Biochem. J. (1982) **204**, 161–170
Printed in Great Britain

Proton-translocating Mg^{2+}-dependent ATPase activity in insulin-secretory granules

John C. HUTTON* and Mina PESHAVARIA
Department of Clinical Biochemistry, University of Cambridge, New Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, U.K.

(*Received 9 November 1981/Accepted 22 December 1981*)

Insulin-secretory granules isolated from a pancreatic islet-cell tumour by centrifugation on Percoll density gradients exhibited a membrane-associated Mg^{2+}-dependent ATPase activity. In granule suspensions incubated in iso-osmotic media, activity was increased 2–3-fold by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, the combination of valinomycin, nigericin and K_2SO_4 or by the addition of a detergent. Permeant anions also increased Mg^{2+}-dependent ATPase activity under iso-osmotic conditions when combined with K^+ and nigericin, or NH_4^+. It was deduced that a major component of the activity was coupled to the translocation of protons into the granule interior. The granule membrane appeared poorly permeable to H^+, K^+, NH_4^+ and SO_4^{2-} but permeable, in increasing order, to phosphate or acetate, Cl^-, I^- and SCN^-. Like the proton-translocating ATPase of mammalian mitochondria the granule enzyme when membrane-bound was inhibited by up to 85% by tributyltin or *NN'*-dicyclohexylcarbodi-imide and was solubilized in a tributyltin-insensitive form after extraction with dichloromethane. It was clearly not a mitochondrial contaminant as evidence by the distribution of marker proteins on density gradients. Unlike mitochondrial activity it was insensitive to oligomycin, efrapeptin, atractyloside, azide and oxyanions. Its properties, however, were indistinguishable from those of the proton-translocating ATPase found in the chromaffin granules of the adrenal medulla. Moreover, insulin granules and chromaffin granules exhibited similar levels of activity. This indicated that in spite of the differences in their internal composition, granules from tissues involved in polypeptide and amine hormone secretion possess catalytic components in common. Only a minor role for the ATPase in amine transport in insulin granules was apparent. Rather, its presence here may relate to the process of secretory vesicle morphogenesis or to the exocytotic mechanism.

The insulin-storage granule of the pancreatic β-cell may be expected to possess a diversity of biochemical activities related to the processing of its secreted product, the maintenance of its internal composition and its recognition of, and interaction with, other cellular elements during exocytosis. As a lipid-membrane delimited vesicle with a protein-containing core, and in its pathway of intracellular formation, it is equivalent to granules in many other tissues, whether they be endocrine or exocrine, or whether the active secreted product is an enzyme, peptide hormone or biogenic amine.

Other parallels are evident, for example, in the capacity of secretory granules from various sources to concentrate bivalent cations and permeant bases (Owman *et al.*, 1973; Hopkins & Duncan, 1979). Biochemical properties that are common to different granule types bear scrutiny since potentially they provide an approach to the elucidation of general mechanisms involved in granule formation and exocytosis.

The transport of solutes by subcellular organelles is, in many instances, linked energetically to chemiosmotic proton gradients. The universal importance of such gradients in transport and energy-transducing systems is reflected also at the enzymic level. The proton-translocating ATPase implicated in amine transport into chromaffin granules of the adrenal medulla, for example, exhibits subunit structure and immunological properties similar to the $(F_0–F_1)$ complex involved in ATP synthesis and

* To whom correspondence and reprint requests should be addressed.

0306-3283/82/040161-10$01.50/1

ATP-driven solute accumulation in mitochondria (Apps & Schatz, 1979). The mitochondrial enzyme in turn closely resembles the membrane ATPases of prokaryotic cells in which proton gradients can support nutrient transport as well as ATP synthesis and motility (for reviews, see Downie *et al.*, 1979; Fillingame, 1980). The present paper investigates the presence in insulin-secretory granules of such proton-translocating Mg^{2+}-dependent ATPase activity.

Studies of the biochemical properties of isolated insulin-secretory granules has been impeded by the small quantities of material available when using pancreatic islets as a source. Present studies were performed with a transplantable rat islet-cell tumour (Chick *et al.*, 1977), which has an insulin content similar to that of isolated islets and whose cellular morphology is dominated by β-cells as assessed by electron microscopy and immunofluorescent staining for insulin. The tumour releases insulin in response to leucine, theophylline and glucagon by a process indistinguishable from that in islets with respect to its dependence on extracellular Ca^{2+}, cellular energy, cyclic nucleotides, calmodulin and microskeletal elements (Sopwith *et al.*, 1981).

Experimental

Preparation of tumour homogenates

The rats and tumours used were derived from material supplied by Professor W. L. Chick, New England Deaconess Hospital, Boston, MA, U.S.A. Tumour fragments (10–20mg wet wt.) suspended in physiological medium (Hanks & Wallace, 1949) were injected subcutaneously in the flank or groin of 6-week-old mixed sex recipients fed *ad libitum*, and harvested as 0.3–1g wet wt. tumours 3–6 weeks later. The fibrous capsules of 8–10 tumours were removed, the tissue chopped into 1–2mm fragments and blood and cellular debris removed by rinsing in saline then in homogenization medium. All subsequent procedures were performed at 4°C unless specified. The tissue (4–5g wet wt.) was homogenized in a 2.5cm-diameter Potter homogenizer by six strokes of a close-fitting Teflon pestle driven at 600rev./min. The homogenization medium (20ml) contained 0.27M-sucrose, 10mM-4-morpholine-ethanesulphonic acid and 1mM-EGTA and was adjusted with Tris base to pH6.5.

