The first indications that Ca\(^{2+}\) is an essential requirement for secretion of insulin were obtained in perfused pancreas preparations by Grodsky & Bennett (1966), and it has subsequently been shown that an adequate extracellular Ca\(^{2+}\) concentration is essential for sustained secretion of insulin in vitro by any of the pancreas or isolated islet preparations that are now in common use. More recent work, especially by Malaisse and his collaborators, has explored the fluxes of 45Ca\(^{2+}\) into and out of intact cells in isolated islets in a wide variety of situations, and these studies are discussed elsewhere in this colloquium (Malaisse, 1977). The present paper reviews some investigations of the localization of calcium within B-cells, and of factors which may alter Ca\(^{2+}\) distribution between individual organelles during various phases of insulin secretion. It is obviously of critical importance to the interpretation of data on Ca\(^{2+}\) fluxes across plasma membranes to obtain some knowledge of the compartmentation and sites of accumulation of Ca\(^{2+}\) in various organelles. Two approaches have been made to this problem: firstly, to attempt indirect localization of Ca\(^{2+}\) by the use of pyroantimonate procedures, and secondly, to attempt direct analysis of the Ca\(^{2+}\) content of various organelles by means of X-ray micro-analysis.

Pyroantimonate procedures. Treatment of tissues with potassium pyroantimonate during fixation with osmium tetroxide was originally suggested as a procedure for

**Intracellular Localization of Calcium in Pancreatic B-Cells**

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The first indications that Ca\(^{2+}\) is an essential requirement for secretion of insulin were obtained in perfused pancreas preparations by Grodsky & Bennett (1966), and it has subsequently been shown that an adequate extracellular Ca\(^{2+}\) concentration is essential for sustained secretion of insulin in vitro by any of the pancreas or isolated islet preparations that are now in common use. More recent work, especially by Malaisse and his collaborators, has explored the fluxes of 45Ca\(^{2+}\) into and out of intact cells in isolated islets in a wide variety of situations, and these studies are discussed elsewhere in this colloquium (Malaisse, 1977). The present paper reviews some investigations of the localization of calcium within B-cells, and of factors which may alter Ca\(^{2+}\) distribution between individual organelles during various phases of insulin secretion.
localization of Na⁺ (Komnick & Komnick, 1963). It subsequently became clear that other cations in addition to Na⁺ were precipitated by this agent, and latterly the method has been quite widely used for localization of Ca²⁺, since this was shown to be one of the elements present in pyroantimonate precipitates.

This type of method was first applied to isolated islets by Herman et al. (1973) who were able to confirm by X-ray micro-analysis that the electron-opaque precipitates of pyroantimonate did indeed contain calcium. Precipitates were found to be associated with membranes of storage granules, mitochondria, cytoplasmic matrix and nuclei. Assessment of the pattern of precipitation in cells that were secreting insulin rapidly showed increased precipitation in the granule sac and plasma membrane, whereas other sites of deposition were unchanged. These findings were confirmed and extended by Ravazzola et al. (1976) in a similar study. Schäffer & Klöppel (1974) in comparable experiments using mouse islets were able to show that, in B-cells from normoglycaemic mice, pyroantimonate deposits were associated with granule membranes, plasma membranes and cytoplasmic matrix, whereas during hypoglycaemia there was a shift of precipitate into endoplasmic reticulum and mitochondria. In B-cells from hyperglycaemic animals, deposits were increased on the inner surface of the plasma membrane and in the secretory granule sacs.

In each of these three investigations the presence of calcium within the precipitates was confirmed by X-ray micro-analytical methods. Clearly, however, other elements were also present within the precipitates and it has not so far been possible to make micro-analytical studies of the increased deposits present in the granule sacs after incubation in the presence of high glucose concentrations, to confirm that they are in fact richer in calcium. Nevertheless, the results do uniformly suggest a shift of pyroantimonate-precipitable material to the granule sac during conditions in which insulin release is stimulated.

X-ray micro-analysis. An alternative approach to the problem of calcium localization has been attempted by Howell et al. (1975) who used frozen sections of unfixed tissue in order to attempt localization of Ca²⁺ in the various organelles. This has the great advantage of providing a direct measurement of Ca²⁺ concentrations in conditions in which loss and diffusion during fixation should be avoided, but the disadvantage of the technical difficulty of obtaining ultrathin frozen sections of a quality which are usable for micro-analytical purposes. Nevertheless, it has been possible to obtain values for the Ca²⁺ content of various organelles in frozen sections of B-cells by these methods, and the results have suggested not only that the storage granules contain high concentrations of Ca²⁺, but also that the mitochondria represent a second Ca²⁺-rich pool within the cells. Pyroantimonate procedures had not in general shown the mitochondria to be a major store of Ca²⁺ in this cell type and the reason for this anomaly is at present uncertain; perhaps the more labile mitochondrial pool is lost selectively during the fixation procedures involved in the pyroantimonate method. It will be interesting to obtain more accurate information on the distribution of Ca²⁺ and other ions with the aid of technical improvements in micro-analysis, which are now becoming available.

