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Isolation and Characterisation of Insulin Secretory Granules from a Rat Islet Cell Tumour

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Summary. Density gradient centrifugation techniques, using iso-osmotic colloidal silica suspensions (Percoll), were developed for the isolation of insulin secretory granules from a transplantable rat islet cell tumour. These procedures were readily completed within 7 h and from each animal yielded approximately 1 mg of granule protein. The isolated granules were essentially free of other subcellular organelles as evaluated by their contents of marker proteins, electron microscopy and by electrophoretic analyses. Their susceptibilities to lysis at low osmotic strength, at pH values above 7 or in media containing sodium ions were similar to those of granules partially purified from islets. Insulin comprised 50–60% of the total granule protein when determined by immunoassay or by densitometry of electrophoretic profiles. The proinsulin content was marginally higher than that of islets, as was the ratio of insulins I to II. Electrophoretic analyses revealed that the secretory granules contained 150 or more proteins besides insulin-related peptides. The majority of these had acidic isoelectric points and were located both within the granule interior and its enveloping membrane.

Key words: Insulin, proinsulin, granule, vesicle, rat, islet cell tumour, insulinoma, subcellular fractionation, electrophoresis, ultrastructure.

Investigation of the biochemical properties of secretory granules of the pancreatic B cell has been impeded by the small quantity and cellular heterogeneity of material available from pancreatic islets. Moreover, established procedures for the subcellular fractionation of islet tissue [1–3] typically yield granule fractions contaminated with other organelles.

This laboratory is currently investigating a transplantable rat B cell tumour [4] as a model of the secretory process. The tissue has a high insulin content and is available in gram quantities. Tumour cells are well differentiated and closely resemble pancreatic B cells in their ultrastructural and immunochemical staining properties. Glucagon, pancreatic polypeptide, somatostatin, gastrin, neurotensin, vasoactive intestinal polypeptide and B lipotropin have not been detected using immunological techniques in the cell line maintained in our laboratory (T. E. Adrian, S. R. Bloom, I. Doniach, L. H. Rees; personal communications). The tumour releases insulin by a process which is indistinguishable from that in islets [5].

The present communication reports the development of techniques to isolate insulin granules in high yield and purity from this tissue. Electrophoretic analyses of the constituent granule proteins are documented.

Methods

Assay Procedures

Insulin was determined by a modification of the back-titration immunoassay procedure of Wright et al. [6]. Samples were solubilised in 50 µl of phosphate buffer (100 mmol/l, pH 7) containing Triton X-100 (1 g/l), EDTA (10 mmol/l) and bovine serum albumin (10 g/l) and then diluted in buffer containing bovine serum albumin alone to an insulin concentration of 0.5–2.5 U/l. Two hundred µl of the diluted sample was incubated for 30 min at 37 °C with 200 µl of the same buffer containing non-immune horse serum (200 ml/l) and guinea pig anti-bovine insulin antisera sufficient to bind 5 U/l of insulin. Buffer (200 µl) containing 10^8 dpm/l ^{125}I-insulin (Amersham International, Amersham, Bucks, UK) and bovine insulin (7.5 U/l) was then added and a further incubation performed at room temperature for 30 min. Five hundred µl of 250 g/l polyethylene glycol 6000 (Fisons, Loughborough, Leicestershire, UK) was finally added and the samples centrifuged at 4 °C for 20 min at 1700 × *g*. The supernatant was aspirated and the radioactivity of the precipitated immune complex determined with a gamma radiation counter. Rat insulin (Novo, Copenhagen, Denmark) was used as a standard. Proinsulin was determined in the same samples by an indirect two-site immunoradiometric assay [7].

Spectrophotometric assays were performed for aryl sulphatase [8], cytochrome oxidase [9], NADPH-cytochrome c reductase [10], lactate dehydrogenase [11] and catalase [12]. 5′ nucleotidase was determined radiometrically in the presence of 100 mg/l Lubrol 12A9 [13] and DNA was assayed fluorimetrically [14]. Galactosyl transferase activity was determined from the incorporation of UDP-U-^{14}C galactose (200 mCi/mmol; Amersham) into soya bean trypsin inhibitor (Sigma, Kingston-on-Thames, Surrey, UK) [15]. The reaction was terminated

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by the addition of 200 g/l trichloroacetic acid and the precipitated acceptor protein recovered by filtration on cellulose acetate membranes (0.45 μ, type HAWP; Millipore, Bedford, MA, USA). Air-dried membranes were dissolved in 5 ml scintillation counting fluid (cocktail T, Hopkins & Williams, Chadwell Heath, Essex, UK) and their radioactivities determined by liquid scintillation spectrometry.

Density gradient fractions were usually assayed on the same day without prior removal of the gradient media. At the pH values used in some methods, the density gradient material (Percoll) precipitated and so was removed by centrifugation immediately before any optical measurement.

Protein was determined in samples precipitated with 50 g/l trichloroacetic acid using bovine serum albumin fraction V or bovine insulin as a standard. The method of Lowry et al. [16] was used except when samples contained Percoll, in which instance a fluorimetric procedure was adopted [17]. In this case the pelleted material was dissolved in 50 mmol/l sodium borate buffer (1 ml, pH 9) and 200 μl of a solution of fluorescamine (300 mg/l in acetonitrile) added while mixing. The fluorescence at 390 nm excitation/480 nm emission was then determined. Percoll itself produced a fluorescence $\leqslant$ 10% that of the sample and was corrected for by passing standards through the entire procedure. The Percoll content of each gradient fraction and thence its density was determined from its refractive index.