Centrifugation procedures

The homogenate was centrifuged for 10min at 1700*g* and 10ml portions of the supernatant were layered on to 25ml of medium having a composition identical with the homogenization medium, except for the addition of 27% by vol. Percoll [poly-(1-vinylpyrrolidin-2-one)-coated colloidal silica particles; Pharmacia, Uppsala, Sweden]. The tubes (8.5cm × 2.5cm) containing these samples were centrifuged for 45min at 35000*g* in a Beckman type-30 rotor (Beckman Instruments, Palo Alto, CA, U.S.A.). The white turbid zone 0.5–2.5cm from the tube base (fraction A, Fig. 1) was then dispersed in homogenization medium containing 42% (v/v) Percoll and subjected to the same centrifugation protocol. Material now located 2.2–5.8cm from the tube base (fraction C, Fig. 2) was diluted to 50ml with homogenization medium and centrifuged for 15min at 20000*g* on a Sorvall SS-34 rotor (Du Pont Co., Newton, CT, U.S.A.) to separate the granules from residual Percoll. The particulate material sedimented at this step was subjected to three further cycles of resuspension and centrifugation and finally suspended in homogenization medium at 2–10mg of protein/ml and used within 5h or stored at −70°C.

Mg^{2+}-dependent ATPase assays

Investigation of the pH dependency of Mg^{2+}-dependent ATPase activity in osmotically disrupted granules using the buffer series 50mM-4-morpholine-ethanesulphonic acid/Tris, pH6 or 6.5, 50mM-maleic acid/Tris, pH7, 50mM-imidazole/acetic acid, pH7, 50mM-triethanolamine/HCl, pH7.5, 50mM-Tris/HCl, pH7.5 or 8, 50mM-Tris/acetic acid, pH8, and 50mM-Tris/bicarbonate, pH8, revealed no obvious pH optimum or effect of the ionic constituents. A progressive increase in activity of about 50% was observed from pH6 to 8. The pH value of 7 chosen for routine assays with intact granules was a compromise between the activity obtained and documented fragility of insulin granules at higher pH values (Howell *et al.*, 1969; Coore *et al.*, 1969).

Two spectrophotometric Mg^{2+}-dependent ATPase assays that gave identical results on the same preparation were routinely used. The first contained (final concentration) 0.27M-sucrose, 50mM-maleic acid/Tris buffer, pH7, 2.5mM-disodium ATP, 5mM-$MgSO_4$, 5mM-K_2SO_4, 1mM-EGTA, 0.4mM-potassium phosphoenolpyruvate, 0.17mM-NADH, 1 unit of rabbit muscle pyruvate kinase/ml and 1 unit of rabbit muscle lactate dehydrogenase/ml. Enzyme suspensions in $(NH_4)_2SO_4$ were sedimented by centrifugation and dialysed overnight against 10^4 vol. of 10mM-maleic acid/Tris buffer, pH7, before use. Reactions were initiated by the addition of a 5–50μl sample to 1ml of reaction mixture and the $A_{1cm,340}$ at 37°C registered for 5min on a chart recorder.

The reaction mixture for the second assay contained 50mM-KCl, 5mM-$MgSO_4$, 2.5mM-disodium ATP and either the same buffer as above or 50mM-Tris/HCl, pH7.5. Incubations were initiated by the addition of 20μl samples to 180μl of reaction mixture in 3ml-capacity polystyrene tubes (LP3 tubes; W. Sarstedt, Leicester, U.K.) and terminated after 20min at 37°C by adding 0.5ml of ice-cold 7% (w/v) trichloroacetic acid. Phosphate was deter-

mined in the supernatant fluid obtained after centrifugation (Fiske & Subbarow, 1925). Mixtures of samples and reaction media that were not incubated were also analysed to correct for the contribution of endogenous phosphate to the final reading. Sample dilutions were chosen to ensure a linear time course and less than 15% consumption of ATP during the assay. In some instances samples were pre-incubated for 20min at 37°C, ATP then added from a 50mM pH7 stock solution, and the assay performed as above. Further modifications to the standard assay procedures are detailed in the Results section. Compounds of low aqueous solubility were introduced in 100-fold concentrated solutions in ethanol or dimethyl sulphoxide; neither solvent affected the assays.

Marker proteins

Density-gradient fractions were assayed in the presence of 0.1% Triton X-100 for arylsulphatase (Roy, 1953), cytochrome *c* oxidase (Cooperstein & Lazarow, 1951) and NADPH-cytochrome *c* reductase (Sottocasa *et al.*, 1967). In the arylsulphatase assay a centrifugation step was included to remove Percoll precipitated by the final colour reagent.

Protein was determined in samples after precipitation with ice-cold 5% (w/v) trichloroacetic acid with bovine serum albumin (fraction V) or bovine insulin (Sigma) as standard. The method of Lowry *et al.* (1951) was used for Percoll-free samples. Otherwise, the pelleted material was dissolved in 1ml of 50mM-sodium borate buffer, pH9, and then 0.2ml of 0.03% (w/v) fluorescamine {4-phenylspiro[furan-2(3*H*),1-phthalan]-3′,3′-dione} in acetonitrile added while mixing. The fluorescence was determined at 390nm (excitation) and 480nm (emission) with an Aminco Bowman spectrophotofluorimeter (American Instrument Co., Silver Spring, MD, U.S.A.). The blank contributed by Percoll, which was usually less than 10% of the sample fluorescence, was calculated by passing Percoll standards through the entire procedure. The Percoll contents of samples and their densities were determined from their refractive indices related to gravimetrically standardized Percoll solutions in homogenization media.

Insulin was determined by the immunoassay procedure of Wright *et al.* (1968) with the following modifications; reagent volumes were reduced 2.5-fold, 20% (v/v) non-immune horse serum was added to the buffer containing guinea-pig anti-(bovine insulin) antisera and 25% (v/v) Polyethylene glycol 6000 was added instead of a cellulose suspension to separate the bound and free hormone. The radioactivity of the precipitated immune complex was determined after centrifugation for 20min at 1700*g*. Rat insulin (Novo Ind., Copenhagen, Denmark) was used as a standard.

