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Low-molecular-weight constituents of isolated insulin-secretory granules

Bivalent cations, adenine nucleotides and inorganic phosphate

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The concentrations of Zn^{2+}, Ca^{2+}, Mg^{2+}, P_i and adenine nucleotides were determined in insulin-secretory granules prepared from a transplantable rat insulinoma. Differential and density-gradient centrifugation analyses revealed that Zn^{2+} in this tissue was principally localized in the secretory granule, a second major fraction being found in association with cytosolic proteins. P_i was principally recovered in the latter fraction, whereas Ca^{2+} and Mg^{2+} were more widely distributed. Intragranular ion-distribution experiments suggested that Zn^{2+} was complexed mainly to insulin and its precursor forms and remained in the granule in an insoluble state. The Zn^{2+}/insulin ratio (0.54) was greater than that expected for insulin molecules having two centrally co-ordinated Zn^{2+} atoms/hexamer, but less than the maximal Zn^{2+}-binding capacity of the molecule. Most of the granular Ca^{2+}, Mg^{2+} and P_i was released in a soluble form when granules were disrupted by sonication. Simulation *in vitro* of the ionic composition of the granule suggested that up to 90% of its Ca^{2+} was complexed to P_i and adenine nucleotides. Granular macromolecules also bound Ca^{2+}, as shown by equilibrium-dialysis studies of granule lysates. However, such binding was displaced by Mg^{2+}. Examination of the efflux of Ca^{2+} from granules incubated in iso-osmotic suspensions at 37°C suggested that the passive permeability of the granule membrane to Ca^{2+} was very low. Nevertheless, more than 50% of the granular Ca^{2+} was rapidly released in an ionized form on hypo-osmotic or detergent-induced disruption of the granule membrane. This may represent a potentially mobilizable pool of Ca^{2+} *in vivo*.

It has long been recognized that secretory granules of the pancreatic B-cell of many species contain adenine nucleotides (Leitner *et al.*, 1975) and concentrate Zn^{2+} (Pihl, 1968), Ca^{2+} (Herman *et al.*, 1973) and biogenic amines (Ekholm *et al.*, 1971). Comparative studies (Pihl, 1968) and those *in vitro* (Wolters *et al.*, 1979) have related the presence of Zn^{2+} in granules to the capacity of insulin and its precursors to bind this cation with a high affinity. Zn^{2+} favours the formation of hexamers of insulin and promotes its precipitation and crystallization (Cunningham *et al.*, 1955). Under the same conditions it also promotes hexamer formation with proinsulin and the formation of higher-order aggregates, which, unlike insulin, remain in solution (Frank & Veros, 1970; Grant *et al.*, 1972). These phenomena may be important *in vivo* in the transfer of proinsulin from its site of synthesis in the endoplasmic reticulum to its storage site in the granule, in favouring thermodynamically the proteolytic processing of proinsulin, in limiting the osmotic effects of insulin concentration or in protecting intragranular insulin from further proteolysis (see Emdin *et al.*, 1980).

The presence of Ca^{2+} and adenine nucleotides, in contrast with Zn^{2+}, is a general feature of secretory granules from a wide variety of species and tissue sources. Several possible functions have been ascribed to these components in different situations; in the B-cell granular Ca^{2+} conceivably constitutes a dynamic regulatory pool (see Wollheim & Sharp, 1981) or is important in the process of granule morphogenesis (Howell *et al.*, 1978).

This paper presents analytical data on these and other ionic constituents in insulin secretory granules that were isolated from a transplantable rat insulinoma (Chick *et al.*, 1977). Such quantitative data are

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important in predicting the physicochemical form of ionic substances in the granule and provide insight into their possible functional significance in the storage and secretion of insulin.

Experimental

Subcellular fractionation

Tumours were propagated and harvested as previously described (Hutton & Peshavaria, 1982). The tissue (4–8g wet wt.) was chopped into fragments of 2mm or less and rinsed several times in ice-cold medium of the following composition; 137mM-NaCl, 5.5mM-KCl, 1.7mM-$MgSO_4$, 1.3mM-$CaCl_2$, 0.3mM-Na_2HPO_4 and 4.2mM-$NaHCO_3$. This medium was removed after centrifugation of the suspension for 30s at 800**g**, the tissue was rinsed once in homogenization medium and then homogenized as described previously (Hutton & Peshavaria, 1982). The homogenization medium contained 275mM-sucrose, 10mM-4-morpholine-ethanesulphonic acid and 1mM-EGTA and was adjusted to pH6.5 with Tris base. All homogenization and centrifugation steps were conducted as rapidly as possible at 4°C.

Differential centrifugation of tumour homogenates was performed on a Beckman 50.1 rotor ($r_{av.}$ 8cm) (Beckman, Palo Alto, CA, U.S.A.) for the times and gravitational forces specified in Table 1. Pelleted material was resuspended in fresh medium for analysis.