Ultrastructural Analyses

Glass haematocrit capillaries were loaded sequentially with 20 μl fluorochemical oil (type FC43 Beckman, Palo Alto, California, USA), 40 μl of a suspension of subcellular particles in cacodylate buffered paraformaldehyde/glutaraldehyde fixative [18], 2 μl horse serum and 10 μl of fixative. The capillary tip at the oil end was heat-sealed leaving an air space of about 15 μl and the tube then centrifuged for 4 min at 10,000 × *g*. The pellicle of particulate material, which was embedded in a serum plug formed at the interface with the oil, was recovered, post-fixed in OsO_4 and stained with uranyl acetate and lead citrate by conventional procedures.

Electrophoretic Analyses

Single dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed on 19% slab gels (1.5 mm × 15 cm × 15 cm) run in an ammediol-based buffer system [19]. Molecular weight calibration was achieved using phosphorylase (98,000), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (31,000), chicken gizzard myosin light chain (20,000), myoglobin (17,000), cytochrome c (12,000) bovine pancreatic trypsin inhibitor (7,000) and mixed A and B chains of bovine insulin (3,000 mol. wt.). Samples were suspended in ammediol Cl buffer (100 mmol/l, pH 8.4) containing sodium dodecyl sulphate (10 g/l), dithiothreitol (10 mmol/l), EDTA (5 mmol/l), sucrose (250 mmol/l) and bromophenol blue (20 mg/l), heated for 5 min at 100 °C, centrifuged for 5 min at 9,000 × *g* and the supernatant loaded onto the gel.

Two dimensional electrophoretic separations were achieved by isoelectric focussing of samples in the first dimension using pH 3.5–10 ampholytes (LKB, Stockholm, Sweden) followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis in the second dimension on a linear 10–20% gradient gel run in a Tris-HCl based buffer system [20]. Samples were prepared as above but using N-ethylmorpholine acetate (50 mmol/l, pH 8.5) instead of ammediol as the buffer.

All gels were fixed in a 50 g/l trichloroacetic acid solution in 20% methanol and stained with Kenacid blue R (British Drug Houses, Poole, Dorset, UK).

Granule Preparation

Islet cell tumours were propagated in 200 g body weight mixed sex NEDH rats fed ad libitum as described previously [21]. The fibrous capsule of each tumour was removed, the tissue chopped into 1–2 mm fragments and rinsed in Hank's saline then in the homogenisation medium. All subsequent steps were performed at 4 °C unless specified. Tissue from 5–30 animals (2.5–15 g wet weight) was homogenised in a Potter homogeniser (2.5 cm diameter) with six strokes of a close fitting teflon pestle driven at 600 rev/min. The media (5 ml/g tissue) contained sucrose (275 mmol/l), 2 [N-morpholino]ethanesulphonic acid (10 mmol/l) and EGTA (1 mmol/l) and was adjusted to pH 6.5 with Tris base. The homogenate was centrifuged for 10 min at 1700 × *g* to remove unbroken cells and nuclei. The efficiency of homogenisation as judged from the release into the supernatant of lactate dehydrogenase was 85.7 ± 3.8% ($n = 6$). The majority of the tissue DNA (90.5 ± 7.4%, $n = 4$) was pelleted at this stage.

Density Gradient Centrifugation

Aliquots (10 ml) of the above supernatant were layered onto 26 ml of homogenisation media supplemented with 270 ml/l Percoll (Pharmacia, Stockholm, Sweden) contained in polypropylene centrifuge tubes (8.5 cm × 2.5 cm). After centrifugation for 45 min at 35,000 × *g* in a Beckman type 30 rotor, the resulting gradient was distributed into 24 tubes using a peristaltic pump. The material in the major insulin-containing fractions of two such gradients (peak I, Fig. 1) was dispersed in homogenisation media (37 ml) containing 420 ml/l Percoll and centrifuged under the same conditions.

Percoll contained in density gradient fractions was subsequently removed by diluting pooled fractions fivefold in homogenisation media and centrifuging for 15 min at 20,000 × *g* in a Beckman SW 27.1 rotor. The particulate material which sedimented was transferred to another tube and subjected to three further cycles of resuspension and centrifugation. The final pellet was suspended in homogenisation media without EGTA at a protein concentration of 10 mg/ml and either kept at 4 °C until use or stored at −70 °C.

Lysis Experiments

Freshly-prepared granules (0.1 mg protein/ml) were incubated for 30 min at 37 °C in 500 μl of media which contained EGTA (1 mmol/l) and 2[N-morpholino]ethanesulphonic acid (10 mmol/l) adjusted to the desired pH with Tris, together with varying concentrations of other salts and sucrose as described in Figure 3. The media was then centrifuged for 10 min at 25,000 × *g* in a Beckman type sw 50.1 rotor and the insulin content of the supernatant fluid determined.

Granule Subfractionation

Granules (4 mg/ml protein) were subjected to hypo-osmotic lysis in ammonium carbonate buffer (50 mmol/l, pH 9) containing EDTA (5 mmol/l) and separated into a soluble and membrane fraction by centrifugation for 30 min at 100,000 × *g* in a Beckman type sw 50.1 rotor. The pelleted material was washed twice in the same buffer. The soluble material was further fractionated by acidifying with acetic acid to pH 5.3 (the pI of insulin) and centrifuging as before. Approximately 10% of the initial protein was recovered in the membrane pellet, 30% in the final supernatant and 60% in material precipitated at pH 5.3.