The statistical significance of differences was estimated by Student's *t* test.

Materials

Analytical-grade chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K., or Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K., except for Lubrol 12A9 (I.C.I., Blackley, Manchester, U.K.) and Polyethylene glycol 6000 (Fisons, Loughborough, Leics., U.K.). Biochemicals were from Sigma or Boehringer Mannheim, Germany, except nigericin (Roche Products, Welwyn Garden City, Herts., U.K.), tri-n-butyltin chloride (tributyltin) Ralph N. Emanuel, Alperton, Middx., U.K.) and efrapeptin, a gift from Dr. P. Henderson, Department of Biochemistry, University of Cambridge.

Results

Subcellular distribution of Mg^{2+}-dependent ATPase activity

Isopycnic density-gradient centrifugation of a nuclei-free homogenate of the rat islet-cell tumour resolved three principal zones of Mg^{2+}-dependent ATPase activity (Fig. 1). The most dense of these coincided with the distribution of insulin. The lysosomal marker arylsulphatase, although overlapping insulin, was at a higher density. The major zone of activity in the centre of the gradient, which coincided with the major band of particulate protein, was characterized as containing mitochondria, endoplasmic reticulum and virtually all Golgi membranes, plasma membranes and peroxisomes by enzymic markers (Fig. 1; J. C. Hutton & E. J. Penn, unpublished work). Within this region, activity followed NADPH-cytochrome *c* reductase more closely than cytochrome oxidase or any other marker, suggesting that the major component was associated with endoplasmic reticulum rather than mitochondria. A third zone of ATPase activity was found in association with soluble proteins located in the region of sample application.

A further density-gradient centrifugation (Fig. 2) was used to separate insulin granules from lysosomes and residual material of lower density. Two broad overlapping zones of insulin were observed that coincided with the turbidity and protein distribution. This bimodal distribution appeared not to represent distinct granule populations but was the product of the sigmoidal nature of the gradient, there being concentration of material in regions of a steeper density profile. The zones of the gradient containing lysosomal and mitochondrial proteins were not distinguishable visually and these organelles did not appear to contribute significantly to the protein content of these regions as judged by the variable specific activity of their marker proteins.

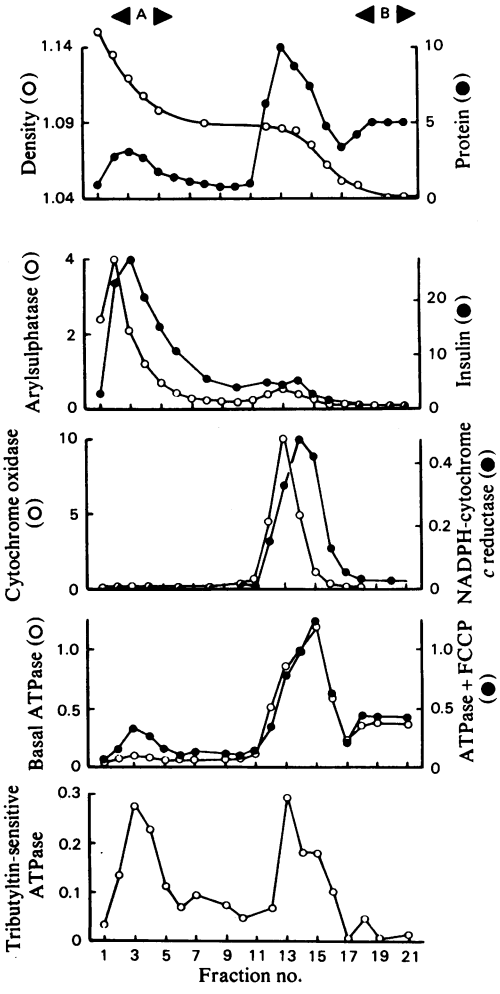

Fig. 1. *Percoll-density-gradient centrifugation of tumour homogenate*

Zone B at the top of the tube indicates the volume initially occupied by the nuclei-free tumour homogenate, which was placed on to 25 ml of 27% (v/v) Percoll solution before centrifugation (see the Experimental section). Mg^{2+}-dependent ATPase activity was determined by the enzymically coupled assay on freshly fractionated material under basal conditions (O), in the presence of 2.5 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; ●) and when this compound and 1 μM-tributylin were present. The tributyltin-inhibitable activity is expressed as the difference between the last two determinations. These and other enzymic activities are expressed in μmol/min per ml, protein as mg/ml, density as g/ml and immunoreactive insulin as units/ml. These data, taken from a single experiment, were representative of a typical analysis.

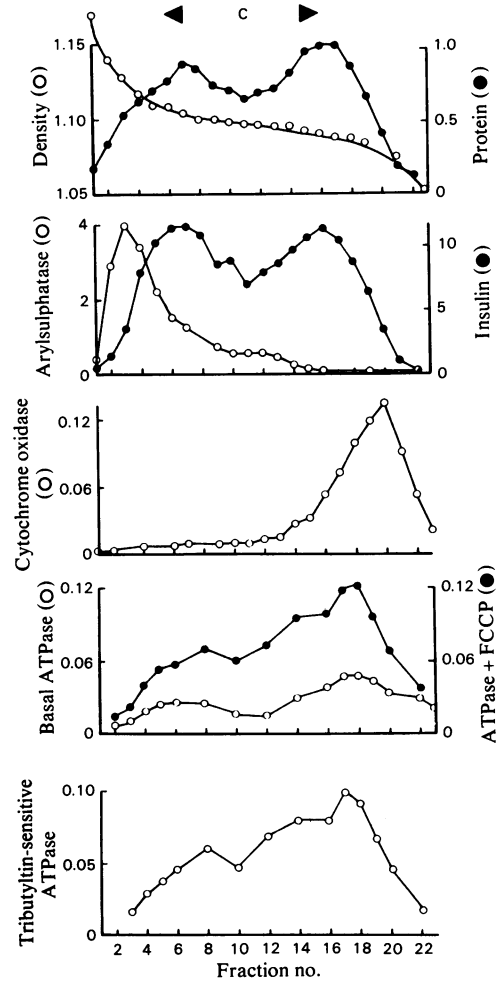

Fig. 2. *Percoll-density-gradient centrifugation of insulin-enriched fractions*

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. Assay conditions and activity units were identical with those shown in Fig. 1. These data, taken from the same experiment, were representative of a typical analysis. The material occupying zone C was recovered by centrifugation for subsequent experiments. Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

