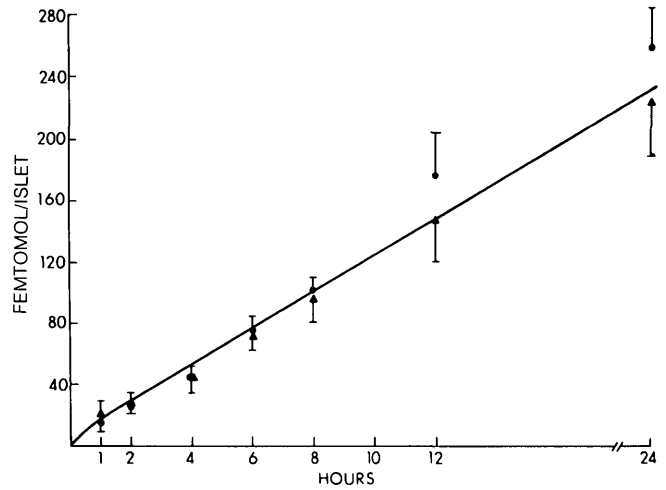
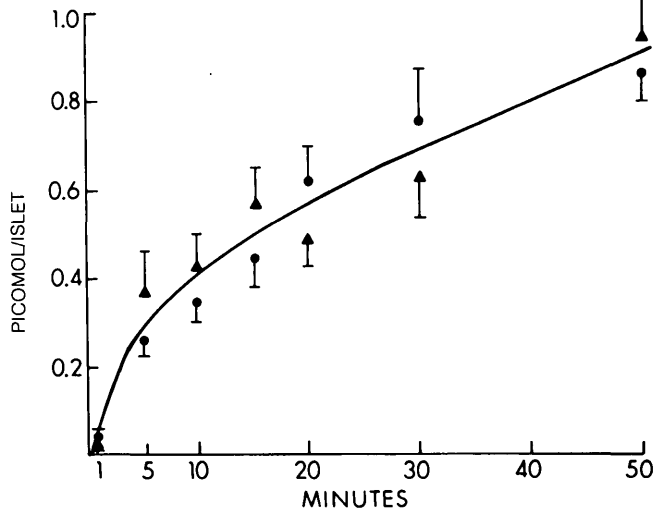
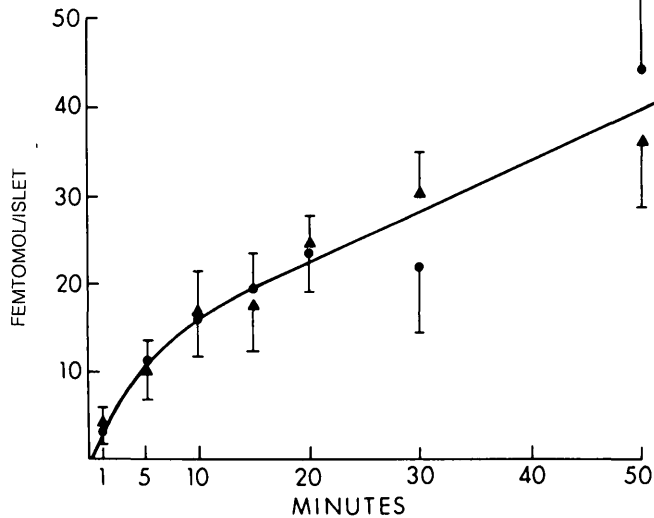


FIGURE 9 (top) and 10. The effect of glucose concentration on long-term zinc uptake. Isolated islets were preincubated in tissue culture media (see METHODS), containing 5.7 mM glucose, for 20 min at room temperature. The preincubation medium was removed and labeled tissue culture medium [⁶⁵Zn (2 μ Ci per vial) and ¹⁴C sucrose (1 μ Ci per vial)], containing either 5.7 mM (●) or 27.5 mM (▲) glucose, were added for the indicated time intervals. Islets were incubated at 37 °C and gassed with 95% air and 5% CO₂. Zinc concentrations were 2 μ M (Figure 9) or 44 μ M (Figure 10). Values are expressed as the mean of three values for each point \pm SEM.

showed no consistent, modulating effect on zinc uptake at any time point (1–24 h) at either zinc concentration (2 or 44 μ M) (Figures 9 and 10).