Results

Granule Preparation

The initial density gradient centrifugation of the nuclei and cell-free homogenate of the tumour resulted in the separation of four discernible bands of turbidity corresponding to the four zones designated I, II, III and IV in Figure 1. These bands were coloured white, yellow,

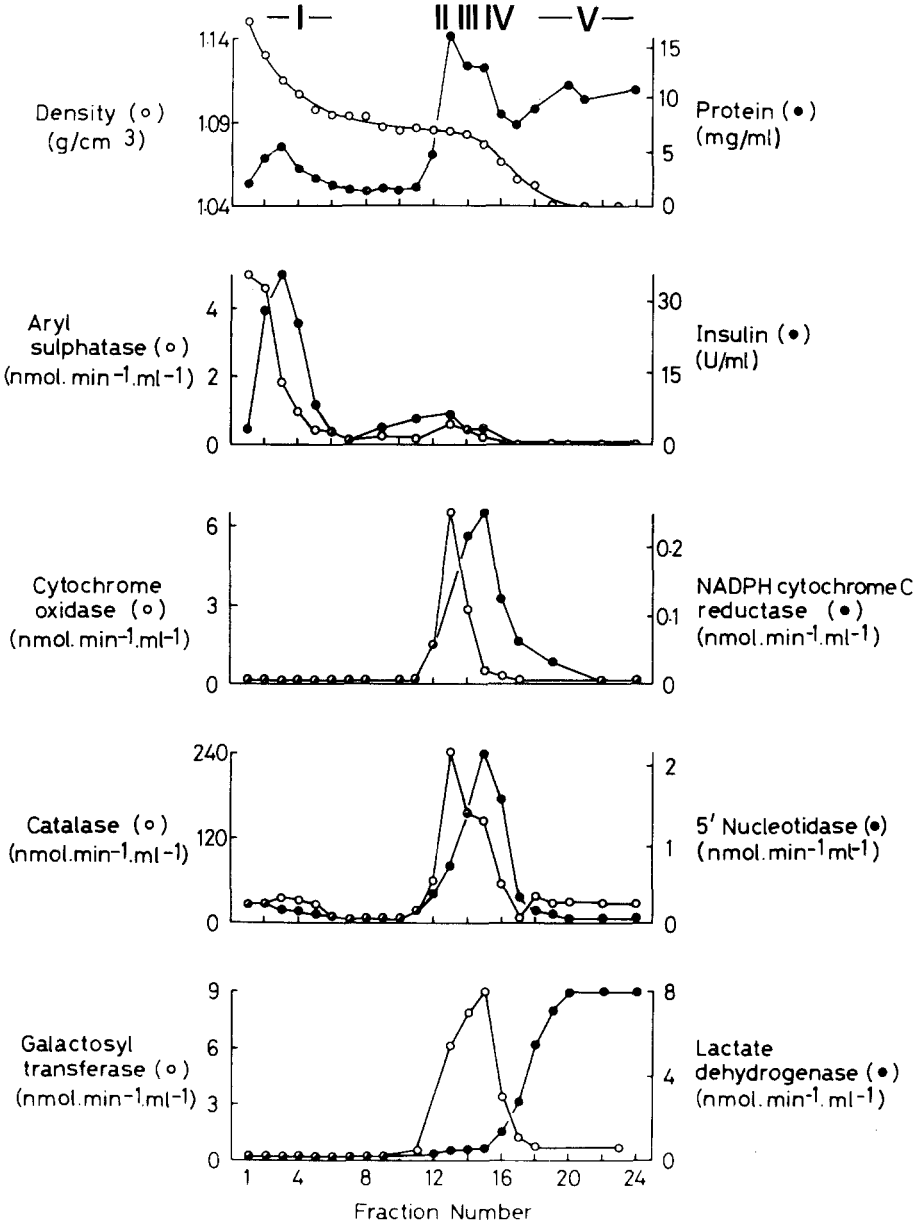

Fig. 1. First density gradient centrifugation. Tumour homogenate was centrifuged to remove nuclei and unbroken cells and then layered on media containing Percoll (270 ml/l) in the region designated V

orange and pink respectively and corresponded to the maximal activities of marker proteins for the insulin granule (insulin), mitochondria (cytochrome oxidase), endoplasmic reticulum (NADPH-cytochrome c reductase) and plasma membrane (5' nucleotidase) respectively. The peroxisomal marker (catalase) and Golgi body marker (galactosyl transferase) were also found principally in zone III. Lysosomes as revealed by the distribution of aryl sulphatase were more dense than insulin granules and not associated with visible banding or a protein peak on the gradient. Other marker proteins which were determined over the course of different experiments included succinate dehydrogenase (mitochondria), acid phosphatase and N-acetylglucosaminidase (lysosomes), glucose 6-phosphatase (endoplasmic reticulum) and proinsulin (secretory granules). Their distribution did not vary significantly from the corresponding marker proteins shown in Figure 1 (unpublished findings).

When the insulin granule-enriched fraction obtained from this gradient was subjected to further gradient centrifugation in Percoll media of a higher initial density, two broad overlapping zones of turbidity were observed. These coincided with the distribution of protein and insulin (Fig. 2). The lysosomal marker aryl sulphatase was found principally in a region of high density where little protein was present. Residual cytochrome oxidase activity appeared at a lower density. Residual 5' nucleotidase activity was distributed in a manner suggestive of a combined association with both granules and lysosomes. Markers for other subcellular organelles shown in Figure 1 could not be detected. The specific activity of insulin did not vary across the gradient in the fractions in which it could be reliably estimated. This