The Mg^{2+}-dependent ATPase activity was distributed in a similar manner to insulin on these gradients. However, both its specific activity and its activity relative to insulin decreased progressively as a function of increasing density. This was not inconsistent with the localization of Mg^{2+}-dependent ATPase activity in the granule; since as the activity was membrane-associated (see below) it may be expected to increase proportionately to the granule lipid/protein ratio, which in turn would be reflected in decreasing granule density.

About 10mg of granule protein was recovered from the final density gradient (zone C, Fig. 2) containing 9–12 units of insulin/mg of protein. This represented 20–25% of the initial tumour content and a purification, expressed in terms of the relative specific activity, of 20-fold. This fraction contained less than 0.3% of the initial cytochrome oxidase activity and 10% of the initial arylsulphatase activity. Neither lysosomes nor mitochondrial membranes were conspicuous in electron micrographs of this preparation (J. C. Hutton & E. J. Penn, unpublished work). Since in addition the Mg^{2+}-dependent ATPase activity was quite low in regions of the density gradient where arylsulphatase and cytochrome oxidase were maximally expressed it was concluded that the contribution of lysosomal or mitochondrial contamination to Mg^{2+}-dependent ATPase activity in the final granule preparation was negligible.

Effect of membrane disruption on granule Mg^{2+}-dependent ATPase activity

Mg^{2+}-dependent ATPase activity in isolated granules was 3-fold greater when assays were performed in the absence of sucrose as an osmotic agent or when Lubrol 12A9 [2.5mg/mg of protein; 0.01% (w/v) in the assay] was added to sucrose-containing media (Table 1). Higher concentrations of this detergent produced progressive reduction in activity. Other detergents that were assessed, namely, 0.1% (w/v) Triton X-100, 0.1% (w/v) zwittergent or 0.1% (w/v) Nonidet P40 caused a 50% or greater decrease in activity. Tris cholate (50mM or 1mmol/mg) produced an effect equivalent to that of Lubrol 12A9. The activity in freeze–thawed preparations assayed in the presence of sucrose was approximately twice the activity of intact granules.

Effect of ionophores

The addition of the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to osmotically stabilized granules increased their Mg^{2+}-dependent ATPase activity about 3-fold (Table 1), an effect not seen in the absence of an osmotic agent or in the presence of Lubrol. The activity induced by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was distributed on density gradients in a manner similar to insulin (Figs. 1 and 2) and in insulin-rich regions bore a constant relationship to the basal Mg^{2+}-dependent ATPase activity. It was not found in association with any other marker protein, an anomaly in that the detection of a mitochondrial ATPase activity sensitive to carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was anticipated.

The effects of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, Lubrol and hypo-osmotic incubation on granule Mg^{2+}-dependent ATPase activity were consistent with the postulate that granules possess an Mg^{2+}-dependent ATPase that translocates protons either to or from the interior and that the granule membrane has a low intrinsic proton permeability. In intact granules ATP hydrolysis would be limited by the thermodynamic restraint imposed by the resultant formation of an electrochemical gradient. This could be removed by rendering the membrane selectively permeable to protons or by physical disruption or dissolution of the membrane.

The addition of K_2SO_4 together with valinomycin and nigericin to osmotically protected granules increased granule Mg^{2+}-dependent ATPase activity 2.5-fold (Table 1), although neither ionophore itself with K_2SO_4 affected activity markedly. Since nigericin permits H^+/K^+ exchange and valinomycin electrogenic K^+ movement, these ionophores com-

Table 1. *Effects of membrane disruption and ionophores on granule Mg^{2+}-dependent ATPase activity*

Rates of ATP hydrolysis by freshly prepared granules were determined by the enzymically coupled assay procedure modified as indicated. Cryogenic disruption of granules was achieved by four cycles of freeze–thawing (−20°C/37°C) over a 30min interval in sucrose-containing medium. Each tabulated value is the mean ± S.E.M. of results obtained in separate experiments, the number of these being shown in parentheses. The statistical significance of differences shown (*$P < 0.001$; **$P < 0.02$; ***$P < 0.05$) refers in each case to the respective control incubation.

Modification or addition	*n*	Mg^{2+}-dependent ATPase activity (nmol/min per mg)		Relative change
		Experimental value	Control value	
Sucrose omission	(4)	132.7 ± 29.4*	42.2 ± 12.4	3.15
Freeze–thaw pretreatment	(3)	55.2 ± 2.4**	33.3 ± 5.7	1.66
Lubrol 12A9 (0.01%)	(3)	111.8 ± 3.8*	28.3 ± 2.9	3.95
Tris cholate (50mM)	(3)	52.8 ± 10.4***	26.4 ± 3.0	2.00
Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (2.5 μM)	(7)	115.6 ± 17.8*	38.2 ± 4.8	3.03
Nigericin (0.1 μM)	(3)	35.0 ± 1.1	27.6 ± 3.4	1.27
Valinomycin (0.13 μM)	(4)	30.9 ± 5.2	24.8 ± 3.8	1.25
Nigericin (0.1 μM) plus valinomycin (0.13 μM)	(3)	69.9 ± 3.4*	28.1 ± 3.5	2.49

bined act as an electrogenic proton translocator like carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Effects of permeant anions

The effects of anion permeability on granule Mg^{2+}-dependent ATPase activity in intact granules were investigated in media containing 0.27M-sucrose and either K^+ salts combined with nigericin or NH_4^+ salts. Concentration-dependent responses were observed in the range 10–80mg ion/litre for most ions. The results tabulated (Table 2) were obtained at an intermediate concentration where differences between the various anions were most apparent.