⁴⁵Ca²⁺ accumulation in islet homogenates

A complementary approach to the study of Ca²⁺ concentrations within organelles of intact cells is to examine the accumulation of ⁴⁵Ca²⁺ by subcellular particles during incubation of islet homogenates or subcellular fractions in suitable conditions, and this approach has been adopted in rat islets by ourselves (Howell & Montague, 1975; Howell et al., 1975), and in obese–hyperglycaemic-mouse islets by Sehlin (1976).

It is clear from these experiments that one or more of the components in homogenates can accumulate Ca²⁺ in a rapid ATP-dependent manner, which requires the presence of a permeant anion (oxalate or phosphate); the characteristics of uptake appear consistent with those of either mitochondria or the microsomal fraction in other tissues. The 8-fold stimulation achieved by 1.25mM-ATP was specific in that GTP, UTP, CTP or AMP were ineffective, whereas ADP or adenylyl imidodiphosphate had only small stimulatory effects. Scatchard analysis suggests the presence of two Ca²⁺-binding
sites, a low-affinity ATP-independent site and a high-affinity ATP-dependent site (Sehlin, 1976). Of some interest was the finding in both studies that addition of cyclic AMP (10 or 100 µM) or of cyclic GMP inhibited the ATP-dependent accumulation of ⁴⁵Ca²⁺ by particulate components of subcellular fractions by as much as 50%. In obese mice, but not in rat islet homogenates, theophylline was effective in decreasing net Ca²⁺ accumulation in a similar way.

These observations appeared to have some counterpart in earlier studies by Borle (1974) of effects of cyclic AMP on Ca²⁺ accumulation by isolated kidney, liver or heart mitochondria, in which it was suggested that the effect of cyclic AMP was to increase the efflux of Ca²⁺ rather than to decrease its uptake, although the significance of these findings has subsequently become less clear (Borle, 1976). Effects on net ⁴⁵Ca²⁺ accumulation of other agents that stimulate insulin secretion from intact B-cells (glucose, adrenaline, noradrenaline, leucine, arginine, glibenclamide) were also examined, but none of these substances, which were used at concentrations known to alter rates of secretion, was effective in these conditions.

**Organelles that accumulate Ca²⁺**

Attempts to identify the organelles responsible for Ca²⁺ accumulation have rested on two main techniques: subcellular fractionation and use of barium as an electron-opaque calcium analogue.

Subcellular-fractionation data have been restricted, because of poor yield obtained after density-gradient fractionation, to studies of fractions obtained by differential centrifugation, and the cross-contamination involved has inevitably resulted in some uncertainty about the organelles that may be involved. Howell et al. (1975) using rat islets found the highest specific radioactivity of accumulated ⁴⁵Ca²⁺ in a pellet (24000g for 10 min) predominantly composed of mitochondria, storage granules and a heavy-microsomal fraction, with much lower activities in nuclei and debris and light-microsomal (105000g for 60 min pellet) fractions. Sehlin (1976), on the other hand, found highest activity in a storage-granule plus microsomal fraction (110000g for 30 min) obtained from obese-hyperglycaemic-mouse islets, and concluded on this basis that microsomal accumulation might be very important in mediating the observed effects. Inhibition of Ca²⁺ uptake as a result of uncoupling of mitochondrial oxidative phosphorylation (2,4-dinitrophenol), or by the apparently specific inhibitor of mitochondrial uptake Ruthenium Red, both suggest that mitochondria might play an important role in regulating Ca²⁺ uptake. Involvement of granules, which were also shown from micro-analysis to contain high Ca²⁺ concentrations, seemed unlikely in view of the finding that homogenates of islets that had been depleted of 50% of their granule store by repeated injection of glibenclamide were equally effective at accumulating ⁴⁵Ca²⁺ as islet homogenates that contained a normal complement of granules (Howell et al., 1975).

Ba²⁺ has been shown to be able to substitute for Ca²⁺ in the insulin-secretory process (Milner & Hales, 1969), and its high atomic number renders it much more electron opaque than calcium. It has thus been possible to identify sites of uptake of Ba²⁺ by organelles directly, after incubation of islet homogenates in conditions identical to those utilized for previous ⁴⁴Ca studies, except for the substitution of 2mM-barium acetate for 10 µM-[⁴⁴Ca]calcium chloride (Howell & Tyhurst, 1976). The results showed clearly that mitochondria are the principal sites of uptake of Ba²⁺ in these conditions, and that this uptake may be considered reasonably specific, since it is prevented by addition of the uncoupling agent dinitrophenol to the incubation medium in exactly the same way that ⁴⁴Ca²⁺ accumulation was inhibited.