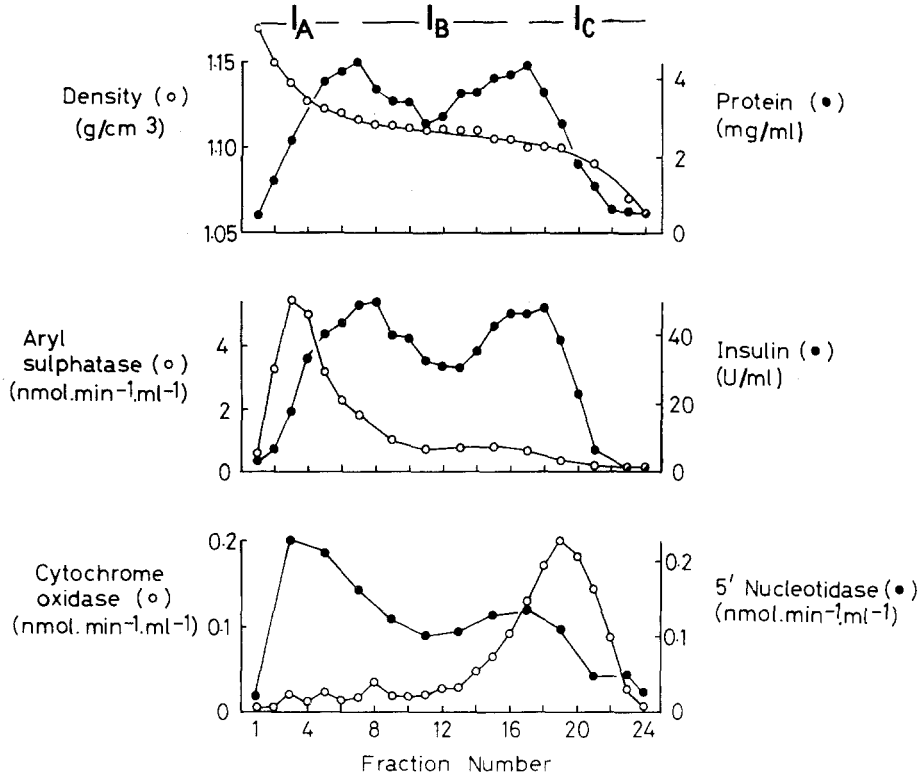

Fig. 2. Second density gradient centrifugation. Material from the first density gradient containing the majority of the tissue insulin (zone II, Fig. 1) was resuspended in media initially containing Percoll (420 ml/l)

Table 1. Recoveries of marker proteins in isolated granules

Marker	Initial homogenate	Isolated granules	Yield (%)	Relative specific activity
Insulin (U/mg protein)	0.71 ± 0.11	14.5 ± 0.8	27.6 ± 3.8	20.4
Aryl sulphatase (nmol · min^{-1} · mg^{-1})	77.4 ± 12.5	714.3 ± 38.4	12.2 ± 1.4	9.2
Cytochrome oxidase (nmol · min^{-1} · mg^{-1})	67.5 ± 4.1	23.4 ± 6.2	0.5 ± 0.2	0.3
NADPH-cytochrome C reductase (nmol · min^{-1} · mg^{-1})	4.1 ± 0.5	1.6 ± 1.0	0.5 ± 0.2	0.4
Catalase (μmol · min^{-1} · mg^{-1})	5.6 ± 0.5	29.1 ± 3.8	6.9 ± 1.2	5.2
5′nucleotidase (nmol · min^{-1} · mg^{-1})	51.0 ± 6.3	7.6 ± 0.7	1.9 ± 0.2	0.2
Lactate dehydrogenase (nmol · min^{-1} · mg^{-1})	144.1 ± 31.2	28.3 ± 1.9	0.3 ± 0.1	0.2

Each tabulated value is the mean ± SEM of results obtained in five different preparations. The percentage of the initial activity of each marker recovered in the final granule pellet is shown as the yield. Protein recovered in the granule fraction was 1.8 ± 0.2% of the initial homogenate value. The relative specific activity is calculated as the ratio of the final to the initial specific activity.

contrasted with the highly variable specific activity of the other marker proteins and suggested that insulin granules were the predominant particles in all fractions.

The bimodality of insulin distribution on this gradient did not reflect the existence of two granule populations but was the product of the sigmoidal density profile, there being concentration of material in the regions of a steeper gradient. The distribution of insulin calculated as a function of a linear increment in density suggested the presence of a single population with a mean density of 1.10 g/l. Fractions comprising zone IB (Fig. 2) were usually recovered for experiments requiring large quantities of granules. The final yields and specific activities of the marker proteins in this fraction are shown in Table 1.

Characterisation of the Granule Fraction

Granules isolated as above were subjected to further isopycnic gradient centrifugation on either a linear 25–50% by weight sucrose or a linear 150–300 g/l urografin (Schering AG, Berlin, FRG) gradient. The insulin and protein co-migrated as a single band in both cases with apparent densities of 1.20 and 1.14 respectively. The specific activity of insulin and electrophoretic profiles were unchanged in comparison with the material initially loaded. Further separation of insulin from residual aryl sulphatase and cytochrome oxidase was not afforded by these procedures (unpublished findings).