Density-gradient centrifugation was carried out on tumour homogenates as described previously (Hutton & Peshavaria, 1982; Hutton *et al.*, 1982); a brief description is set out below. The tissue homogenate was centrifuged for 10min at 1700**g** to remove unbroken cells and nuclei and the supernatant was layered on to 26ml of a 27% (v/v) solution of Percoll (Pharmacia, Stockholm, Sweden) dispersed in homogenization medium and then centrifuged for 45min at 35000**g**. This resulted in the separation of secretory granules from soluble proteins, endoplasmic reticulum, mitochondria, peroxisomes, Golgi and plasma membranes. Further resolution of granules from lysosomes and minor mitochondrial contamination was achieved by resuspending insulin-rich fractions in 36ml of homogenization medium containing 42% (v/v) Percoll and centrifuging as before. Fractions from both these gradients were analysed for enzymic markers without further processing. When required, particulate material in fractions was separated from the density-gradient medium by three cycles of suspension in homogenization medium followed by centrifugation. The last cycle was performed in homogenization medium without chelating agents. This preparation was completed within 4–7h of tissue homogenization. Such granules were kept at 4°C in the final wash medium at a protein concentration of 10mg/ml, or stored at −70°C. Electron microscopy of the final preparation showed the presence of membrane-limited vesicles with electron-dense cores of typical B-cell morphology (Hutton *et al.*, 1982).

All stock solutions used for preparing the medium were pretreated by passage through a column (5cm × 1cm) of bivalent cation-chelating resin (Chelex; Bio-Rad, Richmond, CA, U.S.A.). Glassware was rinsed in 1M-HCl and then in double-distilled water before use.

Analytical procedures

Spectrophotometric assays were performed for the marker enzymes arylsulphatase (Roy, 1953), cytochrome oxidase (Cooperstein & Lazarow, 1951), NADPH-cytochrome *c* reductase (Sottocasa *et al.*, 1967) and lactate dehydrogenase (Nielands, 1955). DNA was determined fluorimetrically (Kissane & Robbins, 1958) and 5′-nucleotidase by a radioisotopic procedure (Avruch & Wallach, 1971). Insulin was determined by a modification (Hutton *et al.*, 1982) of the back-titration method of Wright *et al.* (1968) with rat insulin as standard (lot RC791009; Novo Ind., Copenhagen, Denmark). Protein in density-gradient fractions was determined fluorimetrically (Udenfriend *et al.*, 1972), and in other cases by the method of Lowry *et al.* (1951) with bovine serum albumin (Armour Pharmaceuticals, Eastbourne, Sussex, U.K.) as standard.

Total Zn^{2+}, Ca^{2+} and Mg^{2+} in tissue homogenates and subfractions were determined by atomic-absorption spectroscopy (Pye–Unicam SP90 instrument; Pye Ind., Cambridge, U.K.). Samples diluted with water to 1ml were mixed with 100μl of a solution containing 0.1% (v/v) Triton X-100 and 10mM-EDTA and then with 2ml of a solution of 7.5% (w/v) trichloroacetic acid containing 0.1% $LaCl_3$. After standing for 30min at 4°C samples were centrifuged for 20min at 1700**g** and the resultant supernatant analysed. [P_i] was determined on the same supernatant by a spectrophotometric technique (Itaya & Ui, 1966). Ca^{2+} was determined with a Radiometer Ca^{2+} electrode (Radiometer, Copenhagen, Denmark) coupled to a potentiometric recorder via a Radiometer type 26 pH meter. Solutions to be analysed were placed in a water-jacketted Perspex chamber (O_2 electrode; Rank Bros., Bottisham, Cambs., U.K.) and stirred with a magnetic stirrer. Calibration was achieved with EGTA/Ca^{2+} buffers in the 0.1–1.0μM concentration range of Ca^{2+}, otherwise by appropriate dilution of a standard $CaCl_2$ solution (BDH Chemicals, Poole, Dorset, U.K.). Na^+ and K^+ were determined by flame photometry on samples diluted in water. ATP, ADP and AMP were determined with crude firefly lantern luciferase (Malaisse *et al.*, 1978*a*).

Ca^{2+}-binding studies were performed by equili-

brium dialysis using the micro-method previously described (Hutton *et al.*, 1981). Freeze/thawed granule suspensions were diluted with water to approx. 1 mg of protein/ml and passed through a column (1 cm × 0.5 cm) of bivalent cation-chelating resin (see above) to remove endogenous bivalent cations. Samples (50 μl) were then equilibrated overnight against 25 mM-potassium 4-morpholine-ethanesulphonate buffer (pH 6), which contained 0.25 μCi of $^{45}Ca^{2+}$/ml (11 Ci/g; Amersham International, Amersham, Bucks., U.K.) together with $MgCl_2$ and $CaCl_2$ as indicated. Radioactivities in the non-diffusable material and equilibrating buffers were determined by liquid-scintillation spectrometry using a Triton/toluene-based scintillation counting fluid (Fisofluor 2; Fisons, Loughborough, Leics., U.K.).

Results

Differential centrifugation

Each of the tumour ionic constituents that were analysed exhibited a characteristic subcellular distribution (Table 1). Zn^{2+} was recovered principally in the $5 \times 10^5 g$-min pellet along with the major portion of marker proteins of the secretory granule (insulin), mitochondria (cytochrome oxidase) and lysosomes (arylsulphatase). The Zn^{2+} content (per mg of protein) in this fraction was 2.6-fold that of the crude homogenate; the molar ratio of Zn^{2+} to immunoreactive insulin was 0.9. A second major fraction of Zn^{2+} was found in the final $10^7 g$-min supernatant. However, this contained only a minor proportion of the tissue insulin, the molar ratio of Zn^{2+} to immunoreactive insulin here being 9.6. These two pools of Zn^{2+} overshadowed any association that may have occurred between this ion and the endoplasmic reticulum (NADPH-cytochrome *c* reductase) or plasma membrane (5′-nucleotidase).