DISCUSSION

Zinc uptake in the pancreatic islet is a slow process that continues for hours or perhaps days. Isotopic equilibrium was not reached by 24 h; this may indicate that the pool size for zinc is large and/or the isotopic exchange with these pools is slow. The first consideration—of a large zinc pool—is reasonable considering that the approximately 3.5 mU of insulin per islet²³ is made up of two zinc hexamers,²⁴ which would yield 8 pmol of zinc per islet. The latter value is lower than the total zinc content of 21 pmol per islet found by Falkmer et al.²⁵ Such a high value suggests additional zinc pools, the second fraction of B-cell zinc as described by Lazaris and Meiramov.²⁶ These other pools may be important in enzyme function or zinc storage. Both zinc values are considerably higher than the uptakes observed in these studies, explaining the fact that isotopic equilibrium was not reached. The second consideration—of slow isotopic ex-

change—is also reasonable considering the high affinity of zinc for insulin.²⁷

A component of the uptake of zinc seems to be its facilitation by a carrier. Several findings point toward this conclusion. First, zinc uptake demonstrates saturation kinetics at low zinc concentrations (0.5–7 μM). The initial rates of uptake in relation to zinc concentration become linear at higher zinc concentrations (10–3000 μM), suggesting diffusional mechanisms of uptake at those higher concentrations. Iron uptake also demonstrates this pattern of uptake, with saturation kinetics at low concentrations (1–5 μM) and a more linear relationship of initial rates of uptake to iron concentration at higher levels.²⁸ A second finding to support the hypothesis that a component of zinc uptake is carrier mediated is the demonstration of the counter transport of zinc. This particular procedure distinguishes between binding and transport.²⁹ Preloaded islets (2 mM ZnCl_2) would not be expected to have an accelerated uptake of zinc if this were a binding phenomenon. Conversely, preloaded islets would be expected to show an increased uptake over control islets if unlabeled isotope were competing with newly accumulated label for efflux. The latter—enhanced uptake in preloaded islets—was found at the 2- μM concentration. Zinc uptake at the 44- μM concentration was unaffected by preloading the islets. This finding is consistent with a diffusion mechanism of uptake predominating at high zinc concentrations (above 7 μM) and a facilitated diffusion mechanism of uptake predominating at low zinc concentrations (below 7 μM).

A third piece of evidence to support carrier transport of zinc is the dose-dependent cadmium inhibition of zinc uptake at zinc concentrations that show saturation kinetics. At higher zinc concentrations, where diffusional uptake seems to dominate, cadmium had no effect on zinc uptake. The dose dependency of cadmium inhibition of zinc uptake is suggestive of a competitive type of inhibition, but a detailed analysis cannot be made until initial rates of zinc uptake are viewed in relation to varying cadmium concentrations.

Decreasing temperature progressively inhibited zinc uptake at high zinc concentrations (44 μM), as is expected for a diffusional process. Carrier-mediated processes are less dependent on temperature,²⁹ but, surprisingly, no inhibition of uptake at 20° (compared with 37°) was seen at a zinc concentration (2 μM) that showed saturable kinetics, counter transport, and cadmium inhibition of uptake. Temperatures of 4 °C inhibited zinc uptake at either a high (44 μM) or a low (2 μM) zinc concentration.

The finding that zinc accumulation was not inhibited by dinitrophenol suggests that zinc uptake was not dependent on oxidative phosphorylation and, perhaps, is not an active transport process in the classic sense. An alternate source of energy providing for uptake over extracellular concentration could be provided by the structure of a zinc-binding protein or by the crystallization of insulin to two zinc-insulin hexamers. Also, unabated zinc uptake at 20°, where energy production should be depressed, argues against an active transport mechanism of uptake.

The finding that glucose (5.7 mM or 27.5 mM) did not modulate either short- or long-term zinc uptake implies that (1) the internal (intraislet) stores of zinc are large enough to handle the increased synthesis and secretion that are a result of glucose stimulation without stimulating the uptake

of exogenous zinc or (2) that new insulin biosynthesis and secretion after the ejection of preformed B granules is not dependent on zinc and does not follow the classic B granules' packaging and emiocytosis schemata. With the advent of x-ray microprobe analysis, perhaps internal translocation(s) of zinc pools can be followed subsequent to glucose stimulation.

In summary, it was found that zinc is taken up by isolated islets continuously for 24 h to concentrations some 30-fold higher than those of extracellular zinc. The findings of counter transport, cadmium inhibition, and saturability of uptake is most consistent with a carrier-mediated model of uptake at zinc concentrations below 7 μM . The absence of counter transport, cadmium inhibition of uptake, and nonsaturability of uptake at higher zinc levels (44 μM) is most consistent with the hypothesis that a diffusional mechanism of uptake predominates at the higher zinc concentrations. Zinc uptake was depressed at 4° but not at 20° compared with uptake at 37° (2 μM Zn), and dinitrophenol treatment had no effect on the extent of uptake at 30 min. These two items argue for, albeit not conclusively, a passive, facilitated diffusion rather than active transport of zinc. Glucose, at either 5.2 mM or 27.5 mM, had no effect on either the rate or extent of uptake at short (1–70 min) or longer (1–24 h) times.

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