Electron Microscopy

Electron microscopy of the granule fraction that was used in most subsequent experiments (zone IB; Fig. 2), showed the presence of granules of a size and morphology typical of the B cell (Fig. 3 a). The central electron-dense core of these ranged in appearance from a diffuse

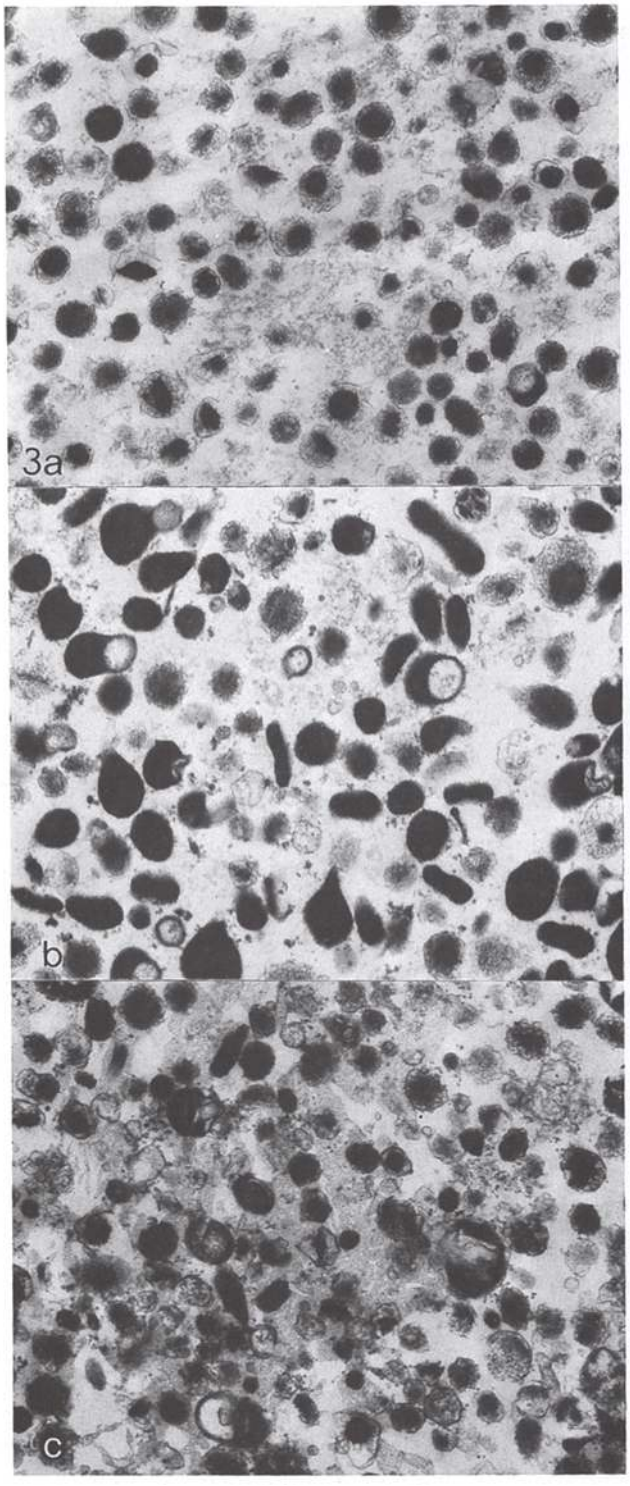

Fig. 3a–c. Electron micrographs of isolated granule fractions. **a** Purified granule fraction (zone IB, Fig. 2); × 22,400. **b** High density granule fraction (zone IA, Fig. 2) containing substantial lysosomal contamination; × 22,400. **c** Low density granule fraction (zone IC, Fig. 2) containing substantial contamination with mitochondrial and other membranes; × 22,400

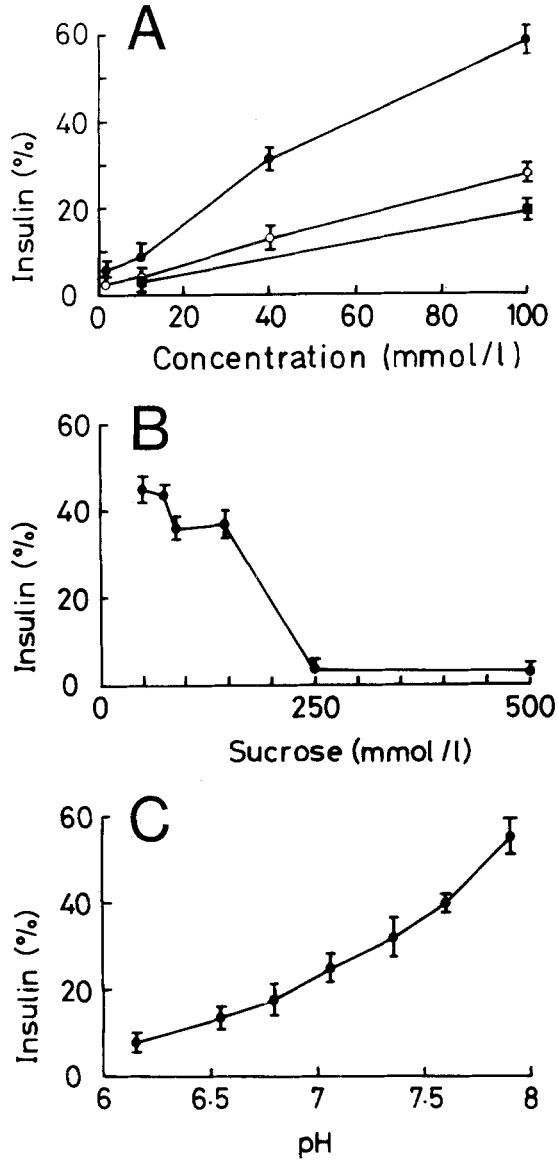

Fig. 4a–c. Granule lysis experiments. The supernatant insulin content is expressed as a percentage of that initially present in each tube. Each value is the mean ± SEM of results obtained with three different granule preparations incubated for 30 min at 37 °C **A** Sodium sulphate (●), potassium sulphate (○) or Tris sulphate (■) were added in iso-osmotic replacement of sucrose. The final pH was 6.5. **B** Reduction of the sucrose concentration of the media was made as indicated. The final pH was 6.5. **C** Media containing 0.25 mol/l sucrose was adjusted to the designated pH value with Tris

to a crystalline structure. A bilaminar membrane was associated with most granules.