Ca^{2+} had a distribution similar to Zn^{2+}, although a greater proportion of it appeared in the soluble protein fraction. The Ca^{2+} content (per mg of protein) in the insulin-rich $5 \times 10^5 g$-min pellet was similar to that in the crude homogenate.

Mg^{2+} and P_i were found predominantly in the soluble protein fraction. The contents of these ions in this fraction expressed per mg of protein were 3.5- and 4.7-fold respectively those of the whole tissue. In the $5 \times 10^5 g$-min pellet, the content of Mg^{2+} expressed per mg of protein was similar to that of intact tissue, whereas that of P_i was considerably lower (0.2-fold).

Density-gradient centrifugation

Zn^{2+} was distributed bimodally on the initial density gradient (Fig. 1). The distribution of the

Table 1. *Differential centrifugation of tumour homogenates*

Washed tumour pieces (2–4 g wet wt.) were homogenized in iso-osmotic sucrose medium (pH 6.5) and then centrifuged for 10 min at 100 *g* ($r_{av.}$ 24 cm) to remove large particulate matter, cellular debris and nuclei. The release of each constituent into the supernatant at this stage is expressed as a percentage of the initial tumour content. This supernatant was centrifuged in a Beckman SW 50.1 rotor ($r_{av.}$ 8 cm) operated at the indicated times and gravitational forces. The recoveries of each constituent in each fraction are expressed as percentages of the content in the first supernatant fraction. Each value is the mean ± S.E.M. of results obtained with different tissue preparations; the numbers of these are shown in parentheses.

			Distribution (%)			
	Initial content	Release (%)	10 min × 5000 *g* pellet	20 min × 25000 *g* pellet	50 min × 200000 *g* pellet	Final supernatant
Protein (mg/g wet wt.)	111.4 ± 8.0 (4)	67.6 ± 4.8	12.5 ± 2.3	20.9 ± 2.7	18.4 ± 2.2	53.5 ± 3.1
DNA (μg/mg of protein)	73.2 ± 8.4 (6)	11.4 ± 1.7	43.0 ± 7.9	2.2 ± 0.4	1.0 ± 0.5	1.5 ± 0.5
Insulin (nmol/mg of protein)	5.10 ± 0.6 (9)	86.8 ± 5.9	19.0 ± 4.2	67.3 ± 4.0	7.0 ± 1.5	3.1 ± 1.0
Arylsulphatase (nmol/min per mg of protein)	64 ± 10 (8)	70.1 ± 4.1	18.2 ± 1.6	64.5 ± 6.1	4.2 ± 1.3	9.2 ± 2.2
Cytochrome oxidase (nmol/min per mg of protein)	115 ± 37 (9)	68.1 ± 6.1	29.6 ± 5.9	58.1 ± 7.4	17.7 ± 7.6	0.7 ± 0.6
NADPH-cytochrome *c* reductase (nmol/min per mg of protein)	8.3 ± 2.3 (8)	65.8 ± 6.1	12.5 ± 3.7	26.0 ± 4.6	53.5 ± 9.8	13.9 ± 3.1
5′-Nucleotidase (nmol/min per mg of protein)	132 ± 20 (9)	73.1 ± 6.0	12.2 ± 1.8	25.8 ± 2.8	21.0 ± 2.3	40.5 ± 5.9
Lactate dehydrogenase (nmol/min per mg of protein)	153 ± 15 (6)	81.2 ± 4.2	4.8 ± 1.0	2.3 ± 0.4	0.4 ± 0.1	77.8 ± 2.3
Zn^{2+} (nmol/mg of protein)	6.9 ± 0.6 (7)	74.8 ± 5.0	9.9 ± 1.0	51.3 ± 4.5	11.1 ± 2.2	21.6 ± 2.9
Ca^{2+} (nmol/mg of protein)	66.9 ± 6.9 (4)	76.7 ± 6.7	11.9 ± 3.1	31.3 ± 1.2	7.0 ± 0.7	35.1 ± 3.1
Mg^{2+} (nmol/mg of protein)	89.3 ± 2.3 (4)	70.3 ± 2.0	4.8 ± 0.3	17.8 ± 2.6	5.9 ± 1.2	71.2 ± 3.6
P_i (nmol/mg of protein)	97.8 ± 4.9 (4)	71.6 ± 5.2	2.8 ± 1.0	4.0 ± 0.3	0.9 ± 0.2	91.9 ± 1.1