The addition of K^+, nigericin and either I^- or SCN^- provoked a marked increase in granule Mg^{2+}-dependent ATPase activity, which almost equalled that in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone in the same experiment. Cl^- was less effective, as was acetate or phosphate, and only a small response was observed with SO_4^{2-} under these conditions. K^+ salts of these anions used alone produced a much lower response. NH_4^+ salts evoked increases in Mg^{2+}-dependent ATPase activity that were similar to those in the presence of nigericin and the corresponding K^+ salt. None of the NH_4^+ or K^+ salts tested produced an increase in Mg^{2+}-dependent ATPase activity in hypo-osmotically incubated granules or in granules incubated in sucrose with 2.5μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Table 2. *Anion effects on Mg^{2+}-dependent ATPase activity in intact granules*

Mg^{2+}-dependent ATPase activity was determined by the enzymically coupled assay procedure on freshly prepared granules. Initial rates were determined in the presence of 2.5mM-K_2SO_4 then the indicated K^+ or NH_4^+ salt added to a final concentration of 40mM from 1M stock solutions, pH7. Nigericin (0.1μM) was finally added to samples containing added K^+ salts. The rates were recorded over the interval 60–180s after each addition. Each tabulated value is the mean (±S.E.M.) percentage of the initial rate observed in each incubation. The results of four such incubations each performed with different granule preparations are shown in each case. The mean initial rate was 30±3nmol/min per mg ($n = 7$), the relative increase induced by nigericin alone was 111±3% and that by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone 293±14%.

	Mg^{2+}-dependent ATPase activity (% of initial rate)		
Anion	K^+ salt	K^+ salt plus nigericin	NH_4^+ salt
Sulphate	99±8	115±11	130±4
Acetate	110±3	144±10	165±12
Phosphate	99±4	153±16	159±17
Chloride	107±2	207±21	186±17
Iodide	115±7	301±25	200±11
Thiocyanate	128±11	307±31	195±8

The effects of these anions did not result from lysis of the granule in so far as this may have been evidenced by an accelerating ATP hydrolysis after addition of the salt. The $A_{1cm,750}$, which was determined on completion of each assay as an index of changes in granule volume or lysis, was reduced by 15% in the presence of nigericin and either KI or KSCN, but was not significantly affected by other incubation conditions. It could, however, be reduced by 80–90% by deliberate membrane disruption.

Proton translocation in the insulin granule in the absence of other ion movements may be expected to generate both a gradient of protons and a transmembrane electrical potential. If inwardly directed and accompanied by a permeant anion the electrical component would be eliminated, resulting in the expression of the protonmotive force entirely as a pH gradient. In the presence of NH_4^+ salts where the base NH_3 is permeant (for review, see Roos & Boron, 1981), or when H^+ is exchanged with K^+ by nigericin, the pH component can be reduced and the protonmotive force expressed as the electrical component. If, in addition, a permeant anion is present, proton translocation becomes electroneutral, leading to the accumulation of solutes in the granule. Such thermodynamic uncoupling, as it is termed, has been extensively studied in submitochondrial particles (for review, see Dawson & Selwyn, 1974).

The above results were consistent with this chemiosmotic model. The difference observed between anions could be attributed to differences in the permeability of the granule membrane, namely, SCN^- or $I^- > Cl^-$ > acetate or phosphate > SO_4^{2-}. Had proton translocation occurred in an outward direction, little effect of these treatments would have been expected. The small decrease observed in A_{750} with I^- and SCN^- may be attributed to particle swelling resultant from solute accumulation, which would be greatest in the presence of these anions.

An additional postulate in the above model was that the K^+ or NH_4^+ accumulated in conjunction with Mg^{2+}-dependent ATPase activity did not readily permeate the granule membrane. If the cation were freely permeant, a membrane potential would not form and ATP hydrolysis would proceed without restraint. The failure to observe a marked increase in Mg^{2+}-dependent ATPase activity in the presence of K_2SO_4 with nigericin or $(NH_4)_2SO_4$ therefore suggested that the granule membrane permeability to either K^+ or NH_4^+ was, like H^+, low.

Inhibitor studies

Mg^{2+}-dependent ATPase activity in freeze–thawed granules incubated under hypo-osmotic

conditions was reduced to $17 \pm 2\%$ ($n = 4$) of its initial rate by tributyltin (Fig. 3*a*). In both the assay procedures used, half-maximal inhibition occurred at 0.7 nmol of tributyltin/mg of protein. The corresponding inhibitor concentration was either 50 or 150 nM in the enzymically coupled and phosphate-release assays respectively.

In intact granules incubated under iso-osmotic conditions, tributyltin reduced Mg^{2+}-dependent ATPase activity to about 50% of the initial rate. Subsequent addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (2.5 μM) did not produce the usual increase in activity. Conversely, tributyltin added after the protonophore reduced the rate of ATP hydrolysis to the same value as that seen in the presence of the inhibitor alone. The magnitude of this inhibitory effect in the presence of the protonophore was thus equivalent to that produced under hypo-osmotic conditions.