Contamination of the granule fraction with other subcellular organelles was low, consisting of mitochondrial membrane fragments and lysosomes, the latter sometimes incorporating granule elements. Fraction IA (Figs. 2 and 3b) as expected from enzymic markers was contaminated with lysosomes, and fraction IC (Figs. 2 and 3c) with mitochondrial and other membrane elements. Some remaining Percoll particles were also evident in all fractions.

Lysis Experiments

Isolated granules incubated at pH 6.5 for 30 min at 37 °C released 14.3 ± 2.0% ($n = 3$) of their insulin con-

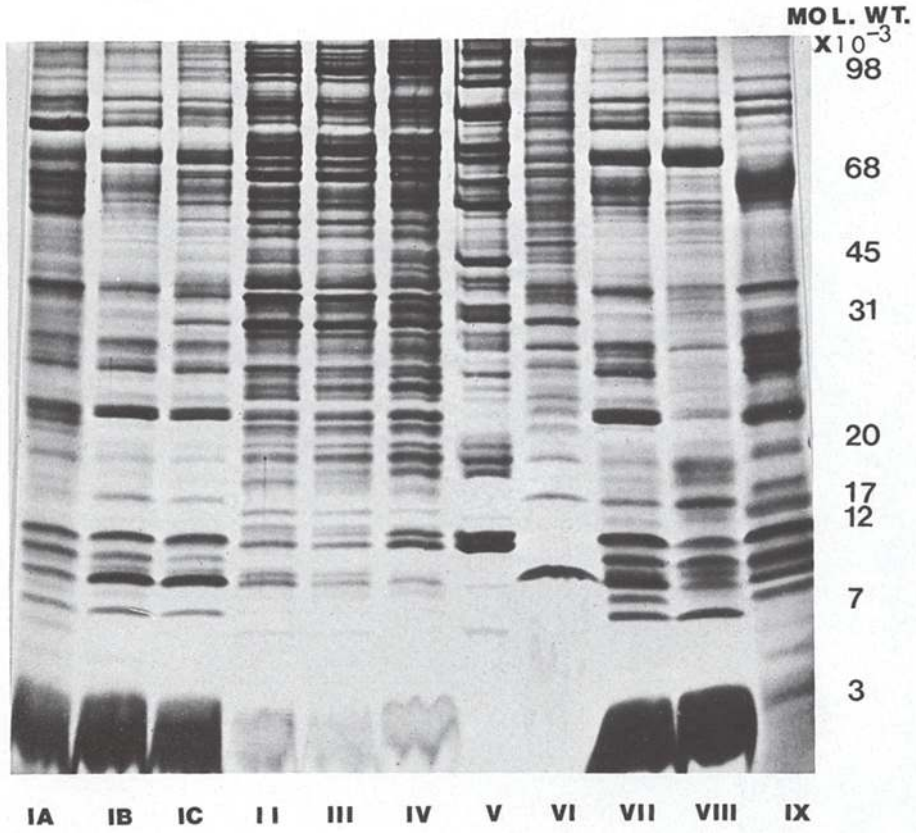

Fig. 5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of subcellular fractions. Lanes designated IA, IB, IC, II, III, IV and V represent material from identically labelled zones on the density gradients shown in Figs. 1 and 2. Lane VI represents granule membranes, and lane VII soluble material prepared from isolated granules at pH 9. Lane VIII was material precipitated from the soluble fraction by adjustment of the pH to 5.3 and lane IX the material still remaining in solution. Approximately 50 μg of protein was loaded in each case. The mol. wt. scale was deduced from the mobilities of marker proteins run on the same gel

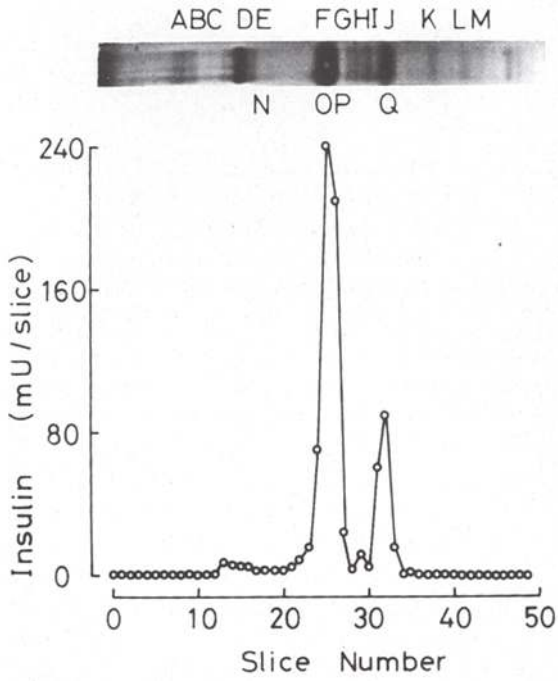

Fig. 6. Non-denaturing polyacrylamide gel electrophoresis of granules. Gel tracks were stained (insert) or sliced in 2.1 mm pieces and then subjected either to immunoassay or sodium dodecyl sulphate polyacrylamide gel electrophoresis. The latter process resolved peptides of the following mol. wt.: A 6,500; B 7,200; C 8,000; D and E 8,000, 21,000 and 22,000; F 3,000, 5,500 and 8,500; G 11,000; H 8,500; I 25,000; J 3,000; K 11,000; L 11,000 and 15,000 and M 12,500. The positions of migration of proinsulin (N and P) and insulin (O and Q) which were purified from the tumour are shown

tent into the medium compared with 4.9 ± 2.1% (n = 3) from granules maintained at 4 °C for the same interval. Complete solubilisation occurred in media containing the detergent Triton X-100 (1 g/l). Granule fragility assessed by this method was also markedly increased by iso-osmotic replacement of sucrose by sodium sulphate and to a lesser extent by potassium or Tris sulphate (Fig. 4a). Incubation at pH values above 7 (Fig. 4b) and hypo-osmotic incubation (Fig. 4c) also caused release into the media.