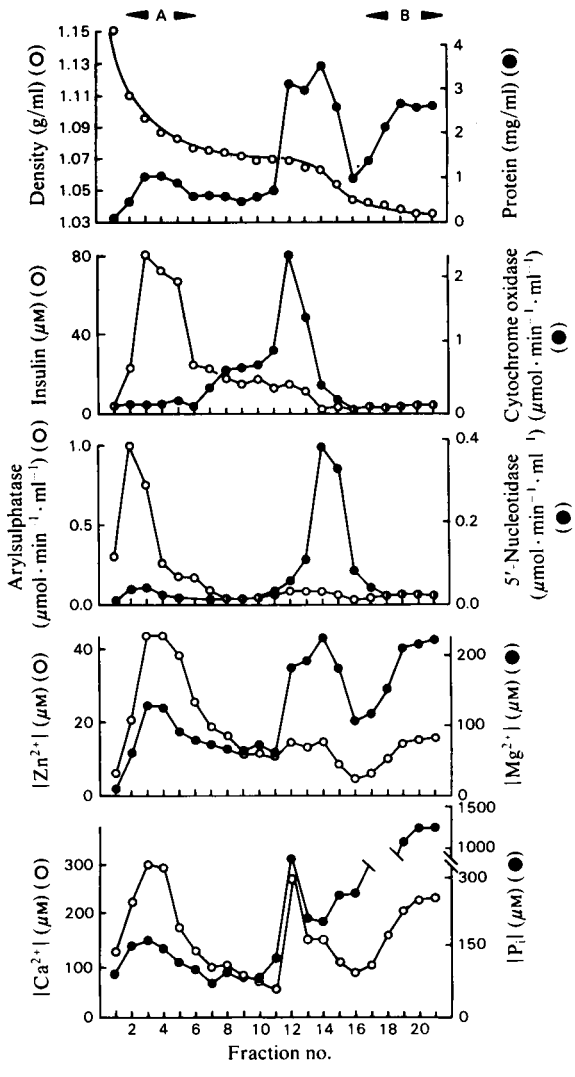

Fig. 1. *Percoll-density-gradient centrifugation of a tumour homogenate*

Zone B at the top of the tube indicates the volume initially occupied by the nuclei-free tumour homogenate before centrifugation (see the Experimental section). The material in zone A was subjected to further fractionation (see Fig. 2). The recoveries of the ionic constituents were 85–105% of the amounts originally transferred.

major Zn^{2+} fraction coincided with that of insulin; a second component was identified in association with the soluble tissue proteins. The molar ratio of Zn^{2+} to immunoreactive insulin in most particulate fractions fell within a narrow range with the exception of the most dense fractions, which contained most of the lysosomes as judged from the distribution of arylsulphatase on the gradient. The molar ratio here was approx. 2-fold that in the principal insulin-containing regions.

Ca^{2+} was recovered in all density-gradient fractions. The sample content of this ion was highest in the region occupied by insulin granules (zone A; Fig. 1) followed by regions in which the soluble proteins (zone B) and mitochondria were located. The Ca^{2+} content was lower again in fractions enriched in plasma membrane, a region of the gradient that also contained endoplasmic reticulum, peroxisomes and Golgi membranes, as was shown by the distribution of marker enzymes in previous studies (Hutton *et al.*, 1982).

Mg^{2+} was found in all regions of the first density gradient, its distribution being similar to that of protein. P_i, on the other hand, was found predominantly in the soluble protein fraction.

The secretory granule fraction from the first density gradient (zone A; Fig. 1) was further subfractionated to remove residual lysosomes and mitochondrial contaminants (Fig. 2). On this gradient Zn^{2+}, Ca^{2+}, Mg^{2+} and P_i were distributed in a manner similar to insulin, except in the low-density region, where Ca^{2+} and P_i, and, to a lesser extent, Mg^{2+} and Zn^{2+} were in a higher molar ratio to insulin.

Ionic composition of isolated granules

Table 2 shows the ionic composition of the granule preparation isolated from zone C of the second density gradient. In spite of the three washing steps involved in the preparative procedure there appeared to be little loss of Zn^{2+}, Ca^{2+}, Mg^{2+} and P_i from the granule by this stage, judging by the similarity of their contents expressed per mg of protein in this fraction with those in peak insulin fractions on the first density gradient (Fig. 1). Na^+, K^+, ATP, ADP and AMP could also be demonstrated in the final granule preparation.

The apparent concentrations of each constituent in the granule could be calculated on the basis of the internal total exchangeable water space (1.97 μl/mg of protein), which was determined from the difference in the spaces of distribution of ^3H_2O and inulin[^{14}C]carboxylic acid in suspensions of isolated granules (Hutton, 1982). Such calculations showed that the granule concentrations of Zn^{2+}, Ca^{2+}, Mg^{2+} and insulin were an order of magnitude or more greater than their apparent concentrations in the tissue as a whole. The total adenine nucleotide content of the granule approximated that of the intact tissue, as did the Na^+ and P_i contents; the K^+ content was lower than that of the tissue as a whole (J. C. Hutton & M. Peshavaria, unpublished work).