It might be argued that the results of these experiments were predictable, since the buffers and conditions used for incubation were those which favoured mitochondrial bivalent cation accumulation and so to some extent did not allow an impartial choice between the organelles present. In an attempt to overcome this objection, further experiments were performed by using intact cells which were incubated with barium acetate in a modified bicarbonate medium and sites of deposition of electron-
Permeability channel

Mitochondria

Endoplasmic reticulum

Diffusion channel

Microtubules

binding proteins

Fig. 1. Regulation of cytosolic Ca^{2+} concentrations in B-cells

Ca^{2+} entry into the cells may occur by diffusion, or via a permeability channel which can be blocked by compound D600 or verapamil (Malaise et al., 1976). Efflux is achieved by pumping against the prevailing concentration gradient. Cytosolic Ca^{2+} concentrations are regulated by accumulation into mitochondria and endoplasmic reticulum, and by binding to unspecified proteins. A rise in cytosolic Ca^{2+} concentration may favour the attachment of granules to microtubules and their contractile function, as well as the fusion of granule and plasma membranes (see the text).

Opaque deposits were again observed. The results showed that mitochondria were the principal sites of barium deposition; almost every mitochondrion of every cell contained multiple deposits. A very small proportion of granules contained a single deposit of approximately similar size in the space between the granule core and its limiting membrane; the only other major site of deposition was on the plasma membrane of the cell, where it occurred intermittently in an apparently random pattern of particles of a much smaller diameter than those seen in mitochondria (Howell & Tyhurst, 1976).

Calcium and the regulation of insulin secretion

The process of secretion is very complex, involving transfer of storage granules from a cytoplasmic pool to the plasma membranes via a network of microtubules, with subsequent fusion of granule and plasma membranes and release of the granule contents, a process termed exocytosis. The exact way in which changes in cytosolic Ca^{2+} concentrations elicit alterations in rates of secretion is still largely unknown (Fig. 1). The calculations of Dean (1975) and Dean & Matthews (1975), about surface charges on chromaffin-granule membranes, together with the findings of Dahl & Gratzxl (1976) and Davis & Lazarus (1975) of increased fusion of granule membranes in the presence of Ca^{2+} have stimulated us to examine directly the distribution of anionic sites on the surface of granule and plasma membranes of B-cells, using the electron-opaque probe cationic ferritin. The results show clearly that excess anionic sites are present on both granule and plasma membranes, and that the number of charges present on the
granule-membrane surface as it undergoes exocytosis is dramatically decreased (Howell & Tyhurst, 1977). Incubation of isolated granules with 1–2 mM-CaCl₂ did not produce a visible decrease in the number of anionic sites, although as shown by Dean (1974), even the small decrease in charge which could be expected after addition of 1 mM-Ca²⁺ would be sufficient to produce a 50-fold increase in the number of fusions of granule and plasma membranes.

It is certainly attractive to speculate that once a granule has been transported to a position very close to the plasma membrane by the action of microtubules, possibly also involving calcium, it will be maintained at a point close to the membrane by the equilibrium between van der Waals forces of attraction and electrostatic repulsion (Dean, 1975). The rise in cytosolic Ca²⁺ concentrations, which results from stimulation of secretion, will then decrease the surface charge and electrostatic repulsion, and allow fusion of granule and plasma membrane to occur.

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**Strontium Ions as a Probe for the Role of Alkaline-Earth Metal Ions in Histamine Secretion**

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The use of a variety of bivalent and tervalent ions to probe the nature of the Ca²⁺-transporting and -binding sites in cells has become commonplace, but often, the information obtained by such studies is limited. In the process of histamine secretion from mast cells, one ion, Sr²⁺ when substituted for Ca²⁺ has given valuable information about the mechanism of secretion.

When mast cells are stimulated by an antigen–antibody reaction on their plasma membrane, the secretion of histamine-containing granules from the cell will proceed only if Ca²⁺ is present in the extracellular medium. The concentration range over which Ca²⁺ will activate this secretory process is 0.1–1.0 mM, with a maximum activation at 1.0 mM (Foreman & Mongar, 1972). Sr²⁺ is able to replace Ca²⁺ in activating the secretory process stimulated by an antigen–antibody reaction. However, in contrast with Ca²⁺, Sr²⁺ is active in the concentration range 1–10 mM (Fig. 1). A further point of