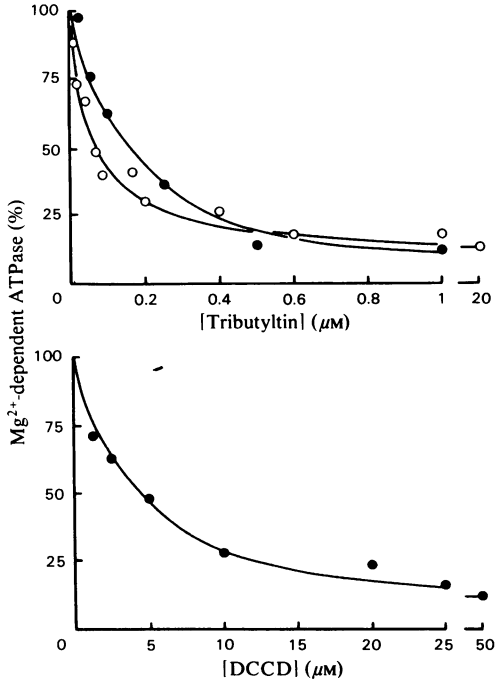

Fig. 3. *Inhibition of granule Mg^{2+}-dependent ATPase activity*

Mg^{2+}-dependent ATPase activity was determined in freeze–thawed samples from the rate of phosphate formation in 50 mM-Tris/HCl buffer, pH 7.5, using the pre-incubation/20 min incubation schedule (●) or in an enzymically coupled assay at pH 7 without sucrose present (○). Each plotted value is the mean of determinations made in at least two separate experiments on different preparations. Each is expressed as a percentage of the mean Mg^{2+}-dependent ATPase activity observed (88 ± 15 nmol/min per mg; $n = 4$). Abbreviation: DCCD, dicyclohexylcarbodi-imide.

On the initial density gradient used in the granule preparation (Fig. 1) the component of the total Mg^{2+}-dependent ATPase activity that was inhibited by tributyltin was distributed in two distinct density regions, one coinciding with insulin, a second with cytochrome oxidase activity. The latter association was consistent with the well-documented effects of this compound on the mitochondrial ATPase (Dawson & Selwyn, 1975). On the second density gradient (Fig. 2) the tributyltin-sensitive Mg^{2+}-dependent ATPase activity followed closely the distribution of the total Mg^{2+}-dependent ATPase activity and insulin, there being little activity associated with either lysosomes or mitochondrial contaminants.

The Mg^{2+}-dependent ATPase activity in hypo-osmotically incubated granules was reduced to $13 \pm 2\%$ ($n = 3$) of its initial rate after incubation with 25 μM-*NN'*-dicyclohexylcarbodi-imide (Fig. 3*b*). Half-maximal inhibition occurred at a 7 μM concentration (3 nmol/mg of protein). These concentrations were comparable with those producing inhibitory effects directed at the F_0 region of the mitochondrial ATPase (Beechey *et al.*, 1967).

Other inhibitors of mitochondrial ATPase activity [oligomycin (10 μg/ml), efrapeptin (10 μg/ml) and NaN_3 (20 mM)], however, were without effect on granule Mg^{2+}-dependent ATPase activity as determined in the enzymically coupled assay in the absence or presence of an osmotic agent. Under identical conditions oligomycin and efrapeptin produced greater than 80% inhibition of Mg^{2+}-dependent ATPase activity assayed in a sonically-disrupted suspension of rat liver mitochondria.

Atractyloside, which inhibits ATPase activity in intact mitochondria by blocking adenine nucleotide transport (Vignais *et al.*, 1973), did not affect granule Mg^{2+}-dependent ATPase activity at either 6 or 600 μM concentration when tested in the presence of 0.27 M-sucrose with or without the addition of 2.5 μm-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Under the same assay conditions 0.01 or 1 mM-sodium orthovanadate, 1 mM-ouabain or 5 mM-5-hydroxytryptamine were without effect on ATP hydrolysis.

Solubilization of granule Mg^{2+}-dependent ATPase activity

Sonication of granules in 50 mM-NH_4HCO_3 containing 5 mM-EDTA resulted in the solubilization of the majority of their insulin and protein content but only a minor proportion of the Mg^{2+}-dependent ATPase activity (Table 3). The insoluble activity was partially solubilized by further sonication in the presence of 0.1% Lubrol, but with attendant reduction in the total activity recovered. This

Table 3. *Detergent solubilization of granule Mg^{2+}-dependent ATPase activity*

Granules were suspended in 1 ml of 50 mM-NH_4HCO_3 buffer, pH 8, containing 5 mM-Tris/EDTA, sonicated for 30 s at 4°C and centrifuged for 40 min at 4°C and 100000 *g* to obtain the first supernatant. The pellet was sonicated in fresh buffer containing 0.1% Lubrol 12A9 and centrifuged as before giving the second supernatant and final pellet. Mg^{2+}-dependent ATPase activity was determined from the rate of phosphate formation at pH 7 as described in the Experimental section. Each tabulated value is the mean of determinations made in two separate experiments on different preparations. Each is expressed as a percentage of the mean initial sample content for each marker (0.55 mg of protein, 5.0 units of insulin and 74 nmol/min for Mg^{2+}-dependent ATPase activity).

	Recovery (%)		
	Protein	Insulin	Mg^{2+}-dependent ATPase
First supernatant	86	87	19
Second supernatant	<5	17	32
Final pellet	12	0.4	16
Total recovery	>98	104	67

suggested that a major component of the granule Mg^{2+}-dependent ATPase was tightly bound to the granule membrane.

Extraction of granule suspensions (5 mg/ml) with 0.02 vol. of dichloromethane by the method of Apps & Glover (1978) resulted in the appearance of Mg^{2+}-dependent ATPase activity in a clear supernatant formed by centrifugation for 5 min at 9000 *g*. The extracted activity was equivalent to up to 60% of that initially present. It was distinguishable by a much reduced sensitivity to inhibition by tributyltin and in being highly labile either at 4°C or at room temperature. Increasing the solvent/granule-protein ratio in the extraction resulted in a progressively lower recovery of Mg^{2+}-dependent ATPase activity but no change in the relative sensitivity to tributyltin.