Ionic distribution within the granule

Sonication of granule suspensions in hypo-osmotic medium at pH6 resulted in the solu-

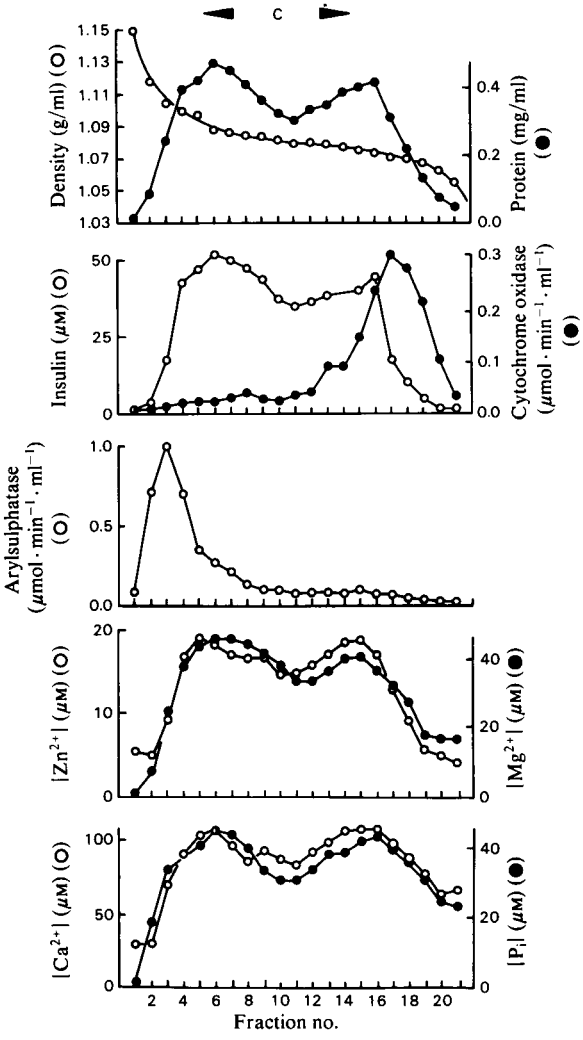

Fig. 2. *Percoll-density-gradient centrifugation of insulin-enriched fractions*

Insulin-containing material (zone A; Fig. 1) from the initial density gradient was dispersed in 42% (v/v) Percoll and centrifuged as detailed in the Experimental section. The material occupying zone C was recovered for subsequent experiments. The recoveries of the ionic constituents were 85–105% of the amounts originally transferred.

bilization of most of the granule contents of Mg^{2+}, Ca^{2+} and P_i (Table 3). Zn^{2+} and insulin, however, were largely insoluble under these conditions and could be recovered by centrifugation. Treatment of the pelleted material at pH 7.4 with a solution containing a high salt concentration and bivalent-cation-chelating agent brought both Zn^{2+} and insulin into solution to a similar extent. This fraction also contained a significant quantity of Mg^{2+} and P_i

Table 2. *Composition of isolated granules*

Granules were isolated from zone C on the second Percoll density gradient (Fig. 2) by the methods described in the Experimental section. The granule content of each constituent is expressed in relation to the total protein content determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. A M_r of 5800 for insulin was assumed in these calculations. The total concentration of each constituent was estimated by dividing its specific activity by the total exchangeable water space of the isolated granules, which was determined by radio-isotopic uptake measurements (Hutton, 1982). Each tabulated value is the mean ± S.E.M. of results obtained with different preparations; the numbers of these are shown in parentheses.

		Content (nmol/mg of protein)	Concentration (mmol/l)
Insulin	(4)	79.4 ± 7.7	41.6 ± 4.0
Zn^{2+}	(14)	43.6 ± 3.4	22.8 ± 1.8
Ca^{2+}	(10)	229.4 ± 19.9	120.1 ± 10.4
Mg^{2+}	(7)	138.3 ± 11.0	72.4 ± 4.8
P_i	(9)	132.3 ± 6.1	69.3 ± 3.2
ATP	(8)	6.6 ± 1.2	3.5 ± 0.6
ADP	(8)	9.8 ± 3.8	5.1 ± 2.0
AMP	(8)	6.4 ± 1.8	3.4 ± 1.9
Na^+	(2)	55.0	28.8
K^+	(2)	30.0	15.8

but little Ca^{2+}; the molar ratio of these ions to insulin was in each case less than 0.3. The addition of a detergent and further sonication resulted in the solubilization of the remaining Zn^{2+} and insulin.

Ca^{2+} electrode experiments

The addition of a 50 μl sample of freshly-prepared osmotically-protected granules (about 8 mg of protein/ml) to 2 ml of iso-osmotic sucrose medium resulted in an increment in the free $[Ca^{2+}]$ of the medium from approx. 1–2 μM to 4–5 μM. Subsequent incubation of such suspensions for 15 min at 37°C did not result in further changes in the free $[Ca^{2+}]$. Addition of Triton X-100 (final concn. 0.1%), however, resulted in an immediate increase in the free $[Ca^{2+}]$ to about 15–25 μM. This change occurred with a time course that was as rapid as the response time of the electrode itself under these conditions (less than 10 s). When granules were diluted into medium without sucrose to achieve the same final protein concentration as above, Ca^{2+} was again rapidly released; the increment in concentration observed being 89.9 ± 8.3% (nine experiments) of that seen in detergent-containing iso-osmotic medium. Under these conditions the further addition of Triton X-100 produced negligible changes in medium $[Ca^{2+}]$. The Ca^{2+} released from the granules in the ionized form amounted to 125 ± 17 nmol/mg of protein (10 preparations), a

Table 3. *Solubilization of proteins and ionic constituents of isolated granules*

Isolated granules were suspended at 1.5–4mg of protein/ml in 400μl of potassium 4-morpholine-ethanesulphonic acid buffer (pH6.5), sonified for 15s at 4°C (MSE sonifier; medium setting) and then centrifuged in a Beckman SW 50.1 rotor for 30min at 50000*g* ($r_{av.}$ 8cm) to produce the first supernatant. The pellet was sonified as before in 400μl of a solution of 0.5M-KCl containing 5mM-EDTA (pH7.4) and centrifuged as before to produce the second supernatant fraction. The pellet was then treated in an identical manner with the same solution containing 0.1% Triton X-100 to produce the third supernatant and final pellet. Tabulated values are means (±S.E.M. where relevant) of results obtained with different granule preparations; the numbers of these are shown in parentheses.