Polyacrylamide Gel Electrophoresis

Insulin was the dominant protein in electrophoretograms of granule fractions on sodium dodecyl sulphate polyacrylamide gels (Fig. 5). Densitometric quantification of the protein band corresponding to mixed insulin A and B chains showed it to be 50–60% of the total stained material, a value consistent with the observed insulin specific activity of the granule fraction (Table 1) given a theoretical maximal specific activity of 25 U/mg protein. A band co-migrating with beef and porcine proinsulins on these gels constituted 5–10% of the insulin content as assessed by densitometry, a value lower than that derived from radioimmunoassays (27 ± 2%, n = 7). This difference was probably due to the presence within the granule of conversion intermediates which would have been detected in the latter analysis.

Apart from insulin-related peptides, a large number of other proteins were evident in granule fractions vary-

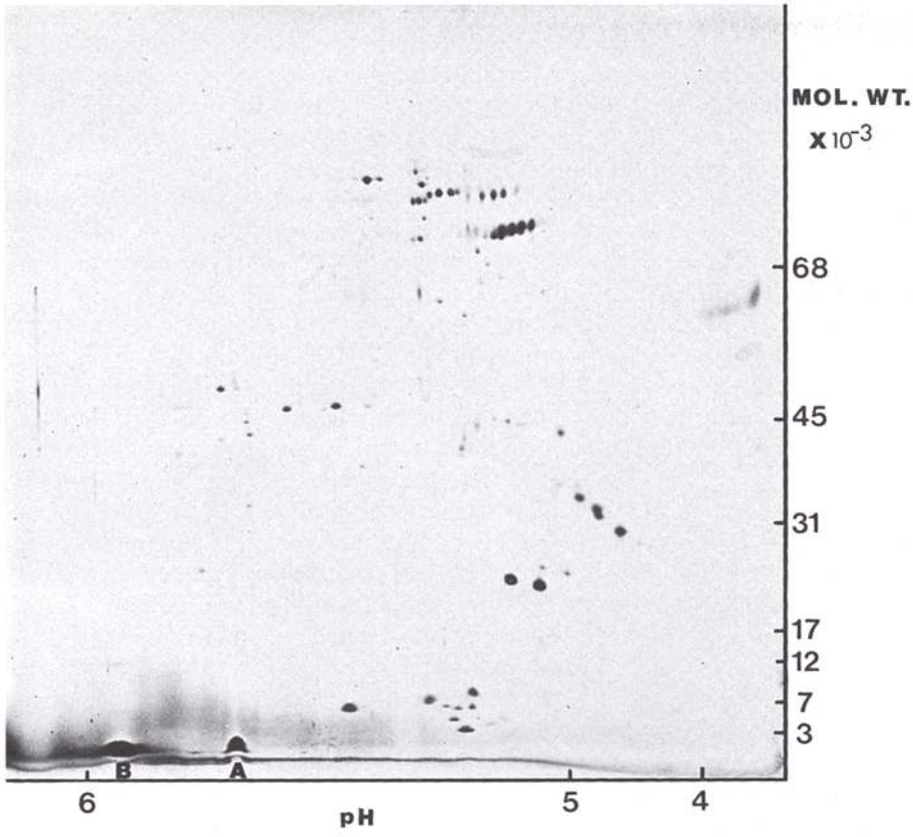

Fig. 7. Two dimensional polyacrylamide gel electrophoresis of granules. The granule sample derived from zone IB (Fig. 2) was subjected to isoelectric focussing in the first dimension (X axis) and in the second dimension to sodium dodecyl sulphate polyacrylamide gel electrophoresis. The migration of insulin A and B chains are indicated. The approximate pH and mol. wt. scales were derived from gels run in parallel

ing in quantity from 1–10% of the insulin content. These were not attributable to contamination since no major protein was found in a disproportionately greater quantity in another tissue fraction (Fig. 5). The insulin-rich fractions which were discarded (fractions IA and IC, Fig. 2) contained additional protein bands consistent with the presence of lysosomes in fraction IA and contamination of fraction IC with peptides seen predominantly in a mitochondrial-rich region of the initial density gradient (zone II; Fig. 1).

The soluble subfraction of granules prepared at pH 9 showed, besides insulin, 25 other components including several peptides in the 5–15,000 mol. wt. range, a doublet band at 21,000 mol. wt. and other major constituents with mol. wts. of 26,000, 28,000, 31,000 and 60,000. Adjustment of the pH of this subfraction to 5.3 resulted in the precipitation of insulin together with several other proteins. This further fractionation procedure permitted visualisation of minor soluble components. Electrophoresis of the granule membrane fraction revealed a large number of proteins of a broad mol. wt. range.

Polyacrylamide gel electrophoresis of granules under non-denaturing conditions at pH 9 (Fig. 6), showed the presence of two dominant bands corresponding in mobilities to rat insulin I and II standards. Such gels were cut into 2.1 mm pieces which were equilibrated for 2 days at 4 °C in phosphate buffer (100 mmol/l, pH 7) containing sodium azide (1 g/l) and bovine serum albumin (1 g/l). Immunoassay of the eluted fractions revealed two major components corresponding to rat insulin I (67.7 ± 2.6%) and rat insulin II (26.9 ± 2.4%, $n = 3$).