Proton-translocating ATPase activities in mitochondrial (Beechey *et al.*, 1975) and chromaffin-granule membranes (Apps & Glover, 1978) have been extracted by similar protocols yielding soluble active ATPases. Such solubilization is thought to involve the separation of the ATPase from hydrophobic subunits of the proton channel, which is the site of trialkyltin inhibition (Kagawa & Racker, 1966). The above observations with insulin granules suggested a parallel to such a model.

Discussion

Indirect evidence suggests that the insulin-secretory-granule interior is more acidic than that of the cytoplasm. Granule-enriched subcellular fractions from pancreatic islets accumulate the fluorescent base 9-aminoacridine in an ATP-independent manner (Abrahamsson & Glyfe, 1980) and *in vivo* granules concentrate the bases of 5-hydroxytryptamine and dopamine (3,4-dihydroxyphenethylamine) (Ekholm *et al.*, 1971; Hellman *et al.*, 1972). Such acidification is consistent with the present findings that granules purified from an islet-cell tumour possess an inwardly-directed proton-translocating Mg^{2+}-dependent ATPase. ATPase activity has been documented previously in partially purified granules (Formby *et al.*, 1976); however, this has been related to Ca^{2+} transport.

Two thirds or more of the total Mg^{2+}-dependent ATPase activity expressed in osmotically-disrupted or detergent-treated granules appeared to be of the proton-translocating type. Protonophores thus increased ATP hydrolysis 3-fold in intact granules, producing a rate equivalent to that seen after membrane disruption. Given that the inhibition observed with tributyltin and *NN'*-dicyclohexylcarbodi-imide is specific to proton-translocating activity, then at least 85% of the Mg^{2+}-dependent ATPase in hypo-osmotically incubated granules was attributable to such an enzyme.

Several properties of the granule Mg^{2+}-dependent ATPase activity, including its inhibition by tributyltin and *NN'*-dicyclohexylcarbodi-imide and solubilization on extraction with dichloromethane, were similar to those of the proton-translocating ATPase of mitochondria (for reviews, see Pedersen, 1975; Criddle *et al.*, 1979). It could be distinguished from this enzyme, however, in not being affected by oxyanions, oligomycin, azide and efrapeptin. The subunit of the mitochondrial ATPase at which high-affinity oligomycin binding occurs is encoded by the mitochondrial genome and therefore probably differs in its primary sequence from any analogous protein in an insulin-granule enzyme. The existence of a functionally equivalent subunit in the granule enzyme is suggested by the inhibitory actions of *NN'*-dicyclohexylcarbodi-imide, which in mitochondria acts on the same subunit as oligomycin, although at a different site. Efrapeptin sensitivity of the mitochondrial ATPase appears to reside on the catalytic subunit of the F_1 region (Lardy *et al.*, 1975). The differences presently observed with the insulin-granule Mg^{2+}-dependent ATPase may relate to the differences in this region or in the accessibility of this high-molecular-weight inhibitor to its site of action.

Comparison of the properties of the insulin-granule Mg^{2+}-dependent ATPase activity with those of the proton-translocating ATPase of chromaffin granules showed even greater similarities than between insulin-granule and mitochondrial enzymes. Its sensitivity to a trialkyltin derivative and *NN'*-dicyclohexylcarbodi-imide, insensitivity to oligo-

mycin, efrapeptin, ouabain and orthovanadate, its solubilization after extraction with dichloromethane, and behaviour in different detergents were identical with reported properties of chromaffin-granule Mg^{2+}-dependent ATPase activity (Trifaró & Warner, 1971; Bashford *et al.*, 1976; Apps & Glover, 1978; Apps *et al.*, 1980; Buckland *et al.*, 1981).

It was deduced from present studies of the effects of ionophores on Mg^{2+}-dependent ATPase activity in osmotically protected granules that the insulin-granule membrane is poorly permeable to H^+, K^+, NH_4^+ and SO_4^{2-}, yet permeable, in increasing order, to phosphate or acetate, Cl^-, I^- or SCN^-. Similar permeabilities have been documented in chromaffin-granule membranes (for review, see Njus & Radda, 1978). This suggested further parallels between the biochemical properties of different granule types. It cannot, however, be inferred that the membranes enveloping these secretory vesicles might be identical as a consequence of an identical pathway of biogenesis. Insulin granules, for example, do not display the characteristic absorption in the visible spectrum produced in chromaffin granules by the presence of cytochrome b_{562} (Banks, 1965; Flatmark *et al.*, 1971; J. C. Hutton, unpublished work).

Since insulin granules, like chromaffin granules, can accumulate biogenic amines (Ekholm *et al.*, 1971; Owman *et al.*, 1973), a similar function of the Mg^{2+}-dependent ATPase in amine uptake may be envisaged. It can be calculated that even if all amines in pancreatic islet tissue (Zern *et al.*, 1980) were localized exclusively within the insulin-secretory granule compartment their content would be one or more orders of magnitude less than the catecholamine content of the chromaffin granule (see Njus & Radda, 1978). Nevertheless these granules exhibited comparable rates of ATP hydrolysis (Table 1; Banks, 1965; Trifaró & Warner, 1971; Bashford *et al.*, 1975). It may thus be inferred that the capacity of the insulin granule for ATP hydrolysis far exceeds any energetic requirement for amine transport. Indirect experimental evidence for this conclusion was seen in the failure of 5-hydroxytryptamine to stimulate Mg^{2+}-dependent ATPase activity in intact granules.