	Protein (3)	Insulin (5)	Zn^{2+} (6)	Ca^{2+} (2)	Mg^{2+} (2)	P_i (2)
Initial content (nmol/mg of protein)		79.6±6.0	38.5±4.1	186	152	142
First supernatant (%)	39.8±6.7	9.7±3.1	21.8±4.3	77	73	87
Second supernatant (%)	33.9±2.5	76.4±2.7	66.7±7.2	<8	12	13
Third supernatant (%)	10.2±2.1	8.9±3.8	10.6±3.5	<8	5	11
Final pellet (%)	7.4±1.4	3.3±1.0	3.9±1.3	<8	4	8
Recovery (%)	91	98	103	—	94	119

value equivalent to 55% of their total Ca^{2+} content as determined by atomic-absorption spectroscopy. The stability of the granule Ca^{2+} expressed as a percentage of the total Ca^{2+} released after incubation in iso-osmotic medium was 9.1±2.5% (10 experiments).

Simulation of the ionic environment of the granule

The mixing of Mg^{2+}, Ca^{2+}, ATP, ADP, AMP and P_i at concentrations equivalent to those in isolated granules resulted in the precipitation of a substantial proportion of the total Ca^{2+} and further formation of nucleotide–Ca^{2+} complexes, with the result that only a small proportion of Ca^{2+} remained in ionized form (Fig. 3). Within the pH range prevailing in the granule interior (pH5.5–6.0; Hutton, 1982) this accounted for about 10% of the total Ca^{2+} added. The inclusion of bovine insulin (200mg/ml) containing endogenously bound Zn^{2+} (67nmol/mg) did not produce any change in the free [Ca^{2+}] under these conditions. Both P_i and the adenine nucleotides contributed to the process of Ca^{2+} binding as evidenced by the effect of nucleotide omission from the mixture. Omission of Mg^{2+} from the formulation decreased the free [Ca^{2+}], indicating that Ca^{2+} and Mg^{2+} competed for available ligands to a quantitatively significant extent under these conditions. When a mixture prepared at pH5.5 containing all components including insulin was diluted to a concentration approximating that used in granule lysis experiments, the extent of dissociation of Ca^{2+} increased from 10 to 65% over a time course that was faster than the electrode response. The extent of dissociation of Ca^{2+} achieved was thus similar to that observed when granules were hypo-osmotically lysed.

Equilibrium-dialysis experiments

The concentration-dependent binding of Ca^{2+} to components within lysed granule extracts was demonstrated by equilibrium-dialysis experiments (Fig. 4). Scatchard plots (Scatchard, 1949) of such data were curvilinear, suggesting that multiple binding sites were involved. The inclusion of 10mM-Mg^{2+} in the equilibration buffer, a concentration severalfold lower than that found in isolated granules, however, reduced binding of Ca^{2+} to insignificant levels (<3nmol/mg of protein).

Control experiments

A series of control experiments was performed to eliminate some possibly trivial explanations for the observed association of P_i and bivalent cations with the isolated secretory-granule fraction.

(*a*) Homogenization of tissue and subsequent preparation of granules in the presence of 5mM-EDTA and 10μM-Ruthenium Red did not alter the levels of Zn^{2+}, Ca^{2+}, Mg^{2+} or insulin content of the isolated granules. These agents reportedly prevent the redistribution of Ca^{2+} by mitochondria (Moore, 1971); EDTA would also remove superficially bound Mg^{2+}.

(*b*) Pre-incubation of tissue for 30min at 37°C in Krebs bicarbonate buffer containing 2.8mM-glucose had no effect on the tissue content of these ions or their distribution on density gradients. Thus redistribution did not seem to occur as a consequence of maintaining the tissue for extended periods at 4°C before analysis.

(*c*) $^{45}Ca^{2+}$, $^{65}Zn^{2+}$ or [^{32}P]P_i, when added to tissue immediately before its homogenization, were each recovered to a negligible degree in particulate fractions separated on density gradients. This indicated that uptake or binding of these ions by intracellular organelles did not occur after tissue disruption.