The apparent molecular sizes of the other components seen in such electrophoretograms were determined by equilibrating gel slices in the sodium dodecyl sulphate polyacrylamide gel electrophoresis sample buffer and electrophoresing them on the sodium dodecyl sulphate polyacrylamide gel electrophoresis system. The majority of peptides so revealed were within the the 5–15,000 mol. wt. range (Fig. 6 legend). Most of the proteins from which these components were derived did not display significant insulin immunoreactivity with the exception of one of 8000 mol. wt. which migrated in both electrophoresis systems in a manner similar to rat proinsulin I prepared from whole tumours.

Two dimensional gel electrophoresis of isolated granules revealed the presence of 18 major, 60 minor and 70 faintly perceptible components (Fig. 7). The majority of constituents possessed acidic isoelectric points. Insulin was resolved into two major spots, one corresponding to the A chain common to rat insulins I and II and the other to the two different B chains which were poorly resolved in this system. The bands prominent in the 5–15,000 mol. wt. region on single dimensional sodium dodecyl sulphate polyacrylamide gels (Fig. 5) resolved into seven major acidic components with similar isoelectric points and a single, more basic, peptide. A

major doublet band at 21,000 mol. wt. seen on single dimensional sodium dodecyl sulphate polyacrylamide gels resolved into two major spots with similar isoelectric points. Some of the higher molecular weight bands which migrated diffusely on single dimensional analysis appeared as a string of closely spaced spots inclining toward higher molecular weights and more acidic isoelectric points. This phenomenon is a feature of glycoproteins in such two dimensional analyses and is attributable to small changes in charge and molecular weight resulting from the addition of successive acidic carbohydrate residues.

Discussion

The preparation of insulin secretory granule fractions from mammalian pancreatic islets has in most investigations relied solely upon differential centrifugation procedures. Although adequate for the separation of granules from nuclei, cystolic proteins and to a certain extent mitochondria, these procedures fail to resolve granules from lysosomes and vesicles formed from internal membranes during homogenisation. Additional use of sucrose density gradients and phase separation techniques have succeeded in producing only a limited improvement in granule purity with a considerable reduction in yield [1, 3]. Such techniques applied to tumour tissue were similarly unsuccessful. Tumour granules behaved identically to those of islets with respect to their sedimentation behaviour on differential centrifugation and their buoyant density on sucrose gradients.

A density gradient procedure using colloidal silica particles met with considerable success in the subcellular fractionation of the tumour tissue. This success was attributed to the ability, using Percoll, to maintain isoosmolarity and thus separate subcellular elements on the basis of their native buoyant densities. Using the technique described, approximately 5–20 mg of purified granules could be obtained within 7 h, a situation which permitted experiments in vitro to be conducted on the same day.

The granules isolated were essentially free from contamination by other subcellular organelles as assessed by marker proteins, ultrastructure and electrophoretic analyses. Part of the lysosomal contamination which was evident was apparently due to crinophagy. A further element may be related to the presence in the granule of lysosomal-like enzymic activities [22, 23]. For most purposes the residual presence of lysosomal proteins was unimportant since they contributed little to the total protein in isolated granule fractions. Granules with a lesser contamination could also be prepared using select gradient fractions albeit with a low final yield.

A particular concern in investigations of the biochemical properties of insulin secretory vesicles from a tumour source is their relatedness to granules of the normal B cell. As in islets, insulin secretion by the tumour appears to occur by an exocytotic mechanism suggesting the presence of equivalent functional elements. Morphologically, tumour granules were identical to islet B cell granules both before and after isolation. They responded to a detergent and changes in media pH and ionic composition in a similar manner to granule-enriched subfractions from islets [1, 2, 24]. The granules were, however, less fragile at 37 °C and more susceptible to osmotic shock than reported by most investigators.

The tumour presently used is reported to have a reduced proinsulin processing capability [25]. This was borne out by the observation of a slightly higher proinsulin content in the tumour granules than in rat islets. The ratio of rat insulins I and II in tumour granules (2.57) also differed from that reported in rat islets (1.38) [26]. Similar quantitative differences between normal and tumour tissue has been recorded in the content of enzymes and zymogens in exocrine pancreatic secretory vesicles [27]. In this case no major qualitative differences were evident from electrophoretic analyses.

Two dimensional electrophoresis suggested that insulin granules may contain 150 or more proteins, many of which were readily solubilised in aqueous alkaline media and, therefore, were probably components of the granule core or loosely associated with the granule membrane. Such proteins are conceivably co-secreted with insulin. Some of these proteins were presumably intermediates of the processing of proinsulin, although many were of a larger molecular size than preproinsulin (12,400 mol. wt.). The majority of the smaller peptides which were separated by electrophoresis under non-denaturing conditions, furthermore, did not cross-react with anti-insulin antisera.

The large number of proteins present in the insulin granule membrane presumably reflects a diversity of biochemical processes. Some of these properties, like the ability to accumulate protons [28, 29], Ca^{2+} [30] and amines [31] are found in other granule types such as that of the chromaffin cell of the adrenal medulla. No striking similarities, however, were evident between the observed spectrum of proteins in the insulin granule membrane and that published for the chromaffin granule [32].

The present techniques provide an opportunity to study the functional properties of the B cell granule and yield sufficient material for small-scale purification of proteins from this source. The procedures also furnish a defined subcellular component suitable for studies in vitro of the molecular events of exocytosis.

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