Hypothetical roles for the participation of proton-translocating ATPases in membrane signalling and recognition (Lazo *et al.*, 1981) and in granule assembly and exocytosis (Pollard *et al.*, 1979) have been formulated. Such models at least may account for the presence of equivalent catalytic activities in granules that package different secretory products and where secretion is activated by different stimuli.

These studies were supported by grants from the Medical Research Council and British Diabetic Association to Professor C. N. Hales. J. C. H. received personal assistance from the N.H. and M.R.C. of Australia and the British Insulin Manufacturers. Professor C. N. Hales, Dr. A. P. Dawson and Dr. J. H. Phillips are thanked for helpful discussion and Mrs. P. Brown for preparing the manuscript.

References

Abrahamsson, H. & Gylfe, E. (1980) *Acta Physiol. Scand.* **109**, 113–114

Apps, D. K. & Glover, L. A. (1978) *FEBS Lett.* **85**, 254–258

Apps, D. K. & Schatz, G. (1979) *Eur. J. Biochem.* **100**, 411–419

Apps, D. K., Pryde, J. G., Sutton, R. & Phillips, J. H. (1980) *Biochem. J.* **190**, 273–282

Banks, P. (1965) *Biochem. J.* **95**, 490–496

Bashford, C. L., Radda, G. K. & Ritchie, G. A. (1975) *FEBS Lett.* **50**, 31–34

Bashford, C. L., Casey, R. P., Radda, G. K. & Ritchie, G. A. (1976) *Neuroscience* **1**, 399–412

Beechey, R. B., Roberton, A. M., Holloway, C. L. & Knight, I. G. (1967) *Biochemistry* **6**, 3867–3879

Beechey, R. B., Hubbard, S. A., Linnett, P. E., Mitchell, A. D. & Munn, E. A. (1975) *Biochem. J.* **148**, 533–537

Buckland, R. M., Radda, G. K. & Wakefield, L. M. (1981) *Biochim. Biophys. Acta* **643**, 363–375

Chick, W. L., Warren, S., Chute, R. N., Like, A. A., Lauris, V. & Kitchen, K. C. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 628–632

Cooperstein, S. J. & Lazarow, A. (1951) *J. Biol. Chem.* **189**, 665–670

Coore, H. G., Hellman, B., Pihl, E. & Taljedal, I.-B. (1969) *Biochem. J.* **111**, 107–113

Criddle, R. A., Johnston, R. F. & Stack, R. J. (1979) *Curr. Top. Bioenerg.* **9**, 89–145

Dawson, A. P. & Selwyn, M. J. (1974) in *Companion to Biochemistry* (Bull, A. T., Lagnado, J. R., Thomas, J. O. & Tipton, K. F., eds.), pp. 553–586, Longman, London

Dawson, A. P. & Selwyn, M. J. (1975) *Biochem. J.* **152**, 333–339

Downie, J. A., Gibson, F. & Cox, G. E. (1979) *Annu. Rev. Biochem.* **48**, 103–131

Ekholm, R., Ericson, L. E. & Lunquist, J. (1971) *Diabetologia* **7**, 339–348

Fillingame, R. H. (1980) *Annu. Rev. Biochem.* **49**, 1079–1113

Fiske, C. H. & Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375–400

Flatmark, T., Terland, O. & Helle, K. B. (1971) *Biochim. Biophys. Acta* **226**, 9–19

Formby, B., Capito, K., Egeberg, J. & Hedeskov, C. J. (1976) *Am. J. Physiol.* **230**, 441–448

Hanks, J. H. & Wallace, R. E. (1949) *Proc. Soc. Exp. Biol. Med.* **71**, 196–200

Hellman, B., Lernmark, A., Sehlin, J. & Taljedal, I.-B. (1972) *Biochem. Pharmacol.* **21**, 695–706

Hopkins, C. R. & Duncan, C. J. (1979) *Symp. Soc. Exp. Biol.* **33**

Howell, S. L., Young, D. A. & Lacy, P. E. (1969) *J. Cell Biol.* **41**, 167–176

Kagawa, Y. & Racker, E. (1966) *J. Biol. Chem.* **241**, 2461–2466
Lardy, H., Reed, P. & Lin, C.-H. C. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 1707–1710
Lazo, P. S., Barros, F., de la Peña, P. & Ramos, S. (1981) *Trends Biochem. Sci.* **7**, 83–86
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Njus, D. & Radda, G. K. (1978) *Biochim. Biophys. Acta* **463**, 219–242
Owman, C., Håkanson, R. & Sundler, R. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 1785–1791
Pedersen, P. L. (1975) *Bioenergetics* **6**, 243–275
Pollard, H. B., Pazoles, C. J., Creutz, C. E. & Zindler, O. (1979) *Int. Rev. Cytol.* **58**, 159–197
Roos, A. & Boron, W. F. (1981) *Physiol. Rev.* **61**, 296–434
Roy, A. B. (1953) *Biochem. J.* **53**, 12–15
Sopwith, A. M., Hutton, J. C., Naber, S. P., Chick, W. L. & Hales, C. N. (1981) *Diabetologia* **21**, 224–229
Sottocasa, G. L., Kuylenstierna, B., Ernster, L. & Bergstrand, A. (1967) *J. Cell Biol.* **32**, 415–438
Trifaró, J. M. & Warner, M. (1971) *Mol. Pharmacol.* **8**, 159–169
Vignais, P. V., Vignais, P. M. & Defaye, G. (1973) *Biochemistry* **12**, 1508–1519
Wright, P. H., Makulu, D. R., Malaisse, W. J., Roberts, N. M. & Yu, P.-L. (1968) *Diabetes* **17**, 537–546
Zern, R. T., Bird, J. L. & Feldman, J. M. (1980) *Diabetologia* **18**, 341–346