(*d*) Tumour pieces incubated in Krebs bicarbonate buffer for 10min at 37°C incorporated significant quantities of $^{45}Ca^{2+}$ and [^{32}P]P_i into

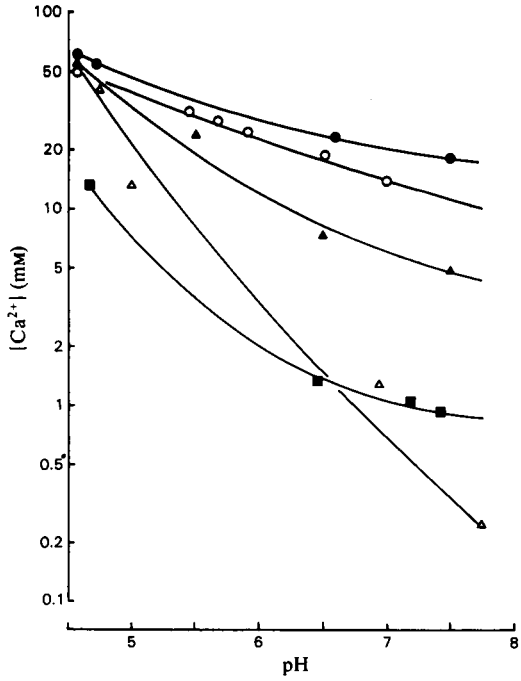

Fig. 3. *Simulation of the intragranular ionic environment*

Solutions of 0.57 M-$CaCl_2$, 0.35 M-$MgCl_2$, 0.33 M-KH_2PO_4, and a composite mixture of ATP (Na^+ salt), ADP (monocyclohexylammonium salt) and AMP (Na^+ salt), each at five times their apparent concentration in isolated granules (Table 2), were prepared. These were mixed in various proportions, the last addition in all cases being KH_2PO_4. KOH was then added to change the pH and the mixtures were left at room temperature overnight. The final pH and free [Ca^{2+}] were then determined (see the Experimental section). Results are shown for a mixture with a composition resembling that of isolated granules (▲), similar ones in which the initial P_i content was doubled (△), or where omissions were made of Mg^{2+} (■), Mg^{2+} and adenine nucleotides (○), or adenine nucleotides alone (●). The electrode response to $CaCl_2$ solutions was predicted by the Nernst equation over the range of Ca^{2+} concentrations examined and was not affected by pH.

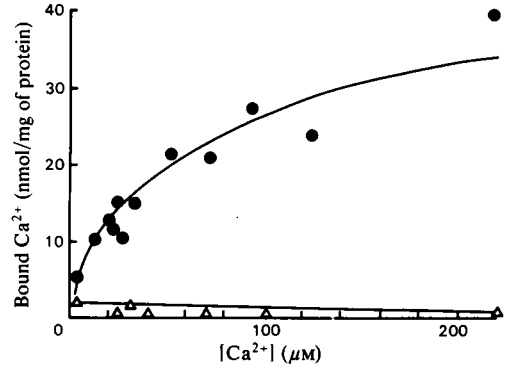

Fig. 4. *$^{45}Ca^{2+}$ equilibrium-dialysis experiments*

Granules were hypo-osmotically lysed, their endogenous bivalent cations removed with a bivalent cation-exchange resin and the remaining material dialysed overnight against buffer (pH 6) containing $^{45}Ca^{2+}$ at the indicated free [Ca^{2+}] (see the Experimental section). The Mg^{2+} concentration was maintained at either <10 μM (●) or 10 mM (△). Two different granule preparations were used in establishment of each curve; each point represents the results of duplicate analyses on single incubations.

intracellular organelles. For both ions the distribution of radioactivity on density gradients was not altered when the interval from homogenization to the commencement of the centrifugation procedures was extended from 5 min to 3 h. It appeared unlikely, therefore, that redistribution of these ions occurred during granule isolation.

Discussion

Current information on the subcellular localization of Zn^{2+} and Ca^{2+} in the pancreatic B-cell comes primarily from data obtained from islets by ultrastructural and histochemical techniques of limited specificity and from radioisotope incorporation experiments performed under non-equilibrium conditions (Herman *et al.*, 1973; Howell *et al.*, 1975; Ravazzola *et al.*, 1976; Kohnert *et al.*, 1979). The present analyses relied on direct chemical measurements and are not subject to these technical limitations. They were, however, performed in B-cells of a tumour origin, and are subject conceivably to artefacts of ion redistribution occurring during tissue disruption. The contents of Zn^{2+}, Ca^{2+}, and Mg^{2+} in the insulinoma, were within the range expected from analyses performed on islets of Langerhans (Havu *et al.*, 1977; Malaisse *et al.*, 1978*b*; Berggren *et al.*, 1978; Figlewicz *et al.*, 1980; Wolters *et al.*, 1982). The subcellular distribution of Zn^{2+} accorded with ultrastructural data obtained in islets in suggesting that the major pool is the secretory granules. A second pool was identified in association with a soluble tissue fraction. A recent study with rat islets (Figlewicz *et al.*, 1980) has similarly suggested the existence of a non-granular Zn^{2+} pool. However, the extent of this was considerably greater than that presently defined.

The distribution of Ca^{2+} amongst different subcellular compartments in the tumour tissue was consistent with reported results of X-ray microprobe analyses (Herman *et al.*, 1973; Howell *et al.*, 1975)

and similar to that deduced by differential centrifugal analysis of $^{45}Ca^{2+}$ taken up by islets (Kohnert *et al.*, 1979). The endogenous granular Ca^{2+} content, however, was considerably greater than may have been anticipated from $^{45}Ca^{2+}$ data. This was most likely due to the homogeneity of the granule fraction analysed and the fact that, in radioisotopic experiments, granules are unlikely to reach isotopic equilibrium owing to the poor exchangeability of their Ca^{2+} content (Borowitz & Matthews, 1980).

The contents of ATP, ADP and AMP in isolated tumour granules were in close agreement with those calculated from radioisotopic incorporation data (Leitner *et al.*, 1975) and their insulin content conformed with values deduced from morphological data obtained in pancreatic B-cells (Howell, 1974).

Given that virtually all of tissue insulin was located in the secretory granule it was possible to calculate, from the ionic composition of isolated granules, the minimum relative contribution of the granular pool to the total tissue contents. It thus appeared that 40% of the total Zn^{2+}, 26% of the Ca^{2+}, 16% of the Mg^{2+} and 6% of the P_i was in the granules.

The intragranular storage of Zn^{2+}

X-ray crystallography and equilibrium-dialysis studies have demonstrated that Zn^{2+}–insulin and Zn^{2+}–proinsulin hexamers commonly possess two centrally co-ordinated Zn^{2+} atoms linked to B10 histidine residues. Three further atoms may be bound as a consequence of the pairing of B13 glutamate carboxy groups during dimer formation, making, in total, five sites per hexamer of potentially high affinity. Additional isolated carboxylate side chains, particularly in proinsulin, may support further low-affinity binding (see Emdin *et al.*, 1980).

Isolated granules contained approx. 0.5 atoms of Zn per mol of immunoreactive insulin, a value that is greater than the capacity of the central co-ordination sites but less than the 0.83 atom predicted for all the high-affinity sites described above. It seemed unlikely that the high-capacity low-affinity sites prominent in proinsulin were occupied. This would have been reflected as a much greater Zn^{2+} to immunoreactive insulin ratio than was presently observed in the first supernatant obtained after disruption of the granule (Table 3). The concomitant presence of high concentrations of Ca^{2+} and Mg^{2+} in the granule may preclude the occupation of these sites. Recent ^{113}Cd n.m.r. studies on insulin performed at alkaline pH values (Sudmeier *et al.*, 1981) suggest that Zn^{2+} binding to the sites associated with B13 glutamate residues may be displaced by Ca^{2+}. This could explain the submaximal high-affinity Zn^{2+} binding seen for granules.

The intragranular form of Ca^{2+}

The ratio of Ca^{2+} to P_i of 1.73 observed in isolated granules approximated the stoichiometry of crystalline hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, or amorphous $Ca_3(PO_4)_2$, two storage forms of Ca^{2+} commonly found in biological tissues. This initially suggested that all the intragranular Ca^{2+} was sequestered as insoluble phosphates. Investigation of the intragranular distribution of Ca^{2+}, however, revealed that it was rapidly solubilized by osmotic or detergent lysis in a form that was readily dissociable at pH 7. Such findings do not preclude the existence of more soluble forms such as $Ca(H_2PO_4)_2$ and $CaHPO_4$; indeed these would be more compatible with the acidic interior of this organelle (Hutton, 1982). Not all the granule Ca^{2+}, however, could be in this form since a higher P_i to Ca^{2+} stoichiometry is required than was observed. The intragranular adenine nucleotides were further potential Ca^{2+} ligands, although minor ones in quantitative terms.

Other bivalent cation binding sites conceivably exist among the granule proteins. Insulin, proinsulin and C-peptide collectively could contribute 10 times as many negatively charged species as P_i, and there are possibly as many sites again amongst the remaining peptides within the granule (Hutton *et al.*, 1982). Equilibrium-dialysis experiments, however, suggested that the binding capacity of the granule proteins, or for that matter the membrane phospholipids, did not account for substantial Ca^{2+} binding. In this case, and also with P_i and the adenine nucleotides, it has to be considered that granular Mg^{2+} will also compete for Ca^{2+}-binding.

The above considerations were borne out in experiments in which bivalent cations, insulin, adenine nucleotides and P_i were mixed at concentrations equivalent to those found in the granule. Such experiments indicated that the intragranular free $[Ca^{2+}]$ concentration may be as high as 10mM. In the cell this could mean that the transmembrane free $[Ca^{2+}]$ gradient is as high as 10^4, a situation that implies the existence of an energy-linked Ca^{2+}-transporting system operating either within the granule itself or at some stage of granule morphogenesis. Modulation of such a process *in vivo* could account for the observed increase in granular Ca^{2+} after stimulation of secretion in pancreatic islets (Herman *et al.*, 1973; Kohnert *et al.*, 1979).

Experiments in which the free $[Ca^{2+}]$ surrounding isolated granules in suspension was measured suggested that the passive permeability of the granule membrane to Ca^{2+} was very low. These studies also suggested that an increase in the permeability *in vivo* would result in a substantial efflux of Ca^{2+} from the granule interior. There is little evidence for this occurring in the granule population at large since the turnover of the granule Ca^{2+} pool is low (Borowitz & Matthews, 1980).

However, it could take place in a localized cellular environment, for example, at the site of exocytosis. An increase in the free $[Ca^{2+}]$ here could underly the mechanism whereby exocytosing granules undergo so-called chain fusion, a phenomenon that has been described in many secretory cells including the pancreatic B-cell (Orci & Malaisse, 1980).

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